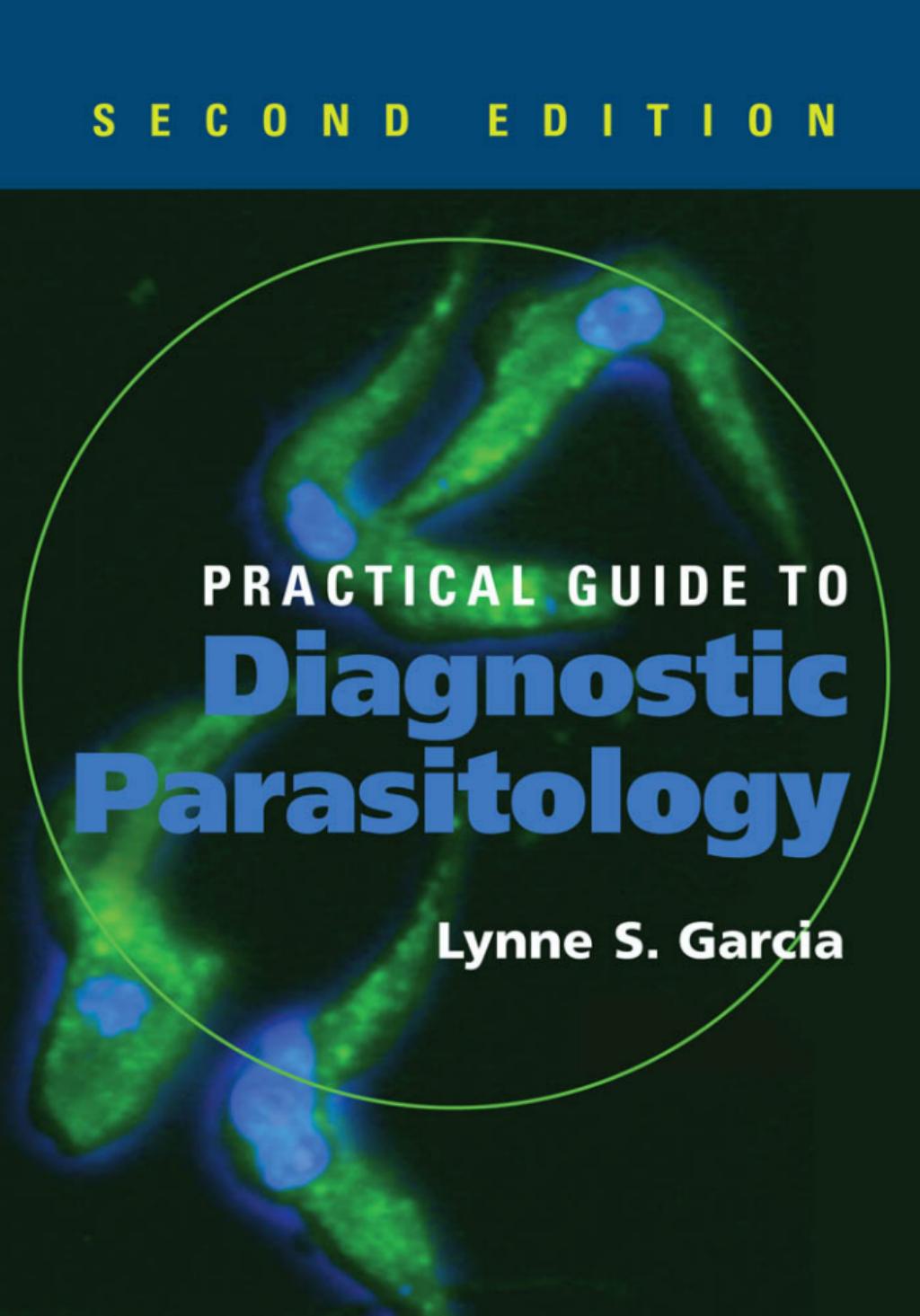


S E C O N D   E D I T I O N



PRACTICAL GUIDE TO  
**Diagnostic  
Parasitology**

Lynne S. Garcia

PRACTICAL GUIDE TO  
**Diagnostic**  
**Parasitology**  
SECOND EDITION

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**Diagnostic  
Parasitology**

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*Cover figure:* Bloodstream forms of the African trypanosome, *Trypanosoma brucei*, stained for a surface membrane protein (in green) and counterstained with the DNA intercalating dye DAPI, thus revealing the parasite's central nucleus and posterior kinetoplast. (Courtesy of Keith Matthews and Sam Dean, University of Edinburgh.)



*I dedicate the second edition of this practical guide to Robyn Shimizu. Robyn and I have worked together for many years, performing bench work, training students, presenting workshops and seminars, handling consults, performing studies, and preparing manuscripts for publication. A very special thanks to Robyn for her collaboration and support—it's been quite an adventure, and we're looking forward to continued collaboration.*

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# Preface

As we move into the 21st century, the field of diagnostic medical parasitology continues to see some dramatic changes, including newly recognized pathogens, the spread of familiar pathogens, successes and failures related to disease control, geographic and climate changes that support the spread of parasitic disease, new methodology, regulatory requirements that impact on diagnostic testing, and an overall review of the approach to and clinical relevance of this type of diagnostic testing on patient care within the managed care environment, as well as the world as a whole.

The second edition of the *Practical Guide to Diagnostic Parasitology* is organized to provide maximum help to the user, particularly from the bench use perspective. New aspects of the field have been addressed in these sections, and many new figures and plates have been added. Section 1 on the philosophy and approach to diagnostic parasitology has been expanded to include discussions on the possible impact of global warming, population movements, potential outbreak testing, the development of laboratory test menus, and the risk management issues related to STAT testing. The discussion of organism classification and relevant tables has been expanded to provide the user with current information related to changes in nomenclature and overall importance of the various parasite categories to human infection.

In Section 3, expanded information on stool specimen fixatives and testing options has been provided. This information is valuable for any laboratory reviewing collection and testing options related to fixative compatibility with the routine ova and parasite examination, as well as the newer fecal immunoassays. Although some fecal immunoassay reagents are now commercially available for *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*, they are not FDA approved; always check the literature from the relevant company to confirm the FDA status of any new product. The discussion on blood collection, including the pros and cons of current changes from finger stick blood to venipuncture, has been

greatly enhanced, particularly related to potential problems with blood parasite morphology and lag time issues. Additional tables serve to summarize much of this new information.

Additional tables and information have been added to Sections 4 and 5, including a number of new algorithms. Section 6 is one of the most important sections in the book, with extensive revisions related to the most commonly asked questions regarding diagnostic parasitology methods. Additional techniques have been included, as well as new information related to reporting results and the importance of report comments. This section format makes it easy for the reader to use the expanded information.

Section 7 has been greatly expanded; information on each organism has been formatted on facing pages for easy access and reference. Figures have also been expanded and updated. Section 8 on identification aids has been expanded with additional tables and new plates. All of the changes for this edition are based on the need for the readers to update their information related to diagnostic medical parasitology and specifically the issues mentioned below.

With continued emphasis on regulatory requirements related to chemical disposal and the use of mercury compounds, laboratories are being required to develop skills using substitute fixatives that are prepared without the use of mercury-based compounds. Although continued work with these substitute fixatives has not produced the exact same quality organism morphology, the more relevant question is whether the intestinal parasites can be identified by using these alternative fixatives. In most cases, organism identification is comparable; an example of a rare exception is one in which the number of organisms present is quite low. This is a prime example of a change where “different” has been acceptable and relevant, not necessarily “good” or “bad.”

Many laboratories are reviewing all microbiological services, and some specific questions are being asked related to diagnostic parasitology options. Some of these questions include the following: what laboratories should be performing this type of testing, when should testing be performed, what tests should be performed, and what factors should be considered when developing test menus.

Laboratories are also reviewing specimen collection options, particularly as they relate to their geographic area and types of patients serviced. This kind of analysis is beneficial to all concerned, not only in helping laboratories to understand the specimen collection options, but how they relate to test orders, diagnostic testing, and results impacting patient care.

With changes in collection, testing, reporting, and interpretation options, it is critical to remember that this information needs to be shared with the laboratory's client base, particularly if the test orders and results are to be used for the best-quality patient care. Although there are many ways to approach diagnostic parasitology testing, it is mandatory that the laboratory and user both understand the pros and cons of the methods selected. The use of different approaches to parasitology diagnostic testing is acceptable; however, the benefits and drawbacks must be thoroughly understood by all participants. There may be legitimate reasons why dif-

ferent approaches are used by different laboratories; however, cost containment must not be the sole factor in selecting methods.

Another consideration is the fact that not all clinical laboratories will continue to perform diagnostic parasitology testing. This may be due to financial considerations, lack of skilled personnel, etc. With increased emphasis on cross-trained individuals, the technical expertise required to identify these parasites by using routine microscopy may be lacking. Even with the use of molecular diagnostics, these tests are not capable of covering the entire spectrum of organisms that may be present as pathogens. However, as more of these methods become commercially available, the use of nonmicroscopic methods will increase in scope. Many laboratories now include both the ova and parasite examination and various fecal immunoassays on their routine test menus; on the basis of patient histories and symptoms, appropriate orders may focus on one or the other of these options. An important consideration in deciding whether to send out parasitology testing or maintain the testing in-house relates to STAT testing (collection, processing, testing, reporting of thick and thin blood films, and the examination of cerebrospinal fluid and other specimens for the presence of free-living amebae). These tests must be handled as STATs; the time required from collection to reporting must be considered prior to moving these procedures off-site.

Based on the many changes in clinical laboratories within the past few years and many years' experience with teaching and diagnostic bench work, it is my hope that the information contained in this practical guide to diagnostic parasitology will provide some valuable information for the user. This guide is not designed to serve as a diagnostic parasitology text or to contain all possible diagnostic test options but to help the user make some sense of a field for which training has become almost non-existent. I have included a section on commonly asked questions about diagnostic medical parasitology and hope that this discussion will be of practical value to the user; the answers to some of these questions are often difficult to find, even in a more comprehensive book. Again, let me emphasize that different approaches to laboratory work are not always "good" or "bad." The key to success is making sure that the laboratory and clients both understand the pros and cons of each collection, testing, and reporting option and that educational information is provided for all clients on an ongoing basis. Two of the most important functions of the clinical laboratory in the future will be educational and consultative, particularly when laboratory services are within the microbiology area.

A final point is that infectious diseases, particularly parasitic infections, play a huge role in the overall world's health and economy. As travel increases, we anticipate seeing many more people who will be infected with parasites that may not be endemic to the specific area where they live. Continued vector and disease control efforts will remain on the high-priority list, especially when seen within the context of global health. Hopefully, parasitologists, including those who have diagnostic skills, will be available to support these global initiatives.

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# Acknowledgments

I thank the hundreds of colleagues over the past years who have shared their thoughts and suggestions regarding this fascinating field of diagnostic parasitology. There are too many of you to name—you all know who you are and we all recognize the pitfalls, as well as the fun, in providing diagnostic services in this field of microbiology.

I also thank the many bench techs and microbiologists who have tackled this field over the last 30+ years, including those who attended workshops and seminars; your contributions to the growth and expansion of diagnostic parasitology have been significant. Discussions of questions asked, problems for resolution, and reviews of testing options have been invaluable in shaping our approach to diagnostics in this field. This type of interaction, including the many discussions with the UCLA microbiology staff in the parasitology area, helped all of us keep an open mind when reviewing options and possible new ways to approach the work.

Over the years, our association with many companies has also been extremely valuable in helping understand test development, test trials, and relevance of results to the ultimate user within the diagnostic laboratory. Again, these interactions have helped maintain some balance and perspective on new options and their relevance to improved patient care.

A challenge to all of us who are still actively working in this area of diagnostic microbiology: serve as a mentor to some of the young people entering the field of microbiology. The number of personnel trained in this field continues to decline. Until parasite morphology is no longer required for differentiation and diagnosis, skilled microscopists will remain valuable members of the microbiology team and mandatory for the practice of diagnostic medical parasitology.

I thank Sharon Belkin for the new illustrations; she is always capable of translating my verbal descriptions into clear and accurate depictions of the various organisms. This is a true art, and she brings considerable expertise to the process; pictures are always a tremendous addition to verbal descriptions.

I also thank members of the editorial staff of ASM Press, including Susan Birch, Jeff Holtmeier, and our copy editor, Yvonne Strong; they are outstanding professionals. Susan's expertise always helps the authors "look good," and I appreciate her collaboration not only as an editor but also as a friend and colleague.

Above all, my very special thanks go to my husband, John, for his love and support for this and other projects over the years. I could never have taken on these challenges without his help, understanding, and wonderful sense of humor.

## S E C T I O N   1

# Philosophy and Approach to Diagnostic Parasitology

## Why Perform This Type of Testing?

### Travel

With the increase in world travel and access to varied populations and geographic areas, we continue to see more “tropical” diseases and infections outside areas of endemicity due to the rapidity with which people and organisms can be transmitted from one place to another. Travel has also become accessible and more affordable for many people throughout the world, including those whose overall health status is in some way compromised. The increased transportation of infectious agents and potential human carriers, particularly via air travel, has been clearly demonstrated during the last few years. It has also been well documented that vectors carrying parasitic organisms can be transported via air travel in baggage and in the unpressurized parts of the plane itself; once released, these infected vectors can then transmit these parasites to humans, even in areas where the infections are not endemic.

### Population Movements

In many parts of the world, particularly where conflict is ongoing, there continue to be large population movements. Many of these individuals become a part of refugee migrations to and from areas of endemic parasitic diseases. Often in refugee situations, living conditions are very poor and medical limitations may lead to high levels of parasitic disease and severe illness. Also, migrants may move into countries and geographic areas where serious parasitic infections are generally nonendemic, including Europe and parts of North America. Even if these individuals are uninfected when entering these areas, travel home to visit relatives may result in infections that can be imported when they return.

### Control Issues

Control of parasites that cause disease is linked to a number of factors including geographic location, public health infrastructure, political stability, available funding, social and behavioral customs and beliefs, trained laboratory personnel, health care support teams, environmental constraints, degree of understanding of organism life cycles, and opportunities for control and overall commitment. Often control efforts do not cross political or geographic boundaries; unfortunately, vectors and other carriers of infectious agents do not play “by the rules”; as a result, these boundaries become meaningless.

### Global Warming

With continued increase in the global temperature, worldwide climate changes are leading to an overall increase in infectious diseases, vector populations, and endemic ranges of both parasites and vectors. Global warming enhances the potential spread of tropical parasitic infections, specifically those due to parasites such as *Plasmodium* spp., *Leishmania* spp., and *Trypanosoma* spp. Examples of vectors whose range is increasing include *Anopheles*, *Aedes*, and *Culex* mosquitoes, hard ticks, and triatomid bugs.

## **Epidemiologic Considerations**

When newer infectious agents and/or diseases are recognized, there is often very little information available regarding the organism life cycle, potential reservoir hosts, and environmental requirements for survival. Priorities may change, and epidemiologic considerations may have been moved lower on the priority list in areas of the world where they were considered important in the past; unfortunately, funding often plays a role in decisions that impact disease control measures.

## **Compromised Patients**

With the tremendous increase in the number of patients whose immune systems are compromised through underlying illness, chemotherapy, transplantation, AIDS, or age, we are much more likely to see increasing numbers of opportunistic infections, including those caused by parasites. Also, we continue to discover and document organisms that were thought to be nonpathogenic but, when found in the compromised host, can cause serious disease. In assessing the possible cause of illness in this patient population, the possibility of parasitic infections must be considered as part of the differential diagnosis.

## **Approach to Therapy**

As new etiologic agents are discovered and the need for new therapeutics increases, more sensitive and specific diagnostic methods to assess the efficacy of newer drugs and alternative therapies will become mandatory. Skilled laboratorians, physicians, public health personnel, and other health care team members will be required to think globally in terms of infectious diseases caused by bacterial, fungal, parasitic, and viral etiologies, particularly when certain parasitic infections require very specific therapeutic regimens.

## **Who Should Perform Diagnostic Parasitology Testing?**

### **Laboratory Personnel**

Diagnostic procedures in the field of medical parasitology require a great deal of judgmental and interpretative experience and are, with very few exceptions, classified by the Clinical Laboratory Improvement Act of 1988 (CLIA '88) as high-complexity procedures. Very few procedures can be automated, and organism identification relies on morphologic characteristics that can be very difficult to differentiate. Although morphology can be "learned" at the microscope, knowledge about the life cycle, epidemiology, infectivity, geographic range, clinical symptoms, range of illness, disease presentation depending on immune status, and recommended therapy is critical to the operation of any laboratory providing diagnostic services in medical parasitology. As laboratories continue to downsize and reduce staff, cross-training will become more common and critical to financial success. Maintaining expertise in fields such as diagnostic parasitology has become more difficult, particularly when using standard manual methods. Also, the lower the positive rate for parasitic infections,

the more likely it is that the laboratory will generate both false-positive and false-negative laboratory reports. It is important for members of the health care team to thoroughly recognize areas of the clinical laboratory that require experienced personnel and why various procedures are recommended above others.

### **Nonlaboratory Personnel**

Health care delivery settings where physicians provide parasitology diagnostic testing occasionally provide “simple” test results (CLIA '88 waived tests) based on wet mount examinations. However, in spite of the CLIA classification of these diagnostic methods, wet mount examinations are often very difficult to perform, and results are often incomplete or incorrect. Currently, there are no specific “over the counter” testing methods for parasitic infections; however, the future may see some newer diagnostic developments in this area. The key to performance of diagnostic medical parasitology procedures is formal training and experience. As the laboratory setting continues to change during the 21st century, it is important to recognize that these changes will require a thorough understanding of the skills required to perform diagnostic parasitology procedures and the pros and cons of available diagnostic methods. Laboratories will have a number of diagnostic options; whatever approach is selected by an individual laboratory, the clinical relevance of the approach must be thoroughly understood and conveyed to the “client” user of the laboratory services.

## **Where Should Diagnostic Parasitology Testing Be Performed?**

### **Inpatient Setting**

Most diagnostic parasitology procedures can be performed either within the hospital setting or in an offsite location. There are very few procedures within this discipline that must be performed and reported on a STAT basis. Two procedures fall into the STAT category: request for examination of blood films for the diagnosis of malaria or other blood parasites and examination of cerebrospinal fluid (CSF) for the presence of free-living amebae, primarily *Naegleria fowleri*. Any laboratory providing diagnostic parasitology procedures must be prepared to examine these specimens on a STAT basis 7 days a week, 24 h a day. Unfortunately, these two procedures can be very difficult to perform and interpret; cross-trained individuals with little microbiology training or experience will find this work difficult and subject to error, and this will cause severe risk management issues for the laboratory. It has been well documented that automated hematology instrumentation lacks the sensitivity to diagnose malaria infections, particularly since most patients seen in an emergency room have a very low parasitemia. However, even a low parasitemia can be life-threatening in an infection with *Plasmodium falciparum*.

## **Outpatient or Referral Setting**

Diagnostic laboratories outside of the hospital setting are very appropriate settings for this type of diagnostic testing; the test requests, for the most part, are routine and are "batch" tested rather than tested singly. With very few exceptions, STAT requests are not relevant and are not sent to such laboratory locations; therefore, immediate testing and reporting are not required.

## **Decentralized Testing**

Point-of-care testing within the hospital (ward laboratories, intensive care units, emergency rooms, and bedside) is usually not considered appropriate for diagnostic parasitology testing; one exception might be the emergency room, where patients with malaria may first present with fever and general malaise. Alternative sites (outpatient clinics, shopping malls, senior citizen groups and others) are generally not considered appropriate settings for diagnostic parasitology testing, although relevance might be dictated by geographic location and the development of newer, less subjective methods.

## **Physician Office Laboratories**

As mentioned above, the majority of physician office laboratories are not involved in diagnostic parasitology testing; however, as more molecular biology-based (nonmicroscopic) methods are developed, they may become more widely used in this setting. One example would be the fecal immunoassay rapid-lateral-flow cartridges, specifically designed to detect antigen of *Cryptosporidium* spp., *Giardia lamblia*, the *Entamoeba histolytica*/ *E. dispar* group, or *Entamoeba histolytica*. Rapid tests for the detection of *Plasmodium* spp., particularly *P. falciparum*, are also currently available.

## **Over-the-Counter (Home Care) Testing**

Currently no diagnostic tests for medical parasitology are available for this potential market. However, outside of the United States, some of these options are more likely to be available.

## **Field Sites**

Field sites are very relevant for diagnostic parasitology testing, particularly in many areas of the world where instrumentation and automation are not routinely found within clinical laboratories. As the methodology becomes less expensive and easier to use and interpret, testing sites outside of the routine laboratory may become more relevant, particularly when associated with epidemiologic studies.

## **What Factors Should Precipitate Testing?**

### **Travel and Residence History**

Although travel history is generally considered in terms of weeks and/or months, a number of parasitic infections involve potential exposure many

years earlier. The patient may become symptomatic years after having left the area of endemic infection. Therefore, it is important to consider long-range history, as well as the previous few weeks or months. This is particularly true when considering various places the patient may have lived prior to becoming symptomatic. The more information the laboratory has regarding past organism exposure, the more likely it is that the causative agent will be identified and the infection confirmed. **It is often imperative that the laboratory follow up with specific questions for the physician; information received with the test request may be minimal, at best.**

### **Immune Status of the Patient**

Certain parasites can cause severe illness in debilitated patients and should be considered when these patients present with relevant symptoms. Infections that may cause few to no symptoms in an immunocompetent host may cause prolonged illness in an immunocompromised patient. Unfortunately, this information is not always readily available. Client education can help to increase awareness of possible infections with human parasites.

### **Clinical Symptoms**

When infectious diseases are suspected, there are a number of possible etiologic agents. When a patient presents with gastrointestinal symptoms, it is difficult to tell whether the cause is infectious and, if so, which microbe might be responsible. These symptoms often have many non-infectious causes, so laboratory findings can be extremely valuable in confirming a suspected infectious organism. The same diagnostic procedures can also be used to rule out specific etiologic agents.

### **Documented Previous Infection**

Many parasitic infections are extremely difficult to cure or may not cause symptoms on a continual basis; past exposure or prior documented disease will provide valuable information to the laboratory. Knowing that the patient may be experiencing a relapse can guide the laboratory in detecting the suspected organism.

### **Contact with Infected Individuals**

In situations where multiple reports of symptomatic patients are confirmed, contacts with these infected patients should be tested, particularly during a potential outbreak. An example of this type of situation would be outbreaks of diarrhea in the nursery school setting. When *Cryptosporidium* is identified as the causative agent, all nursery school attendees, employees of the school, and family members are often tested for infection. Another example might involve a group of individuals who experience the same symptoms at similar times after attending a function where food was served; the causative agent might be confirmed as *Cyclospora cayetanensis*.

## Potential Outbreak Testing

In potential outbreak situations, laboratories that perform certain tests on request only may revise their protocol and begin to test all specimens for a particular suspected parasite. A potential *Cryptosporidium* outbreak might require a change from testing on request only to screening all fecal specimens submitted for parasite testing for this particular organism. Often this occurs after consultation among various groups such as health care providers, public health personnel, water company personnel, and pharmacy purchasing agents (who may report an increase in the purchase of antidiarrheal medication).

## Occupational Testing

The most common example of occupational testing would involve food handlers and routine testing for intestinal parasites. This practice is less common than in the past, probably due to financial constraints. Each city, county, and state has specific regulations and/or recommendations.

## Therapeutic Failure

With few exceptions, patients are generally retested after therapy to confirm therapeutic efficacy. If testing reveals the infection has not been eradicated, there may be several reasons. In some cases, the patient may not have taken the medication correctly or may have failed to take the number of recommended doses; these reasons are more likely than the presence of a drug-resistant organism. However, there are certainly examples where drug resistance is possible and is the more likely reason, often depending on the geographic area involved. Another reason might involve the timing between therapy and posttreatment checks for cure; if the time lag is extended, the patient's infection may also represent reinfection. This is particularly true if the patient's living conditions, site, and other epidemiological considerations are not modified.

## What Testing Should Be Performed?

### Routine Tests

"Routine" can imply a widely used, well-understood laboratory test; it can also imply a low or moderately complex method, rather than a high complexity procedure. Routine diagnostic parasitology procedures could include the ova and parasite examination (O&P exam), preparation and examination of blood films and pinworm tapes/paddles, occult blood tests, and examination of specimens from other body sites (urine, sputum, duodenal aspirates, urogenital specimens, etc.).

The selection and use of routine test procedures often depend on a number of factors including geographic area, population served, overall positivity rate, client preference, number of test orders, staffing, personnel experience, turnaround time requirements, epidemiology considerations, clinical relevance of test results, and cost. Routine tests generally have a wide range in both sensitivity and specificity. As an example, the O&P

exam (direct wet mount, concentration, permanent stained smear) could be considered a routine test method for the detection of a number of different intestinal protozoa and helminth infections; this procedure is moderately sensitive but relatively nonspecific. Monoclonal antibody-based test methods tend to be very specific (generally for a single organism such as *Giardia lamblia*) and more sensitive than the routine O&P exam for specific intestinal protozoa. However, the test results are limited in scope; either the organism is present or it's not, and none of the other possible etiologic agents have been ruled in or out!

Diagnostic laboratories generally offer tests "on request"; an example would be testing for the presence of *Cryptosporidium* spp. However, if a potential waterborne outbreak was suspected, this laboratory might change its approach and begin testing all stool specimens submitted for an O&P exam rather than testing only specimens accompanied by a specific test request for *Cryptosporidium* spp. These decisions require close communication with other entities, as described above for potential outbreak testing.

### **Special Testing**

Special procedures, such as parasite culture, are often performed in limited numbers of laboratories. These procedures require the maintenance of positive control cultures used for quality control checks on all patient specimens; they also require special expertise and time. Many clinical laboratories do not meet these requirements. Although some standardized reagents are now commercially available, many clinical laboratories choose to send their requests for serologic testing for parasitic diseases to other laboratories. Often, the Centers for Disease Control and Prevention (CDC) perform serologic testing on specimens submitted to each state's Department of Public Health. Generally, specimens for parasitic serologic testing are not submitted directly to CDC but instead are submitted through state public health laboratories. In an emergency situation, consultation with your county or state public health laboratory may allow shipment of a specimen directly to CDC.

### **Other (Nonmicrobiological) Testing**

Test results from other procedures performed in a clinical laboratory can be very helpful when trying to diagnose a parasitic infection. Specific examples might be a routine urinalysis, hematology procedures including a complete blood count, or various chemistry profiles. These results often provide supporting data consistent with a suspect parasitic infection.

## **What Factors Should Be Considered When Developing Test Menus?**

### **Physical Plant**

Provided that equipment requirements are met, most clinical laboratory space designed for microbiology procedures can be used for diagnostic parasitology testing. In smaller facilities, this work can be incorporated

into a routine microbiology laboratory. Another consideration is the physical location of the laboratory with respect to the source of clinical specimens. If distance is a consideration, the use of appropriate specimen preservatives must be incorporated into patient specimen collection protocols to ensure that accurate laboratory results will be obtained.

## **Client Base**

Recognition and identification of groups of clients served may dictate the methodology and range of diagnostic testing available. Requests for testing for parasites may be minimal within a hospital setting where many procedures are related to elective surgery. In contrast, test requests originating from a large medical center with extensive outpatient clinics may require a broader range of testing and expertise.

## **Customer Requirements and Perceived Levels of Service**

Depending on the client base, patient complexity, history of test requests, and physician interests, the laboratory may be required to provide minimal testing that includes the most commonly performed parasitology procedures. This type of laboratory would generally not be considered a consultative resource; it would need to identify a consultative laboratory to assist with more unusual tests and/or test interpretations. The range and complexity of available tests would also depend on the laboratory's definition of its role in the local, regional, national, or international health care arenas.

## **Personnel Availability and Level of Expertise**

Most procedures performed in the diagnostic parasitology laboratory require extensive microscopy training and experience. They are categorized as high-complexity tests by CLIA '88 and are frequently performed by licensed technologists. Based on microscopy examinations, these procedures require a great deal of interpretation. Although cross-training provides some help with certain procedures, including specimen processing, the necessary interpretive skills are not learned in a week or two and can be easily lost without practice. For this reason, it is important to have a minimum of one or two people who are not only skilled at performing the procedures but also capable of interpreting the findings and providing client training and consultation.

## **Equipment**

The level of equipment required for diagnostic parasitology work is very minimal; however, the one expense that should not be limited would be for one or more microscopes with good optics. Each microscope should be equipped with high-quality (flat-field) objectives ( $10\times$ ,  $40\times$ ,  $50\times$ , or  $60\times$ , and  $100\times$  oil immersion objectives). The oculars should be a minimum of  $10\times$ .

Depending on the range of immunoassay testing available, a fluorescence microscope or enzyme immunoassay reader might be desirable. The availability of this equipment varies tremendously from one laboratory

to another, and the equipment may be shared with other groups within the laboratory.

Another option would be a fume hood, in which the staining could be performed; this is not required, but recommended, particularly if the laboratory is still using xylene for dehydration of permanent stained fecal smears.

The rest of the equipment is quite common and can be shared with other areas within the laboratory. Equipment would include refrigerators, freezers, and pipette systems. Often, these can also be shared.

### Budget

In general, approximately 70% of a microbiology laboratory budget is related to personnel costs. Although diagnostic procedures in the parasitology area are labor-intensive and may require a microscope with good optics, in general budget costs are minimal. Costs tend to increase if the newer immunoassay procedure kits are brought into the laboratory; however, these increased supply costs may be balanced out by diminished labor costs. Each laboratory will have to decide which procedures to offer, which tests can be performed in a batch mode, how many procedures will be ordered per month, what length of turnaround time is required, whether STAT testing is possible, and what options exist for referral laboratories, as well as taking educational initiatives and client preferences into consideration.

Although diagnostic parasitology can be an important part of the microbiology laboratory, it is just one section within the total laboratory context and should be analyzed as such for cost containment and clinical relevance.

## Risk Management Issues Associated with STAT Testing

There are two circumstances in diagnostic medical parasitology that represent true STATs. One is a suspected case of primary amebic meningoencephalitis (PAM) caused by *Naegleria fowleri* or granulomatous amebic encephalitis (GAE) caused by *Acanthamoeba* spp., *Balamuthia mandrillaris*, or *Sappinia diploidea*, and the other situation is any case where thick and thin blood films are requested for testing for blood parasites, possibly those that cause malaria. Extensive discussions of these organisms can be found in the following reference (L. S. Garcia, *Diagnostic Medical Parasitology*, 5th ed., ASM Press, Washington, DC, 2007).

### Primary Amebic Meningoencephalitis

Amebic meningoencephalitis caused by *N. fowleri* is an acute, suppurative infection of the brain and meninges. With extremely rare exceptions, the disease is rapidly fatal in humans. The period between contact with the organism and onset of clinical symptoms such as fever, headache, and rhinitis may vary from 2 to 3 days to as long as 7 to 15 days.

The amebae may enter the nasal cavity by inhalation or aspiration of water, dust, or aerosols containing the trophozoites or cysts. The organisms then penetrate the nasal mucosa, probably through phagocytosis

of the olfactory epithelium cells, and migrate via the olfactory nerves to the brain. Data suggest that *N. fowleri* directly ingests brain tissue by producing food cups or amebostomes, in addition to producing a contact-dependent cytolysis which is mediated by a heat-stable hemolytic protein, heat-labile cytolysis, and/or phospholipase enzymes. Cysts of *N. fowleri* are generally not seen in brain tissue.

Early symptoms include vague upper respiratory distress, headache, lethargy, and occasionally olfactory problems. The acute phase includes sore throat, stuffy blocked or discharging nose, and severe headache. Progressive symptoms include pyrexia, vomiting, and stiffness of the neck. Mental confusion and coma usually occur approximately 3 to 5 days prior to death. The cause of death is usually cardiorespiratory arrest and pulmonary edema.

PAM can resemble acute purulent bacterial meningitis, and these conditions may be difficult to differentiate, particularly in the early stages. The CSF may have a predominantly polymorphonuclear leukocytosis, increased protein concentration, and decreased glucose concentration like that seen with bacterial meningitis. Unfortunately, if the CSF Gram stain is interpreted incorrectly (identification of bacteria as a false positive), the resulting antibacterial therapy has no impact on the amebae and the patient usually dies within several days.

Extensive tissue damage occurs along the path of amebic invasion; the nasopharyngeal mucosa shows ulceration, and the olfactory nerves are inflamed and necrotic. Hemorrhagic necrosis is concentrated in the region of the olfactory bulbs and the base of the brain. Organisms can be found in the meninges, perivascular spaces, and sanguinopurulent exudates.

Clinical and laboratory data usually cannot be used to differentiate pyogenic meningitis from PAM, so the diagnosis may have to be reached by a process of elimination. A high index of suspicion is often mandatory for early diagnosis. All aspects of diagnostic testing (specimen collection, processing, examination, and reporting) should be considered STAT. Although most cases are associated with exposure to contaminated water through swimming or bathing, this is not always the case. The rapidly fatal course of 3 to 6 days after the beginning of symptoms (with an incubation period of 1 day to 2 weeks) requires early diagnosis and immediate chemotherapy if the patient is to survive.

Analysis of the CSF shows decreased glucose and increased protein concentrations. Leukocyte counts may range from several hundred to  $>20,000$  cells per  $\text{mm}^3$ . Gram stains and bacterial cultures of CSF are negative; however, the Gram stain background can incorrectly be identified as bacteria, thus leading to incorrect therapy for the patient.

A definitive diagnosis could be made by demonstration of the amebae in the CSF or in biopsy specimens. Either CSF or sedimented CSF should be placed on a slide under a coverslip and observed for motile trophozoites; smears can also be stained with Wright's or Giemsa stain. CSF, exudate, or tissue fragments can be examined by light microscopy or phase-contrast microscopy. Care must be taken not to mistake leukocytes for actual organisms or vice versa. It is very easy to confuse leukocytes and amebae, particularly when one is examining CSF by using a counting

chamber, hence the recommendation to use just a regular slide and coverslip. Motility may vary, so the main differential characteristic is the spherical nucleus with a large karyosome.

Specimens should never be refrigerated prior to examination. When centrifuging the CSF, low speeds ( $250 \times g$ ) should be used so that the trophozoites are not damaged. Although bright-field microscopy with reduced light is acceptable, phase microscopy, if available, is recommended. Use of smears stained with Giemsa or Wright's stain or a Giemsa-Wright's stain combination can also be helpful. If *N. fowleri* is the causative agent, only trophozoites are normally seen. If the infecting organism is *Acanthamoeba* spp., cysts may also be seen in specimens from individuals with central nervous system (CNS) infection. Unfortunately, most cases are diagnosed at autopsy; confirmation of these tissue findings must include culture and/or special staining with monoclonal reagents in indirect fluorescent-antibody procedures. Organisms can also be cultured on non-nutritive agar plated with *Escherichia coli*.

In cases of presumptive pyogenic meningitis in which no bacteria are identified in the CSF, the computed tomography appearance of basal arachnoiditis (obliteration of basal cisterns in the precontrast scan with marked enhancement after the administration of intravenous contrast medium) should alert the staff to the possibility of acute PAM.

The amebae can be identified in histologic preparations by indirect immunofluorescence and immunoperoxidase techniques. The organism in tissue sections looks very much like an *Iodamoeba bütschlii* trophozoite, with a very large karyosome and no peripheral nuclear chromatin; the organisms can also be seen with routine histologic stains.

### **Granulomatous Amebic Encephalitis and Amebic Keratitis**

The most characteristic feature of *Acanthamoeba* spp. is the presence of spine-like pseudopods called acanthapodia. Several species of *Acanthamoeba* (*A. culbertsoni*, *A. castellanii*, *A. polyphaga*, *A. astronyxis*, *A. healyi*, and *A. divionensis*) cause GAE, primarily in immunosuppressed, chronically ill, or otherwise debilitated persons. These patients tend to have no relevant history involving exposure to recreational freshwater. *Acanthamoeba* spp. also cause amebic keratitis, and it is estimated that to date approximately 1,000 cases of *Acanthamoeba* keratitis have been seen in the United States.

GAE caused by freshwater amebae is less well defined and may occur as a subacute or chronic disease with focal granulomatous lesions in the brain. The route of CNS invasion is thought to be hematogenous, with the primary site being the skin or lungs. In this infection, both trophozoites and cysts can be found in the CNS lesions. An acute-onset case of fever, headache, and pain in the neck preceded by 2 days of lethargy has also been documented. The causative organisms are probably *Acanthamoeba* spp. in most cases, but it is possible that others are involved such as *Balamuthia mandrillaris* and *Sappinia diploidea*.

Cases of GAE have been found in chronically ill or immunologically impaired hosts; however, some patients apparently have no definite predisposing factor or immunodeficiency. Conditions associated with GAE

include malignancies, systemic lupus erythematosus, human immunodeficiency virus (HIV) infection, Hodgkin's disease, skin ulcers, liver disease, pneumonitis, diabetes mellitus, renal failure, rhinitis, pharyngitis, and tuberculosis. Predisposing factors include alcoholism, drug abuse, steroid treatment, pregnancy, systemic lupus erythematosus, hematologic disorders, AIDS, cancer chemotherapy, radiation therapy, and organ transplantation. This infection has become more widely recognized in AIDS patients, particularly those with a low CD4<sup>+</sup> cell count.

Laboratory examinations similar to that for *N. fowleri* can be used to recover and identify these organisms; the one exception is recovery by culture, which has not proven to be as effective with GAE patients infected with *B. mandrillaris*.

### **Request for Blood Films**

Malaria is one of the few parasitic infections considered to be immediately life-threatening, and a patient with the diagnosis of *P. falciparum* malaria should be considered a medical emergency because the disease can be rapidly fatal. Any laboratory providing the expertise to identify malarial parasites should do so on a STAT basis (24 h/day, 7 days/week).

Patients with malaria can present for diagnostic blood work when they are least expected. Laboratory personnel and clinicians should be aware of the STAT nature of such requests and the importance of obtaining some specific patient history information. On microscopic examination of the blood films, the typical textbook presentation of various *Plasmodium* morphologies may not be seen by the technologist. The smears should be examined at length and under oil immersion. The most important thing to remember is that even though a low parasitemia may be present on the blood smears, the patient may still be faced with a serious, life-threatening disease.

It is important for both physicians and laboratorians within areas where malaria is not endemic to be aware of the problems associated with malarial diagnosis and to remember that symptoms are often non-specific and may mimic other medical conditions. Physicians must recognize that travelers are susceptible to malarial infection when they visit a country where malaria is endemic, and that they should receive prophylactic medication.

With the tremendous increase in the number of people traveling from the tropics to malaria-free areas, the number of imported malaria cases is also on the rise. There have been reports of imported infected mosquitoes transmitting the infection among people who live or work near international airports. It is also possible that mosquitoes can reach areas far removed from the airports. This situation has been termed "airport malaria," malaria that is acquired through the bite of an infected anopheline mosquito by persons with apparently no risk factors for the disease. Unfortunately, unless a careful history is obtained, the diagnosis of malaria can be missed or delayed. Tests to exclude malaria should be considered for patients who work or live near an international airport and who present with an acute febrile illness. The potential danger of

disseminating the mosquito vectors of malaria via aircraft is well recognized; however, modern disinfection procedures have not yet eliminated the risk of vector transportation. Not only can insects survive nonpressurized air travel, but also they may be transported further by car or other means after arriving at the airport.

We usually associate malaria with patients having a history of travel within an area where malaria is endemic. However, other situations that may result in infection involve the receipt of blood transfusions, use of hypodermic needles contaminated by prior use (for example, by drug addicts), possibly congenital infection, and transmission within the United States by indigenous mosquitoes that acquired the parasites from imported infections.

Malaria is usually associated with patients having a history of travel within an area where malaria is endemic, although other routes of infection are well documented. Frequently, for a number of different reasons, organism recovery and subsequent identification are more difficult than the textbooks imply. It is very important that this fact be recognized, particularly when one is dealing with a possibly fatal infection with *P. falciparum*. It is important to ensure that clinicians are familiar with the following issues.

### **Automated Instrumentation**

Potential diagnostic problems with the use of automated differential instruments have been reported. Some cases of malaria, as well as *Babesia* infection, have been completely missed by these methods. The number of fields scanned by a technologist on instrument-read smears is quite small; thus, failure to detect a low parasitemia is almost guaranteed. In cases of malaria and *Babesia* infection, after diagnosis had been made on the basis of smears submitted to the parasitology division of the laboratory, all previous smears examined by the automated system were reviewed and found to be positive for parasites. Failure to make the diagnosis resulted in delayed therapy. These instruments are not designed to detect intracellular blood parasites, and the inability of the automated systems to discriminate between uninfected erythrocytes and those infected with parasites may pose serious diagnostic problems in situations where the parasitemia is  $\leq 0.5\%$ .

### **Patient Information**

When requests for malarial smears are received in the laboratory, some patient history information should be made available to the laboratorian. This history can be obtained by asking the ordering physician important questions such as the following:

1. Where has the patient been, and what was the date of return to the United States? (Where do you live and where do you work? ["airport malaria"])
2. Has malaria ever been diagnosed in the patient before? If so, which species was identified?

3. What medication (prophylaxis or otherwise) has the patient received, and how often? When was the last dose taken?
4. Has the patient ever received a blood transfusion? Is there a possibility of other needle transmission (drug user)?
5. When was the blood specimen drawn, and was the patient symptomatic at the time?
6. Is there any evidence of a fever periodicity?

Answers to such questions may help eliminate the possibility of infection with *P. falciparum* or *P. vivax*, usually the only two species that can cause severe disease and, in the case of *P. falciparum*, can rapidly lead to death.

### **Conventional Microscopy**

Often, when the diagnosis of malaria is considered, only a single blood specimen is submitted to the laboratory for examination; however, **single films or specimens cannot be relied upon to exclude the diagnosis**, especially when partial prophylactic medication or therapy is used. Partial use of antimalarial agents may be responsible for reducing the numbers of organisms in the peripheral blood and lead to a blood smear that contains few organisms and a conclusion that reflects a low parasitemia when in fact serious disease is present. Patients with a relapsing case or an early primary case can also have few organisms in the blood smear. It is recommended that both thick and thin blood films be prepared immediately, and at least 300 oil immersion fields should be examined on both films before a negative report is issued. Since one set of negative smears does not rule out malaria, additional blood specimens should be examined over a 36-h period. **Although Giemsa stain has been recommended for all parasitic blood work, the organisms can also be seen if other blood stains, such as Wright's stain or any of the rapid blood stains, are used.** Blood collected with the use of EDTA anticoagulant is preferred over heparin; however, if the blood remains in the tube for approximately an hour or more, true stippling might not be visible within the infected erythrocytes (e.g., those infected with *P. vivax*). Using EDTA, if blood is held for more than 2 h prior to blood film preparation, several artifacts may be seen; after 4 to 6 h, some of the parasites will be lost. During the time when the parasites are in the tube of blood, they continue to grow and change according to the life cycle for that species. Also, when using anticoagulants, it is important to remember that the proper ratio between blood and anticoagulant is necessary for good organism morphology—fill the tube with blood. Both thick and thin blood films should be prepared immediately after receipt of the blood. If the specimen is sent to a reference laboratory, both the thick and thin blood films, as well as the tube of blood (room temperature), should be sent. Since this test is always considered a STAT request, it is also important to know what turnaround times are available from the reference laboratory.

**All requests for malaria diagnosis are considered STAT requests, and specimens should be collected, processed, examined, and reported accordingly.** Although other diagnostic tests can be ordered, any request

for examination of blood films should include a possible diagnosis of malaria; thus, these requests are always considered STAT. Not only should the blood collection be considered STAT, but also the processing and examination of both thick and thin blood films should be performed immediately on receipt of the blood. Often immunologically naive individuals with no prior exposure to malaria can present to the emergency room or clinic with symptoms such as fever and malaise and a relevant travel history to an area of the world where malaria is endemic. These patients can have very vague symptoms, but they have the potential to become very ill with malaria, even with a low parasitemia (0.0005% to 0.1%).

S E C T I O N   2

Parasite Classification and  
Relevant Body Sites

Although common names are often used to describe parasites and parasitic infections, these names may refer to different parasites in different parts of the world. To eliminate these problems, a binomial system of nomenclature is used in which the scientific name consists of the genus and species.

Based on life cycles and organism morphology, classification systems have been developed to indicate the relationship among the various parasite species. Closely related species are placed in the same genus, related genera are placed in the same family, related families are placed in the same order, related orders are placed in the same class, and related classes are placed in the same phylum, one of the major categories in the animal kingdom. As one moves up the classification schema, each category becomes more broad; however, each category still has characteristics in common.

Parasites of humans are classified in six major divisions (Table 2.1). These include the Protozoa (amebae, flagellates, ciliates, sporozoans, coccidia, and microsporidia), the Nematoda or roundworms, the Platyhelminthes or flatworms (cestodes, trematodes), the Pentastomids or tongue worms, the Acanthocephala or thorny-headed worms, and the Arthropoda (insects, spiders, mites, ticks, and so on). Although these categories appear to be clearly defined, there may be confusion in attempting to classify parasites, often due to the lack of known specimens. If organisms recovered from humans are very rare, it is difficult to determine their correct taxonomic positions. Type specimens must be deposited for study before a legitimate species name can be given. Also, even when certain parasites are numerous, they may represent strains or races of the same species with slightly different characteristics.

Reproductive mechanisms have been used as a basis for determining species definitions, but there are many exceptions within parasite groups. Another difficulty in species recognition is the ability and tendency of the organisms to alter their morphologic forms according to age, host, or nutrition, which often results in several names for the same organism. An additional problem involves alternation of parasitic and free-living phases in the life cycle; these organisms may be very different and difficult to recognize as belonging to the same species. However, newer molecular methods of grouping organisms have often confirmed taxonomic conclusions reached hundreds of years earlier by experienced taxonomists. As studies in parasitic genetics, immunology, and biochemistry continue, the species designation will be defined more clearly by highly sophisticated molecular techniques.

No attempt has been made to include every possible organism; only those that are considered to be clinically relevant in the context of human parasitology are addressed. Some human infections are represented by very few cases; however, they are well documented and are included here. Further information is provided in the tables at the end of this section.

## Protozoa (Intestinal)

### Amebae

Amebae are single-celled organisms characterized by having pseudopods (motility) and trophozoite and cyst stages in the life cycle. The cell's

organelles and cytoplasm are enclosed by a cell membrane, such that the cell obtains its food through phagocytosis. However, there are some exceptions in which a cyst form has not been identified. In environments which are potentially lethal to the cell, an ameba may become dormant by surrounding itself with a protective membrane to become a cyst. The cell remains in this form until it encounters more favorable conditions, at which time the organism excysts to release trophozoites. While in the cyst form, amebae do not replicate and may die if unable to excyst for a lengthy period.

Amebae are usually acquired by humans via fecal-oral transmission or mouth-to-mouth contact (*Entamoeba gingivalis*). In most species, after several nuclear divisions occur, comparable division of the cytoplasm follows excystation. *Entamoeba histolytica* is the most significant organism within this group.

Although *Blastocystis hominis* is an enteric protozoan parasite that is commonly found worldwide, the classification is undergoing review. *B. hominis* has extensive genetic diversity and infects humans and many other animals. Statistically, it may be the most common intestinal parasite recovered. Some of the subtypes are considered to be pathogens while others are probably nonpathogenic, a situation that leads to different opinions regarding pathogenicity. Unfortunately, these subtypes cannot be differentiated on the basis of microscopic morphology. Since there may be a relationship between numbers present and symptoms, this is one of the few parasites whose numbers should be specified in the report (rare, few, moderate, many, packed). It is recommended that quantitation be determined from the permanent stained smear.

Representative organisms include *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba coli*, *Entamoeba hartmanni*, *Entamoeba gingivalis*, *Endolimax nana*, *Iodamoeba bütschlii*, and *Blastocystis hominis*.

## Flagellates

Flagellates move by means of flagella and are acquired through fecal-oral transmission. With the exception of *Dientamoeba fragilis* (internal flagella) and organisms in the genus *Pentatrichomonas*, flagellates have both trophozoite and cyst stages in the life cycle. Reproduction is by longitudinal binary fission. *Giardia lamblia* is the most common pathogen in this group and is one of the most commonly found intestinal parasites. However, when permanent stained smears are routinely performed, *D. fragilis* is also found to be more common than suspected. Representative organisms include *G. lamblia*, *D. fragilis*, *Pentatrichomonas hominis*, *Chilomastix mesnili*, *Enteromonas hominis*, and *Retortamonas intestinalis*.

## Ciliates

Ciliates are single-celled protozoa that move by means of cilia and are acquired through fecal-oral transmission. *Balantidium coli* is the only human pathogen in the group. Hosts include pigs, wild boars, rats, primates (including humans), horses, cattle, and guinea pigs. Infection is transmitted within or between these species by fecal-oral transmission of the infective cysts. Pigs are the most significant reservoir hosts, although they

show few if any symptoms. Following ingestion, excystation occurs in the small intestine, and the trophozoites colonize the large intestine. The cilia beat in a coordinated rhythmic pattern, and the trophozoite moves in a spiral path. They have both trophozoite and cyst stages in the life cycle, and both stages contain a large macronucleus and a smaller micronucleus. These protozoa have a distinct cell mouth (cytostome), cytopharynx, and less conspicuous cytophyge (anal pore). These organisms are considerably larger than the majority of the intestinal protozoa and can be mistaken for debris or junk when seen in a permanent stained smear. The concentration wet preparation examination is recommended.

### Coccidia

Coccidia are microscopic, spore-forming, single-celled, obligate intracellular parasites, which means that they must live and reproduce within an animal cell. These protozoa are acquired by ingestion of various meats or through fecal-oral transmission via contaminated food and/or water. In some cases, coccidia disseminate to other body sites, particularly in the severely compromised patient. These protozoa have both asexual and sexual cycles, the most common infective stage being the oocyst, containing sporocysts and/or sporozoites, all of which can be acquired through fecal-oral transmission. Representatives within this group include *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Isospora belli*, and *Sarcocystis* spp.

### Microsporidia

Currently, the most difficult intestinal protozoa to diagnose are the microsporidia (size range, 1 to 2.5  $\mu\text{m}$ ); the development of molecular biology-based methods should provide more specific and sensitive methods. These organisms have also been documented to disseminate from the intestinal tract to other body sites, including the kidneys and lungs. Routine parasitology stains are not useful; modified trichrome stains have been developed specifically for these organisms. Compared with the more common Wheatley's trichrome for routine stool staining, the modified trichrome stain contains a 10-fold higher concentration of the main dye, chromotrope 2R. The infective form is called the spore; each spore contains a polar tubule that is used to penetrate new host cells, thus initiating or continuing the life cycle. Infections are acquired through ingestion, inhalation, or direct inoculation of spores from the environment. Currently at least two genera have been documented to cause human infection in the intestinal tract (*Encephalitozoon intestinalis* and *Enterocytozoon bieneusi*).

## Protozoa (Other Body Sites)

### Amebae

With the exception of *Entamoeba gingivalis* (found in the mouth), non-intestinal amebae are pathogenic, free-living organisms that may be associated with warm, freshwater environments. They have been found in

the central nervous system, the eyes, and other body sites. Amebae that invade the central nervous system (*Naegleria fowleri*) can cause severe, life-threatening infection that often ends in death within a few days. Other amebae in this group can cause more chronic central nervous system disease (*Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Sappinia diploidea*, particularly in the immunocompromised patient). *Acanthamoeba* can also cause keratitis; untreated cases can result in blindness.

## Flagellates

*Trichomonas vaginalis* is found in the genitourinary system and is usually acquired by sexual transmission. *Trichomonas tenax* can be found in the mouth and is considered to be nonpathogenic.

## Coccidia

Coccidian parasites are particularly important in the compromised patient and can cause life-threatening disease. These organisms can disseminate from the intestinal tract to other body sites. They may also infect many individuals who have relatively few symptoms. In the immunocompetent patient, symptoms may be minimal or absent; however, in the compromised patient sequelae may be very serious and even life-threatening.

## Microsporidia

As mentioned above, microsporidia are the most difficult protozoa to diagnose (size range, 1 to 2.5  $\mu\text{m}$ ). Dissemination from the intestine to other body sites has been well documented. Modified trichrome stains have been developed specifically for detection of these organisms, since routine parasitology stains for fecal specimens are not very effective for microsporidial spores. Optical brightening agents such as Calcofluor are also recommended; although they are very sensitive, they are nonspecific. Currently, at least 14 species have been identified as human parasites. Genera include *Brachiola*, *Pleistophora*, *Trachipleistophora*, *Vittaforma*, *Microsporidium*, *Nosema*, *Encephalitozoon*, and *Enterocytozoon*.

## Protozoa (Blood and Tissue)

### Sporozoa

All sporozoa are arthropod borne. The genus *Plasmodium* includes parasites that undergo exoerythrocytic and pigment-producing erythrocytic schizogony in vertebrates and a sexual stage followed by sporogony in mosquitoes. *Babesia* spp. are tick borne and can cause severe disease in patients who have been splenectomized or otherwise immunologically compromised. Diagnosis may be somewhat more difficult than for the intestinal protozoa, particularly if automated blood differential systems are used; the microscopic examination of both thick and thin blood films is recommended. Note: examination of blood for these parasites is considered a STAT test request. Representatives in the genus *Plasmodium* include *P. vivax*, *P. ovale*, *P. malariae*, *P. falciparum*, and *P. knowlesi*. Or-

ganisms in the genus *Babesia* include *B. microti*, *B. divergens*, and additional organisms from the West Coast of the United States that are not yet classified to the species level.

## Flagellates

### **Leishmaniae**

The leishmaniae have undergone extensive classification revisions. However, from a clinical perspective, recovery and identification of the organisms are still related to body sites such as the macrophages of the skin (cutaneous), the skin and mucous membranes (mucocutaneous), and the whole reticuloendothelial system (visceral—bone marrow, spleen, liver). Recovery of leishmanial amastigotes is limited to the site of the lesion in infections other than those caused by the *Leishmania donovani* complex (visceral leishmaniasis). These protozoa have both amastigote (mammalian host) and promastigote (sand fly) stages in the life cycle. Reproduction in both forms occurs by binary longitudinal division. Their primary hosts are vertebrates; *Leishmania* commonly infects hyraxes, canids, rodents, and humans. Representative organisms include *L. tropica*, *L. major*, *L. mexicana*, *L. braziliensis*, and *L. donovani*.

### **Trypanosomes**

The trypanosomes are normally identified to the species level based on geographic exposure history and clinical symptoms. These protozoa are characterized by having, at some time in the life cycle, the trypomastigote form with the typical undulating membrane and free flagellum at the anterior end. Unfortunately, the longer the duration of the infection, the more difficult it may be to confirm the diagnosis. The organisms that cause African sleeping sickness (*T. b. gambiense* and *T. b. rhodesiense*) generally cause different disease entities; one tends to be chronic and more typically the patient appears to have sleeping sickness (*T. b. gambiense*), and the other causes a more fulminant disease often leading to death before typical sleeping sickness symptoms can develop (*T. b. rhodesiense*).

The etiologic agent of American trypanosomiasis (formerly South American trypanosomiasis) is *T. cruzi*, which contains amastigote and trypomastigote stages (in the mammalian host) and the epimastigote form (in the arthropod host). Human American trypanosomiasis, or Chagas' disease, is a potentially fatal disease of humans. It has two forms, a trypomastigote found in human blood and an amastigote found in tissues. The acute form usually goes unnoticed and may present as a localized swelling at the site of entry of the parasites in the skin. The chronic form may develop 10 to 20 years after infection. This form affects internal organs (e.g., the heart, esophagus, colon, and peripheral nervous system), and patients may die from heart failure. In 2007, the Red Cross began screening donor blood units for antibody to this parasite. Also, the geographic range of *T. cruzi* within the United States continues to expand and now includes all of Texas. Infected triatomid bug vectors are also present in other states such as California.

## **Nematodes (Intestinal)**

The largest number of helminthic parasites of humans belongs to the roundworm group. Nematodes are elongate-cylindrical and bilaterally symmetrical with a triradiate symmetry at the anterior end. They have an outer cuticle layer, no circular muscles, and a pseudocoelom containing all systems (digestive, excretory, nervous, and reproductive). These organisms are normally acquired by ingestion of their eggs or skin penetration by larval forms from the soil. Nematodes commonly parasitic in humans include *Trichuris trichiura*, *Necator americanus*, *Ancylostoma duodenale*, *Enterobius vermicularis*, *Ascaris lumbricoides*, *Strongyloides stercoralis*, and *Trichostongyulus* spp.

## **Nematodes (Tissue)**

Some tissue nematodes are rarely seen within the United States; however, some are more important and are found worldwide. Diagnosis may be difficult if the only specimens are obtained through biopsy and/or autopsy, and interpretation must be based on examination of histologic preparations. Examples include infections caused by *Trichinella* spp., *Toxocara* spp., *Baylisascaris procyonis* (raccoon roundworm), *Ancylostoma* spp., *Angiostrongylus* spp., and *Gnathostoma* spp.

## **Nematodes (Blood and Tissue)**

Blood and tissue nematodes (filarial worms) are arthropod borne. The adult worms tend to live in the tissues or lymphatics of the vertebrate host. Diagnosis is made on the basis of the recovery and identification of the larval worms (microfilariae) in the blood, other body fluids, or skin. While circulating in peripheral blood or cutaneous tissues, the microfilariae can be ingested by blood-sucking insects. After the larvae mature, they can escape into the vertebrate host's skin when the arthropod takes its next blood meal. The severity of disease due to these nematodes varies; however, elephantiasis may be associated with some of the filarial worms. Specific organisms include *Wuchereria bancrofti*, *Brugia* spp., *Loa loa*, *Mansonella* spp., *Onchocerca volvulus*, and *Dirofilaria* spp.

## **Cestodes (Intestinal)**

The adult form of the tapeworm is acquired through ingestion of the larval forms contained in poorly cooked or raw meats or freshwater fish (*Taenia* spp., *Diphyllobothrium* spp.). *Dipylidium caninum* infection is acquired by the accidental ingestion of dog fleas infected with the larval tapeworms. Both *Hymenolepis nana* and *H. diminuta* are transmitted via ingestion of certain infected arthropods (fleas and beetles). Also, *H. nana* can be transmitted through egg ingestion (its life cycle can bypass the intermediate beetle host). The adult tapeworm consists of a chain of egg-producing units called proglottids, which develop from the neck region

of the attachment organ, the scolex. Food is absorbed through the worm's integument. The intermediate host contains the larval forms, which are acquired through ingestion of the adult tapeworm eggs. Humans can serve as both the intermediate and definitive hosts in *H. nana* and *Taenia solium* infections.

## Cestodes (Tissue)

The ingestion of certain tapeworm eggs or accidental contact with certain larval forms can lead to tissue infection with *Taenia solium*, other *Taenia* spp., *Echinococcus* spp., *Diphyllobothrium* spp., and *Spirometra mansonioides*.

## Trematodes (Intestinal)

Trematodes are flatworms and are exclusively parasitic. With the exception of the schistosomes (blood flukes), flukes are hermaphroditic. They may be flattened, and most have oral and ventral suckers. All of the intestinal trematodes require a freshwater snail to serve as an intermediate host; these infections are foodborne (freshwater fish, mollusks, or plants) and are emerging as a major public health problem (more than 40 million people are infected with intestinal and liver/lung trematodes). Specific examples include *Fasciolopsis buski*, *Heterophyes heterophyes*, and *Metagonimus yokogawai*.

## Trematodes (Liver and Lungs)

The hermaphroditic liver and lung trematodes also require a freshwater snail to serve as an intermediate host; these infections are foodborne (acquired by ingestion of freshwater fish, crayfish or crabs, or plants). Public health concerns include cholangiocarcinoma associated with *Clonorchis* and *Opisthorchis* infections, severe liver disease associated with *Fasciola* infections, and the misdiagnosis of tuberculosis in those infected with *Paragonimus* spp.

## Trematodes (Blood)

The sexes of blood trematodes (schistosomes) are separate, and infection is acquired by skin penetration by the cercarial forms that are released from freshwater snails. The males are characterized by having an infolded body that forms the gynecophoral canal in which the female worm is held during copulation and oviposition. The adult worms reside in the blood vessels over the small intestine, large intestine, or bladder. Although these parasites are not endemic within the United States, patients are seen who may have acquired schistosomiasis elsewhere. Schistosomiasis is a chronic disease, and many infections are subclinically symptomatic, with mild anemia and malnutrition being common in areas where the infection is endemic. Acute schistosomiasis (Katayama's fever) may occur weeks after the initial infection, especially infection by *S.*

*mansoni* and *S. japonicum*. Signs may include abdominal pain, cough, diarrhea, high eosinophilia, fever, fatigue, and hepatosplenomegaly. Representative species include *S. mansoni*, *S. japonicum*, and *S. haematobium*.

## Pentastomids

Pentastomids are found in a separate phylum, Pentastomida, and are called tongue worms. Human infections have been reported from Africa, Europe, Asia, and the Americas. When humans serve as the intermediate hosts, the infective larvae die in situ. However, when mature larvae (often encysted) are ingested, they may migrate from the stomach, attach themselves to nasopharyngeal tissues, develop into adult pentastomids, and cause symptoms of the halzoun syndrome. Symptoms include throat discomfort, paroxysmal coughing, sneezing, and occasionally dysphagia and vomiting. Pentastomids isolated from humans include *Armillifer* spp., *Linguatula serrata*, and *Sebekia* spp.

Rare eye infections have been reported; inflammation is minimal, and the infection is probably the result of direct eye contact with water containing pentastomid eggs. Even more rare is human infection caused by a pentastomid larva belonging to the genus *Sebekia*. The adult worms are parasites found in the respiratory tract of reptiles, and the larval forms are found in the viscera, in the muscles, and along the spinal cord of freshwater fish. There may be serpiginous burrows surrounded by an intense erythematous zone and a 30% eosinophilia. The infection was possibly acquired by the ingestion of water containing eggs of the parasites or the ingestion of raw or improperly cooked fish.

Diagnosis is made by identifying the pentastomid in a biopsy specimen or at autopsy.

## Acanthocephala

The Acanthocephala (thorny-headed worms) are closely related to the tapeworms. These worms are diecious and tend to have a retractable proboscis which is usually armed with spines. The larvae require an arthropod intermediate host, and the adult worms are always parasites in the intestine of vertebrates. Two of these organisms are parasitic in humans: *Macracanthorhynchus hirudinaceus* and *Moniliformis moniliformis*. Human infection is acquired from the ingestion of infected insects (various beetles and cockroaches). Very few cases have been reported in the literature; however, symptoms included abdominal pain and tenderness, anorexia, and nausea. In some cases, adult worms have been passed in the stool.

## Suggested Reading

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**Table 2.1** Classification of human parasites

Protozoa	
Amebae (intestinal)	<i>Leishmania (Leishmania) donovani</i>
<i>Entamoeba histolytica</i>	<i>Leishmania (Leishmania) archibaldi</i>
<i>Entamoeba dispar<sup>a</sup></i>	<i>Leishmania (Leishmania) infantum</i>
<i>Entamoeba hartmanni</i>	New World <i>Leishmania</i> species
<i>Entamoeba coli</i>	<i>Leishmania (Leishmania) mexicana</i>
<i>Entamoeba polecki</i>	<i>Leishmania (Leishmania) amazonensis</i>
<i>Endolimax nana</i>	<i>Leishmania (Leishmania) pifanoi</i>
<i>Iodamoeba bütschlii</i>	<i>Leishmania (Leishmania) garnhami</i>
<i>Blastocystis hominis</i>	<i>Leishmania (Leishmania) venezuelensis</i>
Flagellates (intestinal)	<i>Leishmania (Leishmania) chagasi</i>
<i>Giardia lamblia<sup>b</sup></i>	<i>Leishmania (Viannia) braziliensis</i>
<i>Chilomastix mesnili</i>	<i>Leishmania (Viannia) colombiensis</i>
<i>Dientamoeba fragilis</i>	<i>Leishmania (Viannia) guyanensis</i>
<i>Pentatrichomonas hominis</i>	<i>Leishmania (Viannia) lainsoni</i>
<i>Enteromonas hominis</i>	<i>Leishmania (Viannia) naiffi</i>
<i>Retortamonas intestinalis</i>	<i>Leishmania (Viannia) panamensis</i>
Ciliates (intestinal)	<i>Leishmania (Viannia) peruviana</i>
<i>Balantidium coli</i>	<i>Leishmania (Viannia) shawi</i>
Coccidia, microsporidia (intestinal)	Old World <i>Trypanosoma</i> species
<i>Cryptosporidium parvum</i>	<i>Trypanosoma brucei gambiense</i>
<i>Cryptosporidium hominis</i>	<i>Trypanosoma brucei rhodesiense</i>
<i>Cryptosporidium</i> spp.	New World <i>Trypanosoma</i> species
<i>Cyclospora cayetanensis</i>	<i>Trypanosoma cruzi</i>
<i>Isospora belli</i>	<i>Trypanosoma rangeli</i>
<i>Sarcocystis hominis</i>	Amebae, flagellates (other body sites)
<i>Sarcocystis suis hominis</i>	Amebae
<i>Sarcocystis "lindemanni"</i>	<i>Naegleria fowleri</i>
Microsporidia	<i>Acanthamoeba</i> spp.
<i>Enterocytozoon bieneusi</i>	<i>Hartmanella</i> spp.
<i>Encephalitozoon intestinalis</i>	<i>Balamuthia mandrillaris</i>
Sporozoa, flagellates (blood, tissue)	<i>Sappinia diploidea</i>
Sporozoa (malaria and babesiosis)	
<i>Plasmodium vivax</i>	
<i>Plasmodium ovale</i>	
<i>Plasmodium malariae</i>	
<i>Plasmodium falciparum</i>	
<i>Plasmodium knowlesi</i>	
<i>Babesia</i> spp.	
Flagellates (leishmaniae, trypanosomes)	
Old World <i>Leishmania</i> species	
<i>Leishmania (Leishmania) tropica</i>	
<i>Leishmania (Leishmania) major</i>	
<i>Leishmania (Leishmania) aethiopica</i>	

(continued on next page)

**Table 2.1** Classification of human parasites (*continued*)

<i>Entamoeba gingivalis</i>	<i>Toxocara canis</i> , <i>Toxocara cati</i> (ocular larva migrans)
Flagellates	
<i>Trichomonas vaginalis</i>	<i>Ancylostoma braziliense</i> , <i>Ancylostoma caninum</i> (cutaneous larva migrans)
<i>Trichomonas tenax</i>	
Coccidia, sporozoa, microsporidia (other body sites)	<i>Dracunculus medinensis</i>
Coccidia	<i>Angiostrongylus cantonensis</i>
<i>Toxoplasma gondii</i>	<i>Angiostrongylus costaricensis</i>
Microsporidia	<i>Gnathostoma spinigerum</i>
<i>Nosema ocularum</i>	<i>Anisakis</i> spp. (larvae from saltwater fish)
<i>Pleistophora</i> spp.	<i>Phocanema</i> spp. (larvae from saltwater fish)
<i>Pleistophora ronneafiei</i>	<i>Contraecum</i> spp. (larvae from saltwater fish)
<i>Trachipleistophora hominis</i>	<i>Hysterothylacium</i>
<i>Trachipleistophora anthropophthera</i>	<i>Porrocaecum</i> spp.
<i>Brachiola vesicularum</i>	<i>Capillaria hepatica</i>
<i>Brachiola (Nosema) algerae</i>	<i>Thelazia</i> spp.
<i>Brachiola (Nosema) connori</i>	<i>Ternidens diminutus</i>
<i>Encephalitozoon cuniculi</i>	Blood and tissues (filarial worms)
<i>Encephalitozoon intestinalis</i>	<i>Wuchereria bancrofti</i>
<i>Encephalitozoon hellem</i>	<i>Brugia malayi</i>
<i>Enterocytozoon bieneusi</i>	<i>Brugia timori</i>
<i>Vittaforma corneae (Nosema corneum)</i>	<i>Loa loa</i>
<i>Microsporidium</i> <sup>c</sup>	<i>Onchocerca volvulus</i>
<b>Nematodes (roundworms)</b>	<i>Mansonella ozzardi</i>
Intestinal	<i>Mansonella streptocerca</i>
<i>Ascaris lumbricoides</i>	<i>Mansonella perstans</i>
<i>Enterobius vermicularis</i>	<i>Dirofilaria immitis</i> (usually lung lesion; in dogs, heartworm)
<i>Ancylostoma duodenale</i>	<i>Dirofilaria</i> spp. ( <i>D. tenuis</i> , may be found in subcutaneous nodules)
<i>Necator americanus</i>	
<i>Strongyloides stercoralis</i>	
<i>Strongyloides fuelleborni</i>	
<i>Trichostrongylus colubriformis</i>	
<i>Trichostrongylus orientalis</i>	
<i>Trichostrongylus</i> spp.	
<i>Trichuris trichiura</i>	
<i>Capillaria philippinensis</i>	
Tissue	<b>Cestodes (tapeworms)</b>
<i>Trichinella spiralis</i>	Intestinal
<i>Trichinella</i> spp. ( <i>T. britovi</i> , <i>T. murrelli</i> , <i>T. nativa</i> , <i>T. nelsoni</i> , <i>T. papuae</i> , <i>T. pseudospiralis</i> , <i>T. zimbabwensis</i> )	<i>Diphyllibothrium latum</i>
<i>Toxocara canis</i> , <i>Toxocara cati</i> (visceral larva migrans)	<i>Diplogonoporus</i> spp.
	<i>Dipylidium caninum</i>
	<i>Hymenolepis nana</i>
	<i>Hymenolepis diminuta</i>
	<i>Taenia solium</i>
	<i>Taenia saginata</i>
	Tissue (larval forms)
	<i>Taenia solium</i>
	<i>Echinococcus granulosus</i>
	<i>Echinococcus multilocularis</i>

(continued on next page)

**Table 2.1** (continued)

<i>Echinococcus vogeli</i>	<i>Linguatula serrata</i>
<i>Echinococcus oligarthrus</i>	<i>Sebekia</i> spp.
<i>Multiceps multiceps</i>	Nasopharyngeal tissue
<i>Spirometra mansonioides</i>	<i>Armillifer</i> spp.
<i>Diphyllobothrium</i> spp.	<i>Linguatula serrata</i>
<b>Trematodes (flukes)</b>	<b>Acanthocephalans (Thorny-headed worms)</b>
Intestinal	Intestine
<i>Fasciolopsis buski</i>	<i>Macracanthorhynchus hirudinaceus</i>
<i>Echinostoma ilocanum</i>	<i>Moniliformis moniliformis</i>
<i>Echinochasmus perfoliatus</i>	
<i>Heterophyes heterophyes</i>	
<i>Metagonimus yokogawai</i>	
<i>Gastroducoides hominis</i>	
<i>Phaneropsolus bonnei</i>	
<i>Prosthodendrium molenkempi</i>	
<i>Spelotrema brevicaeca</i>	
<i>Plagiochis</i> spp.	Ticks ( <i>Dermacentor</i> , <i>Ixodes</i> , <i>Argas</i> , <i>Ornithodoros</i> )
<i>Neodiplostomum seoulense</i>	Mites ( <i>Sarcoptes</i> )
Liver and lungs	Crustacea
<i>Clonorchis (Opisthorchis) sinensis</i>	Copepods ( <i>Cyclops</i> )
<i>Opisthorchis viverrini</i>	Crayfish, lobsters, crabs
<i>Opisthorchis felineus</i>	Pentastomida (classification under review)
<i>Dicrocoelium dendriticum</i>	Tongue worms
<i>Fasciola hepatica</i>	Diplopoda
<i>Fasciola gigantica</i>	Millipedes
<i>Paragonimus westermani</i>	Chilopoda
<i>Paragonimus mexicanus</i>	Centipedes
<i>Paragonimus kellicotti</i>	Insecta
<i>Paragonimus africanus</i>	Phthiraptera: lice ( <i>Pediculus</i> , <i>Phthirus</i> )
<i>Paragonimus uterobilateralis</i>	Blattaria: cockroaches
<i>Paragonimus miyazakii</i>	Hemiptera: true bugs ( <i>Triatoma</i> )
<i>Paragonimus caliensis</i>	Coleoptera: beetles
Blood	Hymenoptera: bees, wasps, etc.
<i>Schistosoma mansoni</i>	Lepidoptera: butterflies, caterpillars, moths, etc.
<i>Schistosoma haematobium</i>	Diptera: flies, mosquitoes, gnats, midges ( <i>Phlebotomus</i> , <i>Aedes</i> , <i>Anopheles</i> , <i>Glossina</i> , <i>Simulium</i> , etc.)
<i>Schistosoma japonicum</i>	Siphonaptera: fleas ( <i>Pulex</i> , <i>Xenopsylla</i> , etc.)
<i>Schistosoma intercalatum</i>	
<i>Schistosoma mekongi</i>	
<i>Schistosoma malayi</i>	
<i>Schistosoma mattheei</i>	
<b>Pentastomids (tongue worms)<sup>d</sup></b>	
Tissue (larval forms)	
<i>Armillifer</i> spp.	

(continued on next page)

**Table 2.1** Classification of human parasites (*continued*)

- <sup>a</sup> *Entamoeba histolytica* is used to designate the true pathogenic species, while *E. dispar* is now being used to designate the nonpathogenic species. However, unless trophozoites containing ingested red blood cells (*E. histolytica*) are seen, the two organisms cannot be differentiated on the basis of morphology seen in the permanent stained smears of fecal specimens. Fecal immunoassays are available for detecting the *E. histolytica/E. dispar* group or for differentiating the two species.
- <sup>b</sup> Although some individuals have changed the species designation for the genus *Giardia* to *G. intestinalis* or *G. duodenalis*, there is no general agreement. Therefore, for this listing, the name *Giardia lamblia* will be retained.
- <sup>c</sup> This designation is not a true genus, but a "catch-all" for those organisms that have not been (or may never be) identified to the genus and/or species levels. However, it is now listed as one of the genera of microsporidia infecting humans.
- <sup>d</sup> See the section on arthropods, below.

**Table 2.2** Cosmopolitan distribution of common parasitic infections (North America, Mexico, Central America, South America, Europe, Africa, Asia, and Oceania)

Protozoa	Cestodes
Intestinal	
<i>Blastocystis hominis</i>	<i>Hymenolepis nana</i>
<i>Cryptosporidium parvum</i>	<i>Taenia saginata</i>
<i>Cryptosporidium hominis</i>	
<i>Cyclospora cayetanensis</i>	
<i>Dientamoeba fragilis</i>	
<i>Entamoeba histolytica</i>	
<i>Entamoeba dispar</i> <sup>a</sup>	
<i>Giardia lamblia</i>	
<i>Isospora belli</i>	
Microsporidia	
Tissue	
<i>Toxoplasma gondii</i>	
Microsporidia	
Other	
<i>Acanthamoeba</i> spp.	
Microsporidia	
<i>Naegleria fowleri</i>	
<i>Trichomonas vaginalis</i>	
Nematodes	
Intestinal	
	<i>Ascaris lumbricoides</i>
	<i>Enterobius vermicularis</i>
	Hookworm
	<i>Strongyloides stercoralis</i>
	<i>Trichuris trichiura</i>
Tissue	
	<i>Trichinella</i> spp.

<sup>a</sup> *Entamoeba histolytica* is being used to designate the true pathogenic species, while *E. dispar* is now being used to designate the nonpathogenic species. However, unless trophozoites containing ingested red blood cells (*E. histolytica*) are seen, the two organisms cannot be differentiated on the basis of morphology in the permanent stained smear. Fecal immunoassays are available for detecting the *Entamoeba histolytica/E. dispar* group or for differentiating the two species.

**Table 2.3** Body sites and possible parasites recovered (trophozoites, cysts, oocysts, spores, adults, larvae, eggs, amastigotes, and trypomastigotes)<sup>a</sup>

Site	Parasites	Site	Parasites
Blood Red cells  White cells  Buffy coat Whole blood or plasma Bone marrow	<i>Plasmodium</i> spp. <i>Babesia</i> spp. <i>Leishmania</i> spp. <i>Toxoplasma gondii</i> All blood parasites <i>Trypanosoma</i> spp. Microfilariae <i>Leishmania</i> spp. <i>Trypanosoma cruzi</i> <i>Plasmodium</i> spp.	Intestinal tract (continued)	<i>Cyclospora cayetanensis</i> <i>Isospora belli</i> <i>Enterocytozoon bieneusi</i> <i>Encephalitozoon</i> spp. <i>Ascaris lumbricoides</i> <i>Enterobius vermicularis</i> Hookworm <i>Strongyloides stercoralis</i> <i>Trichuris trichiura</i> <i>Hymenolepis nana</i> <i>Hymenolepis diminuta</i> <i>Taenia saginata</i> <i>Taenia solium</i> <i>Diphyllobothrium latum</i> <i>Clonorchis (Opisthorchis) sinensis</i> <i>Paragonimus</i> spp. <i>Schistosoma</i> spp. <i>Fasciolopsis buski</i> <i>Fasciola hepatica</i> <i>Metagonimus yokogawai</i> <i>Heterophyes heterophyes</i>
Central nervous system	<i>Taenia solium</i> (cysticerci) <i>Echinococcus</i> spp. <i>Naegleria fowleri</i> Acanthamoeba and <i>Hartmanella</i> spp. <i>Balamuthia mandrillaris</i> <i>Sappinia diploidea</i> <i>Toxoplasma gondii</i> Microsporidia <i>Trypanosoma</i> spp. <i>Baylisascaris procyonis</i> <i>Toxocara</i> spp. (visceral larva migrans)	Liver and spleen	<i>Echinococcus</i> spp. <i>Entamoeba histolytica</i> <i>Leishmania</i> spp. Microsporidia
Cutaneous ulcers	<i>Leishmania</i> spp. Acanthamoeba spp. <i>Entamoeba histolytica</i>	Lungs	<i>Cryptosporidium</i> spp. <sup>b</sup> <i>Echinococcus</i> spp. <i>Paragonimus</i> spp.
Eyes	Acanthamoeba spp. <i>Toxoplasma gondii</i> <i>Loa loa</i> Microsporidia <i>Toxocara</i> spp. (ocular larva migrans)	Muscles	<i>Taenia solium</i> (cysticerci) <i>Trichinella</i> spp. <i>Onchocerca volvulus</i> (nodules) <i>Trypanosoma cruzi</i> Microsporidia <sup>c</sup>
Intestinal tract	<i>Entamoeba histolytica</i> <i>Entamoeba dispar</i> <i>Entamoeba coli</i> <i>Entamoeba hartmanni</i> <i>Endolimax nana</i> <i>Iodamoeba bütschlii</i> <i>Blastocystis hominis</i> <i>Giardia lamblia</i> <i>Chilomastix mesnili</i> <i>Dientamoeba fragilis</i> <i>Pentatrichomonas hominis</i> <i>Balantidium coli</i> <i>Cryptosporidium</i> spp.	Skin	<i>Leishmania</i> spp. <i>Onchocerca volvulus</i> Microfilariae <i>Ancylostoma</i> spp. (cutaneous larva migrans)
		Urogenital system	<i>Trichomonas vaginalis</i> <i>Schistosoma</i> spp. Microsporidia Microfilariae

<sup>a</sup> This table does not include every possible parasite that could be found in a body site. However, the most likely organisms have been listed.

<sup>b</sup> Disseminated in severely immunosuppressed individuals.

<sup>c</sup> The genera *Pleistophora* and *Trachipleistophora* have been documented to occur in muscles.

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S E C T I O N     3

## Collection Options

Various collection methods are available for specimens suspected of containing parasites or parasitic elements (Table 3.1). The decision on the method used should be based on a thorough understanding of the value and limitations of each. The final laboratory results are based on parasite recovery and identification and will depend on the initial handling of the organisms. Unless the appropriate specimens are properly collected and processed, these infections may not be detected. Therefore, specimen rejection criteria have become much more important for all diagnostic microbiology procedures. Diagnostic laboratory results based on improperly collected specimens may require inappropriate expenditures of time and supplies and may also mislead the physician. **As a part of any continuous quality improvement program for the laboratory, the generation of test results must begin with stringent criteria for specimen acceptance or rejection.**

Clinically relevant testing also depends on the receipt of appropriate test orders from the physician (Table 3.2). The laboratory is not authorized to order tests; this function is the responsibility of the physician. Depending on the patient's history, very specific diagnostic tests are recommended. It is very important that physician clients become familiar with the test order options available from the laboratory testing menu. They must also have an understanding of the pros and cons of each test when considered within the context of the patient's symptoms and clinical history. Without the appropriate test orders and collection procedures, test results may be misleading or even incorrect. **It is important for the laboratory to provide appropriate and complete information to all clients in order to ensure quality patient care.**

## Safety

All fresh specimens should be handled carefully, since each specimen represents a potential source of infectious material. Safety precautions should include proper labeling of fixatives; specific areas designated for specimen handling (biological safety cabinets may be necessary under certain circumstances); proper containers for centrifugation; acceptable discard policies; appropriate policies for no eating, drinking, or smoking within the working areas; and, if applicable, correct techniques for organism culture and/or animal inoculation. In general, standard precautions as outlined by the Occupational Safety and Health Act must be followed when applicable, particularly when one is handling blood and other body fluids.

## Collection of Fresh Stool Specimens

Stool specimens should always be collected before barium is used for radiological examination. Specimens containing barium are unacceptable for processing and examination, and intestinal protozoa may be undetectable for 5 to 10 days after barium is given to the patient. Certain substances and medications also interfere with the detection of intestinal

protozoa; these include mineral oil, bismuth, antibiotics, antimalarial agents, and nonabsorbable antidiarrheal preparations. After administration of any of these compounds, parasites may not be recovered for one to several weeks. After the administration of barium or antibiotics, specimen collection should be delayed for 5 to 10 days or at least 2 weeks, respectively.

### **Collection Method**

Fecal specimens should be collected in clean, wide-mouth containers; often a waxed cardboard or plastic container with a tight-fitting lid is selected for this purpose. The specimens should not be contaminated with water or urine because water may contain free-living organisms (including arthropod larvae and free-living nematodes) that can be mistaken for human parasites and urine may destroy motile organisms. Stool specimen containers should be placed in plastic bags for transport to the laboratory for testing. The specimens should be labeled with the following information: the patient's name and identification number, the physician's name, and the date and time the specimen was collected (if the laboratory is computerized, the date and time may reflect arrival in the laboratory, not the actual collection time). The specimen must also be accompanied by a request form indicating which laboratory procedures are to be performed; in some cases this information will be computerized and will be entered into the system on the nursing unit or in the clinic. Although it is helpful to have information concerning the presumptive diagnosis or relevant travel history, this information rarely accompanies the specimen. If such information is relevant and necessary to maximize diagnostic testing, the physician may have to be contacted for additional patient history.

### **Number of Specimens To Be Collected**

#### **Standard Approach**

In the past, it has been recommended that a normal examination for stool parasites before therapy include three specimens, consisting of two specimens collected from normal movements and one collected after the use of a cathartic such as magnesium sulfate or Fleet's Phospho-Soda. The purpose of the laxative is to stimulate some "flushing" action within the gastrointestinal tract. A cathartic with an oil base should not be used, and a stool softener (taken either orally or as a suppository) is usually inadequate for obtaining a purged specimen. The use of a laxative prior to collection of the third specimen is much less common than in years past. Also, if the patient already has diarrhea or dysentery, the use of any laxatives would be contraindicated.

When a patient is suspected of having intestinal amebiasis, collection of six specimens may be recommended. The examination of six specimens ensures detection of approximately 90% of amebic infections. However, considering cost containment measures, the examination of six specimens is rarely requested.

## Different Approaches

During the past few years, recommendations regarding the collection, processing, and testing of stool specimens for diagnostic parasitology have been under review. New suggestions and options have arisen as a result of cost containment measures, limited reimbursement, and the elimination of mercury-based compounds for stool preservatives. The number of nonmercury preservative choices, collection systems, concentration devices, and immunoassays has increased dramatically.

It is important to realize that different laboratories will select different approaches. These differences should not be categorized as "right or wrong" or "acceptable or unacceptable"; they are merely different! To assume that there is only one correct approach for the examination of stool specimens is neither appropriate nor realistic. There are many factors to consider before selecting the approach for your own laboratory. In no particular order, some of the considerations include client base, physician ordering patterns, number of specimens received per month, cost, presence or absence of appropriate equipment, current and possible methodologies (including the new immunoassays such as enzyme immunoassay [EIA], fluorescent-antibody assay [FA], and rapid tests [membrane flow cartridge devices]), availability of expert microscopists, collection options, selection of preservative-stain combinations, reimbursement issues, client education, area of the world where the laboratory is located, and emphasis on the most common infections (helminth or protozoa or both) seen in that geographic location.

When laboratory test menus are being developed, the pros and cons of the approaches selected need to be thoroughly understood, and diagnostic tests, potential results, and reporting formats should be carefully explained to all clients. As an example, if the results of a stool examination are based on a concentration sediment examination only, this information must be conveyed to the physician. Many of the intestinal protozoa are missed when this diagnostic test approach is used, and it is important for the physician to recognize the limitations of such testing. Most physicians receive very little, if any, exposure to medical parasitology in medical school, and many newer physicians trained as generalists or family practitioners also have limited parasitology training or experience (Table 3.1).

It is usually realistic to assume that patients are symptomatic if they are submitting stool specimens for diagnostic parasitology testing. In an excellent article by Hiatt et al., the premise tested was that a single stool specimen from a symptomatic patient would be sufficient to diagnose infections with intestinal protozoa. However, with additional stool examinations, the yield of intestinal protozoa from symptomatic patients increased dramatically (*Entamoeba histolytica*, 22.7% increase; *Giardia lamblia*, 11.3% increase; and *Dientamoeba fragilis*, 31.1% increase). This publication again demonstrates the problems associated with performing only a single stool examination (using the ova and parasite examination [O&P exam]). If the patient becomes asymptomatic after examination of the first stool specimen, it may be acceptable to discontinue the series of stool examinations (this should be a clinician decision).

Available options will be compared to the “gold standard,” which includes a series of three stool examinations (direct, concentrate, and permanent stained smear for a fresh specimen; concentrate and permanent stained smear for a preserved specimen). The single-specimen pros and cons are discussed in the previous paragraph (symptomatic versus asymptomatic patients). A suggestion has been made to pool three specimens and to perform a single concentration and a single permanent stained smear on the pooled specimen sample. Depending on the number of positives and negatives, this may be a viable option, but some organisms may be missed. Another testing option for three pooled specimens is to perform a single concentration and three separate permanent stained smears. This approach would probably increase the yield of intestinal protozoa over the previous option. Another suggestion involves placing a sample of each of three stools into a single vial of preservative. This collection approach would require only a single vial, but it is very likely that the vial would be overfilled and that mixing and the ratio of fixative to stool would be inaccurate.

Fecal immunoassays could also be used for *G. lamblia*, the *E. histolytica/E. dispar* group, *E. histolytica*, or *Cryptosporidium* spp. However, information about patient history is rarely received with the clinical specimen; fecal testing based on the risk group or recent activity of the patient is impossible without sufficient information. Testing by immunoassay procedures should be performed on request; however, client education is critical for successful implementation of this approach. Collection and testing options and their pros and cons can be seen in Tables 3.1 and 3.2.

The Clinical Laboratory Standards Institute/National Committee for Clinical Laboratory Standards document *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract* (approved guideline M28-2A) was updated in 2005. Various stool collection, processing, and testing options are also included in that publication.

In summary, laboratories performing diagnostic parasitology testing must decide on the test methods that are relevant for their own operations based on a number of variables mentioned above. It is unrealistic to assume or state that one approach is applicable for every laboratory; however, it is important to thoroughly understand the options within your test menu and to convey this information to your clients once your approach has been selected for implementation. Prior discussion with clients, written educational memos, meetings, and examples of revised report formats are highly recommended prior to implementation. **Based on available testing options, it is recommended that laboratories include both the O&P exam and various fecal immunoassays in their test menus.**

## Collection Times

A series of three specimens should be collected on separate days. If possible, the specimens should be collected every other day; otherwise, the series of three specimens should be collected within no more than 10 days. If a series of six specimens is requested, these specimens should also be collected on separate days or within no more than 14 days. Many

organisms, particularly the intestinal protozoa, are shed sporadically and do not appear in the stool in consistent numbers on a daily basis; thus, the series of two (minimum) or three specimens is recommended for an adequate examination. Multiple specimens should not be collected from the same patient on the same day. One possible exception would be a patient who has severe, watery diarrhea such that any organisms present might be missed because of a tremendous dilution factor related to fluid loss. These specimens should be accepted only after consultation with the physician. It is also not recommended for the three specimens to be submitted one each day for three consecutive days; however, use of this collection time frame would not be sufficient to reject the specimens.

Although three stool specimens are recommended, laboratories have been more willing to accept two specimens, primarily because of cost savings and the assumption that if the patient is symptomatic, the presence of any organisms is likely to be confirmed by testing two specimens. However, it is important that clients understand the pros and cons of the two approaches. Both collection approaches are being used by diagnostic laboratories.

### **Posttherapy Collection**

Patients who have received treatment for a protozoan infection should be checked 3 to 4 weeks after therapy, and those treated for *Taenia* infections should be checked 5 to 6 weeks after therapy; these recommendations have been used for many years. If the patient remains asymptomatic, the posttherapy specimens may not be collected, often as a cost containment measure. If the patient becomes symptomatic again, additional specimens can be submitted. If fecal immunoassays are ordered and parasites are present, the patient should be tested 7 to 10 days posttherapy. It usually takes approximately a week for antigen to be eliminated from the stool. **It is important to remember that if the posttherapy specimens are collected too long after therapy, the presence of parasites or parasite antigen may represent a reinfection.**

### **Specimen Type, Stability, and Need for Preservation**

Fresh specimens are required for the recovery of motile trophozoites (amebae, flagellates, or ciliates). The protozoan trophozoite stage is found in patients with diarrhea; the gastrointestinal tract contents are moving through the system too rapidly for cyst formation to occur. Once the stool specimen is passed from the body, trophozoites do not encyst but may disintegrate if not examined or preserved within a short time after passage. However, most helminth eggs and larvae, coccidian oocysts, and microsporidian spores can survive for extended periods. Since it is impossible to know which organisms might be present, it is recommended that the most conservative time limits be used for parasite preservation and recovery. Liquid specimens should be examined within 30 min of passage, not 30 min from the time they reach the laboratory. If this general time recommendation of 30 min is not possible, the specimen should be placed in one of the available fixatives. Soft (semiformed) specimens may contain a mixture of protozoan trophozoites and cysts and

should be examined within 1 h of passage; again, if this time frame is not possible, preservatives should be used. Immediate examination of formed specimens is not as critical; in fact, if the specimen is examined any time within 24 h after passage, the protozoan cysts should still be intact (Table 3.3).

Currently, fresh or frozen fecal specimens are required for the following fecal immunoassays (either as a single-organism test or when combined with other organisms such as *G. lamblia* or *Cryptosporidium* spp.): *E. histolytica/E. dispar* group and *E. histolytica*.

#### **Summary: Collection of Fresh Stool Specimens**

1. Occupational Safety and Health Act regulations (Standard Precautions) should be used for handling all specimens.
2. Interfering substances (e.g., barium, mineral oil, or antibiotics) should be avoided when stool specimens are collected.
3. Contamination with urine or water should be avoided.
4. Recommendation for collection: two (minimum) or three specimens collected, one every other day or within a 10-day time frame; see Table 3.1 for options and pros/cons.
5. Liquid stool should be examined or preserved within 30 min of passage (trophozoites). Soft stool should be examined or preserved within 1 h of passage (trophozoites and cysts\*). Formed stool should be examined or preserved within 24 h of passage.
6. Fresh or frozen fecal specimens are required for the following fecal immunoassays (either as a single-organism test or combined with other organisms such as *G. lamblia* or *Cryptosporidium* spp.): *E. histolytica/E. dispar* group and *E. histolytica*. Fresh, frozen, or preserved specimens can be used for *G. lamblia* and *Cryptosporidium* spp.; specimens submitted in Cary-Blair transport medium are also acceptable. Note: Due to the freeze-thaw cycle, frozen specimens cannot be used for the FA procedures for *G. lamblia* and/or *Cryptosporidium* spp. (destruction of the actual cysts and/or oocysts that are visual proof of a positive specimen).

\* *Dientamoeba fragilis* trophozoites can be found in formed stool specimens.

## **Preservation of Stool Specimens**

### **Overview of Preservatives**

If there are likely to be delays from the time of specimen passage until examination in the laboratory, the use of preservatives is recommended. To preserve protozoan morphology and to prevent the continued development of some helminth eggs and larvae, the stool specimens can be placed in preservative either immediately after passage (by the patient using a collection kit) or once the specimen is received by the laboratory. Several fixatives are available: formalin, sodium acetate-acetic acid-formalin (SAF), Schaudinn's fluid, polyvinyl alcohol (PVA), and single-vial systems (Table 3.3). **Regardless of the fixative selected, use of the**

**appropriate ratio of fixative to stool (3 parts fixative to 1 part stool) and adequate mixing of the specimen and preservative are mandatory.** Although many products are commercially available, the most commonly used preservatives are discussed below. They are all available from various scientific supply houses.

When selecting an appropriate fixative, keep in mind that a permanent stained smear is required for a complete examination for parasites. If the physician orders a fecal immunoassay such as FA, EIA, or the rapid-flow method, you will need to confirm that the fixative you are using is compatible with the immunoassay you have selected. It is also important to remember that disposal regulations for compounds containing mercury are becoming more strict; each laboratory will have to check applicable state and federal regulations to help determine fixative options.

### **Formalin**

Formalin is an all-purpose fixative that is appropriate for helminth eggs and larvae and for protozoan cysts. Two concentrations are commonly used: 5%, which is recommended for preservation of protozoan cysts, and 10%, which is recommended for helminth eggs and larvae. Although 5% is often recommended for all-purpose use, most commercial manufacturers provide 10%, which is more likely to kill all helminth eggs. To help maintain organism morphology, the formalin can be buffered with sodium phosphate buffers, i.e., neutral formalin. Selection of specific formalin formulations is at the user's discretion. Aqueous formalin permits examination of the specimen as a wet mount only, a technique much less accurate than a stained smear for the identification of intestinal protozoa. The most common preparation is 10% formalin, prepared as follows:

Formaldehyde (USP) ..... 100 ml (or 50 ml for 5%)  
Saline solution, 0.85% NaCl ..... 900 ml (or 950 ml for 5%)

Dilute 100 ml of formaldehyde with 900 ml of 0.85% NaCl solution.  
(Distilled water may be used instead of saline solution.)

**Note:** Formaldehyde is normally purchased as a 37 to 40% HCHO solution; however, for dilution it should be considered to be 100%.

If you want to use buffered formalin, the recommended approach is to mix thoroughly 6.10 g of  $\text{Na}_2\text{HPO}_4$  and 0.15 g of  $\text{NaH}_2\text{PO}_4$  and store the dry mixture in a tightly closed bottle. For 1 liter of either 10 or 5% formalin, 0.8 g of the buffer salt mixture should be added.

Protozoan cysts (not trophozoites), coccidian oocysts, microsporidian spores, and helminth eggs and larvae are well preserved for long periods in 10% aqueous formalin. Hot ( $60^\circ\text{C}$ ) formalin can be used for specimens containing helminth eggs, since in cold formalin some thick-shelled eggs may continue to develop, become infective, and remain viable for long periods; however, this approach is not practical for routine clinical

laboratories. Several grams of fecal material should be thoroughly mixed in 5 or 10% formalin.

Summary: Formalin	
PROS	CONS
<p>Good overall fixative for stool concentrate.</p> <p>Easy to prepare, long shelf life.</p> <p>Formalin-preserved stool can be used with the fecal immunoassay kits for <i>Giardia lamblia</i> and <i>Cryptosporidium</i> spp.</p> <p>Once formalin use has been monitored for formalin vapor, levels do not have to be rechecked unless the number of specimens processed dramatically increases or formalin use within the microbiology laboratory is modified. (Note: <b>Formalin use within microbiology laboratories rarely comes close to the allowable limits.</b>)</p>	<p>Does not preserve trophozoites well.</p> <p>Does not adequately preserve organism morphology for a good permanent stained smear.</p> <p>Currently cannot be used with fecal immunoassay methods for the <i>Entamoeba histolytica/E. dispar</i> group or <i>Entamoeba histolytica</i>.</p>

### Sodium Acetate-Acetic Acid-Formalin (SAF)

Both the concentration and the permanent stained smear can be performed from specimens preserved in SAF, and the formula has the advantage of not containing mercuric chloride, as is found in Schaudinn's fluid and mercuric chloride-based PVA fixatives. It is a liquid fixative, much like the 10% formalin described above. The sediment is used to prepare the permanent smear, and it is frequently recommended that the stool material be placed on an albumin-coated slide to improve adherence to the glass.

SAF is considered to be a "softer" fixative than Schaudinn's or mercuric chloride-based PVA fixatives. The organism morphology is not quite as sharp after staining as are organisms originally fixed in solutions containing mercuric chloride. **The pairing of SAF-fixed material with iron hematoxylin staining provides better organism morphology than does staining of SAF-fixed material with trichrome (personal observation).** Although SAF has a long shelf life and is easy to prepare, the smear preparation technique may be a bit more difficult for less experienced personnel who are not familiar with fecal specimen techniques. Laboratories that have considered using only a single preservative have selected this option. Helminth eggs and larvae, protozoan trophozoites and cysts, and coccidian oocysts and microsporidian spores are preserved using this method.

SAF fixative is prepared as follows:

Sodium acetate.....	1.5 g
Acetic acid, glacial .....	2.0 ml
Formaldehyde, 37–40% solution .....	4.0 ml
Distilled water .....	92.0 ml

To make Mayer's albumin, mix equal parts of egg white and glycerin. Place 1 drop on a microscope slide, and add 1 drop of SAF-preserved fecal sediment (from the concentration procedure). After mixing, allow the smear to dry at room temperature for 30 min prior to staining.

Summary: SAF	
PROS	CONS
<ul style="list-style-type: none"><li>Can be used for concentration and permanent stained smears.</li><li>Contains no mercury compounds.</li><li>Easy to prepare, long shelf life.</li><li>SAF-preserved stool can be used with the fecal immunoassay kits for <i>Giardia lamblia</i> and <i>Cryptosporidium</i> spp.</li></ul>	<ul style="list-style-type: none"><li>Poor adhesive properties, albumin-coated slides recommended.</li><li>Protozoan morphology better if iron hematoxylin stains used for permanent stained smears (trichrome not as good).</li><li>May be more difficult to use; however, this is really not a limiting factor.</li><li>Currently cannot be used with fecal immunoassay methods for the <i>Entamoeba histolytica/E. dispar</i> group or <i>Entamoeba histolytica</i>.</li></ul>

### Schaudinn's Fluid

Schaudinn's fluid (which contains mercuric chloride) was one of the original stool fixatives and is used with fresh stool specimens or samples from the intestinal mucosal surface. Many laboratories that receive specimens from in-house patients (which have fewer problems with delivery times) often select this approach. Permanent stained smears are then prepared from fixed material. A concentration technique using Schaudinn's fluid-preserved material is also available but is not widely used. Due to the difficulties (sources and cost) related to mercury disposal, this fixative is being phased out by most laboratories. Although mercury substitutes are available, the overall protozoan morphology does not tend to be as precise as that seen when mercury-based fixatives are used.

Mercuric chloride, saturated aqueous solution

Mercuric chloride ( $HgCl_2$ ).....	110 g
Distilled water .....	1,000 ml

Use a beaker as a water bath; boil (use a hood if available) until the mercuric chloride is dissolved; let stand several hours until crystals form.

### Schaudinn's fixative (stock solution)

Mercuric chloride, saturated aqueous solution ..... 600 ml  
Ethyl alcohol, 95% ..... 300 ml

Immediately before use, add glacial acetic acid, 5 ml/100 ml of stock solution.

Summary: Schaudinn's Fluid	
PROS	CONS
Used as fixative for smears prepared from fresh fecal specimens or samples from the intestinal mucosal surfaces (sigmoidoscopy).  Provides excellent preservation of protozoan trophozoites and cysts.	Not generally recommended for use in concentration procedures.  Contains mercuric chloride, which is a disposal problem.  Poor adhesive qualities with liquid or mucoid specimens.  Currently cannot be used with any of the fecal immunoassay methods.

### Polyvinyl Alcohol (PVA)

PVA is a plastic resin that can be incorporated into Schaudinn's fixative (or other liquid fixatives). The PVA powder is not a fixative but serves as an adhesive for the stool material; i.e., when the stool-PVA mixture is spread onto the glass slide, it adheres because of the PVA component. Fixation is still accomplished by the Schaudinn's fluid itself. Perhaps the greatest advantage in the use of PVA is the fact that a permanent stained smear can be prepared. PVA fixative solution is highly recommended as a means of preserving cysts and trophozoites for later examination. The use of PVA also permits specimens to be shipped (by regular mail service) from any location in the world to a laboratory for subsequent examination. PVA is particularly useful for liquid specimens and should be used in the ratio of 3 parts PVA to 1 part fecal specimen. The formula is as follows:

PVA..... 10.0 g  
Ethyl alcohol, 95% ..... 62.5 ml  
Mercuric chloride, saturated aqueous ..... 125.0 ml  
Acetic acid, glacial ..... 10.0 ml  
Glycerin ..... 3.0 ml

Mix the liquid ingredients in a 500-ml beaker. Add the PVA powder (stirring is not recommended). Cover the beaker with a large petri dish, heavy wax paper, or foil, and allow the PVA to soak overnight. Heat the solution slowly to 75°C. When this temperature is reached, remove the

beaker and swirl the mixture for 30 s until a homogeneous, slightly milky solution is obtained.

Summary: PVA	
PROS	CONS
<p>Can be used to prepare permanent stained smears and perform concentration techniques (less common).</p> <p>Provides excellent preservation of protozoan trophozoites and cysts.</p> <p>Long shelf life (months to years) in tightly sealed containers at room temperature.</p> <p>Allows specimens to be shipped to laboratory for subsequent examination.</p>	<p><i>Trichuris trichiura</i> eggs and <i>Giardia lamblia</i> cysts are not concentrated as easily from PVA as they are from formalin-based fixatives. <i>Strongyloides stercoralis</i> larval morphology is poor (it is better with formalin-based preservation). <i>Isospora belli</i> oocysts may not be visible from PVA-preserved material (it is better to use formalin-fixed specimens).</p> <p>Contains mercury compounds (Schaudinn's fluid).</p> <p>May turn white and gelatinous when it begins to dehydrate or when refrigerated.</p> <p>Difficult to prepare in the laboratory.</p> <p>Specimens containing PVA cannot be used with the fecal immunoassay detection kits.</p>

### Modified PVA (Mercury Substitutes)

Although preservatives have been developed that do not contain mercury compounds, substitute compounds have not provided the same quality of preservation necessary for good protozoan morphology on the permanent stained smear. Copper sulfate has been tried but does not provide results equal to those seen with mercuric chloride. Zinc sulfate has proven to be an acceptable mercury substitute and is used with trichrome stain. Although zinc substitutes have become widely available, each manufacturer has a proprietary formula for the fixative. Compared with mercuric chloride-based fixatives, there is much less margin for error when using modified PVA fixatives. Rapid fixation, proper stool-to-fixative ratios, and adequate mixing are mandatory for good protozoan morphology, particularly on the permanent stained smear.

**Note:** The important question is not "How beautiful are the organisms?" but "Can you tell which organisms are present?" With some training, microscopists can identify the organisms, although the morphology is not as clear as that seen using mercury compounds. Unfortunately, parasi-

tology microscopy is not a perfect science; we probably miss rare organisms even when using mercury-based fixatives.

#### Summary: Modified PVA (Mercury Substitutes)

PROS	CONS
<p>Can be used for permanent stained smears and concentration techniques.</p> <p>Many workers prefer the zinc substitutes over those prepared with copper sulfate.</p> <p>Does not contain mercury compounds.</p>	<p>Overall protozoan morphology of trophozoites and cysts is poor when preserved in the copper sulfate-based fixative, particularly compared with organisms preserved with mercuric chloride-based fixatives.</p> <p>Zinc-based fixatives are an excellent alternative to copper.</p> <p>Staining characteristics of protozoa not consistent; some are good, but some are poor. Organism identification may be more difficult, particularly with small protozoan cysts (such as <i>Endolimax nana</i>).</p> <p>Rapid fixation, proper stool-to-fixative ratios, and adequate mixing are mandatory for good protozoan morphology. There is much less margin for error when using the modified PVA fixatives than when using mercury-based fixatives.</p>

#### Single-Vial Collection Systems (Other Than SAF)

Several manufacturers now have available single-vial stool collection systems, similar to the SAF or modified PVA methods. Some of these formulations are advertised as "ecologically friendly" and do not contain either mercury or formalin. From the single vial, both the concentration and permanent stained smear can be prepared. It is also possible to perform fecal immunoassays (EIA, FA, or the rapid-flow assay) with samples from some of these vials. Make sure to ask the manufacturer about all three assays (concentrate, permanent stained smear, and immunoassay procedures) and for specific information indicating that there are no formula components that would interfere with any of the three methods. Like the zinc substitutes, these formulas are proprietary.

### Summary: Single-Vial Collection Systems

PROS	CONS
<p>Can prepare permanent stained smears and perform concentration techniques.</p> <p>Can perform immunoassay procedures.</p> <p>Do not contain mercury compounds.</p> <p><b>Unless organism numbers are small, acceptable organism recovery and identification are possible; additional training may be required to recognize the organisms because overall morphology is not comparable to that seen with mercury-based fixatives.</b></p> <p>Tend to be more "ecologically friendly" and do not contain either mercury compounds or formalin. However, as already mentioned, <b>formalin use within microbiology laboratories rarely even comes close to the allowable limits.</b></p>	<p>Overall protozoan morphology of trophozoites and cysts is not as good as that of organisms preserved with mercuric chloride-based fixatives; similar to modified PVA options.</p> <p>Staining characteristics of protozoa not consistent; some are good, but some are poor. Identification of <i>Endolimax nana</i> may be most difficult.</p> <p>Rapid fixation, proper stool/fixative ratios, and adequate mixing are mandatory for good protozoan morphology. There is much less margin for error with the single-vial fixative systems.</p>

### Quality Control for Preservatives

Preservatives for fecal specimens are checked for quality control by the manufacturer prior to sale, generally with living protozoa. If you prepare your own fixatives, you can use the following approach for quality control. The specimen used for quality control presented below is designed to be used with fixatives from which permanent stained smears will be prepared (Schaudinn's fluid, PVA, modified PVA, SAF, or the single-vial systems). However, this quality control specimen can also be used in a concentration; the white blood cells (WBCs) can be seen in the concentrate sediment (sedimentation concentration) or in the surface film (flootation concentration).

1. Obtain a fresh anticoagulated blood specimen, centrifuge, and obtain a buffy coat sample (try to find a specimen with a high WBC count).
2. Mix approximately 2 g of soft, fresh fecal specimen (normal stool, containing no parasites) with several drops of the buffy coat cells.
3. Prepare several fecal smears and fix immediately in Schaudinn's fluid to be quality controlled.
4. Mix the remaining mixture of feces and buffy coat in 10 ml of PVA, modified PVA, or SAF preservative to be quality controlled.
5. Allow 30 min for fixation, and then prepare several fecal smears. Allow to dry thoroughly (60 min at room temperature or 30 to 60 min in an incubator [approximately 35°C]).
6. Stain slides by normal staining procedure.

7. After staining, if the WBCs appear well fixed and display typical morphology and color, one can assume that any intestinal protozoa placed in the same lot number of preservative would also be well fixed, provided that the fecal sample was fresh and fixed within the recommended time limits.
8. Record all quality control results. If the WBC morphology does not confirm good fixation, then describe the results and indicate what corrective action procedures were used (repeated the test, prepared new fixative).

### **Procedure Notes for Use of Preservatives (Stool Fixative Collection Vials)**

1. Most of the commercially available kits have a “fill to” line on the vial label to indicate how much fecal material to add to ensure adequate preservation of the fecal material. However, patients often overfill the vials; remember to open the vials with the vials turned away from your face. There may be excess gas in the vials that may create aerosols once the vial lids are opened.
2. Although the two-vial system (one vial of 5 or 10% buffered formalin [concentration] and one vial of PVA [permanent stained smear]) has always been the “gold standard,” laboratories are beginning to use other options such as the single-vial collection systems. Changes in the selection of fixatives are based on the following considerations.
  - a. Problems with disposal of mercury-based fixatives and the lack of multilaboratory contracts for disposal of such products
  - b. The cost of a two-vial system compared with the cost of a single collection vial
  - c. Selection of specific stains (trichrome, iron hematoxylin) to use with specific fixatives
  - d. Whether the newer immunoassay kits (EIA, FA, rapid cartridges [*Giardia*, *Cryptosporidium*, *E. histolytica*]) can be used with stool specimens preserved with that particular fixative

### **Procedure Limitations for Use of Preservatives (Stool Fixative Collection Vials)**

1. Adequate fixation still depends on the following factors:
  - a. Meeting recommended time limits for lag time between passage of the specimen and fixation
  - b. Use of the correct ratio of fixative to specimen (3:1)
  - c. Thorough mixing of the fixative and specimen (once the specimen is received in the laboratory, any additional mixing will not counteract the lack of fixative-specimen mixing and contact prior to that time)
2. Unless the appropriate stain is used with each fixative, the final permanent stained smear may be difficult to examine (organisms may be hard to see and/or identify). Examples of appropriate combinations are
  - a. Schaudinn's or PVA fixative with trichrome or iron hematoxylin stains

- b. SAF fixative with iron hematoxylin stain
- c. Single-vial mercuric chloride substitute systems with trichrome, iron hematoxylin, or company-developed proprietary stains matched to their specific fixatives

## Collection of Blood

### Collection and Processing

Depending on the parasite life cycle, a number of parasites may be recovered in a blood specimen, either whole blood, buffy coat preparations, or various types of concentrations. These parasites include *Plasmodium*, *Babesia*, and *Trypanosoma* spp., *Leishmania donovani*, and microfilariae. Although some organisms may be motile in fresh, whole blood, species are normally identified from the examination of permanent stained blood films, both thick and thin films (Table 3.4). Blood films can be prepared from fresh whole blood collected with no anticoagulants, anticoagulated blood, buffy coat cells, or sediment from the various concentration procedures (Table 3.5).

Blood can be collected from either finger stick or venipuncture. Venous blood should be collected in a tube containing EDTA. Multiple thick and thin blood films from the blood or buffy coat should be prepared and examined immediately after receipt of the blood by the laboratory, and multiple blood examinations should be performed before blood-borne parasite infection is ruled out. Unless you are positive that you will receive well-prepared thick and thin blood films from finger stick blood, request a tube of anticoagulated blood (EDTA anticoagulant [lavender top] is preferred). The tube should be filled with blood to provide the proper blood/anticoagulant ratio. For detection of stippling, the smears should be prepared within 1 h after the specimen is drawn. After that time, stippling may not be visible on stained films; however, the overall organism morphology is still acceptable. Most laboratories routinely use commercially available blood collection tubes; preparation of EDTA collection tubes in-house is neither necessary nor cost-effective. However, if the need should arise, EDTA (Sequestrene) can be prepared and tubed as follows: dissolve 5 g of EDTA in 100 ml of distilled water, aliquot 0.4 ml into tubes, and evaporate the water. This amount of anticoagulant is sufficient for 10 ml of blood. One can also use 20 mg of EDTA (dry) per tube (20 mg/10 ml of blood).

The time when the specimen was drawn should be clearly indicated on the tube of blood and also on the result report. The physician will then be able to correlate the results with any fever pattern or other symptoms that the patient may have. There should also be some comments on the test result report that is sent back to the physician that one negative specimen does not rule out the possibility of a parasitic infection.

### STAT Test Requests and Risk Management Issues

All requests for malaria diagnosis are considered STAT requests, and specimens should be collected, processed, examined, and reported accordingly. All requests for examination of blood films should include a

possible diagnosis of malaria; thus, these requests are always considered STAT. Not only the blood collection should be considered STAT, but also the processing and examination of both thick and thin blood films should be performed immediately on receipt of the blood (Table 3.6). Immunologically naive individuals with no prior exposure to malaria often present to the emergency room or clinic with symptoms such as fever and malaise and a relevant travel history to an area of the world where malaria is endemic. These patients often have vague symptoms, but have the potential to become very ill with malaria, even with a low parasitemia (0.0005% to 0.1%).

## Collection of Specimens from Other Body Sites

Although clinical specimens for examination can be obtained from many other body sites, these specimens and appropriate diagnostic methods are not as commonly performed as those used for the routine stool specimen. The majority of specimens from other body sites (Table 3.7) would be submitted as fresh specimens for further testing. More information is given in the discussion of specific procedures in Section 5.

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**Table 3.1** Fecal specimens for parasites: options for collection and processing<sup>a</sup>

Option	Pros	Cons
Rejection of stools from inpatients who have been in-house for >3 days	Data suggest that patients who begin to have diarrhea after they have been inpatients for a few days are not symptomatic from parasitic infections, but generally other causes.	There is always the chance that the problem is related to a nosocomial parasitic infection (rare), but <i>Cryptosporidium</i> and microsporidia are possible considerations.
Examination of a single stool (O&P exam)	Some feel that most intestinal parasitic infections can be diagnosed from examination of a single stool. If the patient becomes asymptomatic after collection of the first stool, subsequent specimens may not be necessary.	Diagnosis from a single stool examination depends on the experience of the microscopist, proper collection, and the parasite load in the specimen. In a series of three stool specimens, frequently not all three specimens are positive and/or may be positive for different organisms. Data suggest that 40–50% of organisms present will be found with only a single stool exam.
Two O&P exams (concentration, permanent stained smear)	The examination of two specimens is more sensitive than a single examination; different organisms may be found in the two specimens; one might be negative while the other is positive.	May not be as cost-effective as examination of a single specimen, but is more clinically relevant. Not always as good as three specimens (although it may be a relatively cost-effective approach); any patient remaining symptomatic would require additional testing.
Examination of a second stool only after the first is negative and the patient is still symptomatic	With additional examinations, yield of protozoa increases ( <i>Entamoeba histolytica</i> , 22.7%; <i>Giardia lamblia</i> , 11.3%; and <i>Dientamoeba fragilis</i> , 31.1%). <sup>b</sup>	Assumes the second (or third) stool is collected within the recommended 10-day time frame for a series of stools; protozoa are shed periodically. May be inconvenient for patient. If 10-day time frame is not met, additional stools represent a new 10-day time frame.

(continued on next page)

**Table 3.1** Fecal specimens for parasites: options for collection and processing<sup>a</sup> (*continued*)

Option	Pros	Cons
Examination of a single stool and an immunoassay (EIA, FA, lateral- or vertical-flow cartridge)	If the examinations are negative and the patient's symptoms subside, probably no further testing is required.	Patients may exhibit symptoms (off and on), so it may be difficult to rule out parasitic infections with only a single stool and one fecal immunoassay; immunoassay of two separate specimens may be required to confirm <i>Giardia</i> antigen. If the patient remains symptomatic, then even if two <i>Giardia</i> immunoassays are negative, other protozoa may be missed ( <i>E. histolytica</i> , <i>E. dispar</i> group, <i>E. histolytica</i> , <i>D. fragilis</i> , <i>Cryptosporidium</i> spp., microsporidia). One O&P exam is not the best approach (review the last option below). It is not recommended to automatically perform both the O&P and fecal immunoassay. Depending on the patient's history and clinical symptoms, EITHER the O&P exam OR a fecal immunoassay may be recommended, BUT GENERALLY NOT BOTH.
Pooling of three specimens for examination and performing one concentration and one permanent stain	Three specimens are collected by the patient (three separate collection vials) over 7–10 days, and pooling by the laboratory may save time and expense.	Organisms present in small numbers may be missed due to the dilution factor once the specimens are pooled.
Pooling of three specimens for examination and performing one concentration and three permanent stained smears	Three specimens are collected by the patient (three separate collection vials) over 7–10 days; pooling by the laboratory for the concentration would probably be sufficient for the identification of helminth eggs. Examination of the three separate permanent stained smears (one from each vial) would maximize recovery of intestinal protozoa in areas of the country where these organisms are most common.	Light helminth infection (eggs, larvae) might be missed due to the pooling of the three specimens for the concentration; however, with a permanent stain performed on each of the three specimens, this approach would probably be the next best option after the standard approach (concentration and permanent stained smear performed on every stool). Coding and billing would have to match the work performed; this may present some problems where work performed does not match existing codes.

*(continued on next page)*

**Table 3.1** (continued)

Option	Pros	Cons
Collection of three stools, but with a sample of stool from all three specimens placed into a single vial (patient is given a single vial only)	Pooling of the specimens would require only a single vial.	This would complicate patient collection and very likely result in poorly preserved specimens, especially regarding the recommended ratio of stool to preservative and the lack of proper mixing of specimen and fixative.
Performing immunoassays on specimens from selected patients <sup>c</sup> by methods for <i>G. lamblia</i> , <i>Cryptosporidium</i> spp., and/or the <i>E. histolytica/E. dispar</i> group or <i>E. histolytica</i>	Would be more cost-effective than performing immunoassay procedures on all specimens; however, information required to group patients into categories is often not received with the specimens. <b>This approach assumes that physicians have guidance in terms of correct ordering options (Table 3.2).</b>	Laboratories rarely receive information that would allow them to place a patient in a particular risk group: children <5 yr old, children from day care centers (may or may not be symptomatic), patients with immunodeficiencies, and patients from outbreaks. Performance of immunoassay procedures on every stool is not cost-effective, and the positive rate is low unless an outbreak situation is involved.
Performing immunoassays and O&P exams on request <sup>a</sup> for <i>G. lamblia</i> , <i>Cryptosporidium</i> spp., and/or <i>E. histolytica/E. dispar</i> group or <i>E. histolytica</i>	This approach limits the number of stools on which immunoassay procedures are performed for parasites. Immunoassay results do not have to be confirmed by any other tests (such as O&P exams or modified acid-fast stains). If specific kit performance problems have been identified, individual laboratories may prefer to do additional testing. <b>However, the fecal immunoassays are more sensitive than the O&amp;P exam and special stains (modified acid-fast stains).</b> Also, this may be considered duplicate testing and may not be approved for reimbursement unless specifically ordered by the physician.	This approach requires education of physician clients regarding appropriate times of collection and patients for whom immunoassays should be ordered. Educational initiatives must also include information on the test report indicating the pathogenic parasites that will <b>not</b> be detected using these methods. It is critical to ensure clients know that further testing may not be required if patients have become asymptomatic. <b>However, if the patient remains symptomatic, further testing (O&amp;P exam) is required.</b> Remember, a single O&P exam may not reveal all organisms present.

<sup>a</sup> A number of variables determine the approach to immunoassay testing and the O&P exam (geography, parasites recovered, positive rate, physician requests). Immunoassays and/or O&P exams should be separately ordered, reported, and billed. The plan should be presented to physicians for approval: immunoassays or O&P exams, procedure discussion, report formats, clinical relevance, and limitations of each approach.

<sup>b</sup> See Hiatt et al. (1995) and Cartwright (1999) in Suggested Reading.

<sup>c</sup> See Table 3.2. It is difficult to know when you may be in an early outbreak situation where testing of all specimens for either *G. lamblia*, *Cryptosporidium* spp., or both, may be relevant. Extensive efforts to encourage communication among laboratories, water companies, pharmacies, and public health officials regarding the identification of potential or actual outbreaks are under way. If it appears that an outbreak is in the early stages, then performing the immunoassays on request can be changed to testing all stools.

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**Table 3.1** Fecal specimens for parasites: options for collection and processing<sup>a</sup> (continued)**Suggested Reading**

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**Table 3.2** Approaches to stool parasitology: test ordering

Patient and/or situation	Test ordered <sup>a</sup>	Follow-up test ordered
Patient with diarrhea and AIDS or other cause of immune deficiency OR Patient with diarrhea involved in a potential waterborne outbreak (municipal water supply)	<i>Cryptosporidium</i> or <i>Giardia</i> / <i>Cryptosporidium</i> immunoassay	If immunoassays are negative and symptoms continue, special stains for microsporidia (modified trichrome stain) and other coccidia (modified acid-fast stain) and O&P exam should be performed.
Patient with diarrhea (nursery school, day care center, camper, backpacker) OR Patient with diarrhea involved in a potential waterborne outbreak (resort setting) OR Patient from areas within the United States where <i>Giardia</i> is the most common parasite found	<i>Giardia</i> or <i>Giardia</i> / <i>Cryptosporidium</i> immunoassay	If immunoassays are negative and symptoms continue, special stains for microsporidia and other coccidia (see above) and O&P exam should be performed.
Patient with diarrhea and relevant travel history outside of the United States OR Patient with diarrhea who is a past or present resident of a developing country OR Patient in an area of the United States where parasites other than <i>Giardia</i> are found (large metropolitan centers such as New York, Los Angeles, Washington, DC, and Miami)	O&P exam, <i>Entamoeba histolytica</i> / <i>E. dispar</i> immunoassay; immunoassay for confirmation of <i>E. histolytica</i> ; various tests for <i>Strongyloides</i> may be relevant (even in the absence of eosinophilia), particularly if there is any history of pneumonia (migrating larvae in lungs) or of sepsis or meningitis (fecal bacteria carried by migrating larvae); agar culture plate is the most sensitive diagnostic approach for <i>Strongyloides stercoralis</i> .	The O&P exam is designed to detect and identify a broad range of parasites (amebae, flagellates, ciliates, <i>Isospora belli</i> , helminths); if exams are negative and symptoms continue, special tests for coccidia (fecal immunoassays, modified acid-fast stains, autofluorescence) and microsporidia (modified trichrome stains, Calcofluor white stains) should be performed; fluorescent stains are also options.

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**Table 3.2** (continued)

Patient and/or situation	Test ordered <sup>a</sup>	Follow-up test ordered
Patient with unexplained eosinophilia	Although the O&P exam is recommended, the agar plate culture for <i>Strongyloides stercoralis</i> (more sensitive than the O&P exam) is also recommended, particularly if there is any history of pneumonia (migrating larvae in lungs) or of sepsis or meningitis (fecal bacteria carried by migrating larvae).	If tests are negative and symptoms continue, additional O&P exams and special tests for microsporidia (modified trichrome stains, Calcofluor white stains, fluorescent stains) and other coccidia (modified acid-fast stains, autofluorescence, fluorescent stains) should be performed.
Patient with diarrhea (suspected food-borne outbreak)	Test for <i>Cyclospora cayetanensis</i> (modified acid-fast stain, autofluorescence, fluorescent stains).	If tests are negative and symptoms continue, special procedures for microsporidia and other coccidia and O&P exam should be performed.

<sup>a</sup> Depending on the particular immunoassay kit used, various single or multiple organisms may be included. Selection of a particular kit depends on many variables: clinical relevance, cost, ease of performance, training, personnel availability, number of test orders, training of physician clients, sensitivity, specificity, equipment, time to result, etc. Very few laboratories handle this type of testing in exactly the same way. Many options are clinically relevant and acceptable for good patient care. It is critical that the laboratory report indicate specifically which organisms could be identified by the kit; a negative report should list the organisms relevant to that particular kit. **It is important to remember that sensitivity and specificity data for all of these fecal immunoassay kits (FA, EIA, and cartridge formats) are comparable.**

**Table 3.3** Preservatives and procedures commonly used in diagnostic parasitology (stool specimens)

Preservative	Concentration	Permanent stained smear	Immunoassays ( <i>G. lamblia</i> , <i>Cryptosporidium</i> spp.)	Comments
5% or 10% formalin	Yes	No	Yes	EIA, FA, cartridge
5% or 10% buffered formalin	Yes	No	Yes	EIA, FA, cartridge
MIF	Yes	Polychrome IV stain	ND <sup>e</sup>	No published data
SAF	Yes	Iron hematoxylin	Yes	EIA, FA, cartridge
PVA <sup>a</sup>	Yes, but rarely used	Trichrome or iron hematoxylin	No	PVA interferes with immunoassays
Modified PVA <sup>b</sup>	Yes, but rarely used	Trichrome or iron hematoxylin	No	PVA interferes with immunoassays
Modified PVA <sup>c</sup>	Yes	Trichrome or iron hematoxylin	Some but not all (rinse step may remove problematic compounds)	PVA interferes with immunoassays
Single-vial systems <sup>d</sup>	Yes	Trichrome or iron hematoxylin	Some, but not all	Check with the manufacturer
Schaudinn's fluid (without PVA) <sup>a</sup>	Yes, but rarely used	Trichrome or iron hematoxylin	No	Mercury interferes with immunoassays

<sup>a</sup> PVA and Schaudinn's fluid use the mercuric chloride base in the Schaudinn's fluid; this formulation is still considered to be the gold standard against which all other fixatives are evaluated (organism morphology after permanent staining). Additional fixatives prepared with non-mercuric-chloride-based compounds are used; however, the overall organism morphology is not as good.

<sup>b</sup> This modification uses a copper sulfate base rather than mercuric chloride.

<sup>c</sup> This modification uses a zinc base rather than mercuric chloride and works well with both trichrome and iron hematoxylin stains.

<sup>d</sup> These modifications use a combination of ingredients (including zinc) but are prepared from proprietary formulas. The aim is to provide a fixative that can be used for the fecal concentration, permanent stained smear, and available immunoassays for *G. lamblia*, *Cryptosporidium* spp., and *E. histolytica* (or the *E. histolytica/E. dispar* group).

<sup>e</sup> ND, no data.

**Table 3.4** Advantages of thin and thick blood films

<b>Advantages of thin blood films</b>	<b>Advantages of thick blood films</b>
RBC morphology (size, shape, stippling) can be seen after fixation with methanol prior to staining (Giemsa) or as a part of the staining process (Wright). RBCs are laked in the thick film.	Larger number of parasites are seen per field than in the thin blood film. RBCs are laked, so WBCs, platelets, and parasites are visible after staining.
Identification to <i>Plasmodium</i> spp. easier, since the parasite can be seen within the RBC. The size of the parasites within the RBCs can provide information necessary for identification to species.	Phagocytized malaria pigment can be seen within the WBCs, even with a low parasitemia.
Parasitemia (%) can be calculated from the thin film; determination of parasitemia is mandatory for all <i>Plasmodium</i> spp. and is particularly important to monitor therapy for <i>P. falciparum</i> . Parasitemia should be reported with every set of blood films positive for <i>Plasmodium</i> or <i>Babesia</i> .	Stippling may be seen in a well-stained thick film. However, this depends on how long the blood has been in contact with anticoagulant if not collected as a fresh specimen (finger stick).

**Table 3.5** Advantages and disadvantages of buffy coat films

<b>Advantages of buffy coat film</b>	<b>Disadvantages of buffy coat film</b>
Volume of blood cells is considerably larger than with both thick and thin films prepared from whole blood.	Practice is needed to remove the correct blood layers from the centrifuged blood to prepare thick and thin films.
More sensitive than thick films for diagnosis of malaria; detection of infected RBCs is more likely.	Same potential problems may occur as with traditional thick or thin blood films.
Malaria pigment, which is phagocytized by the WBCs, may be seen more easily in the concentrated WBCs.	Some organisms and/or stages of parasites might be damaged due to high centrifugation; however, centrifugation at $500 \times g$ for 15–20 min should not cause damage.
Detection of parasitemia is easier for larger stages such as schizonts and gametocytes.	Increased number of platelets may be confusing in terms of parasite differentiation.

**Table 3.6** Potential problems of using EDTA anticoagulant for the preparation of thin and thick blood films

Potential problem	Comments
Adhesion to the slide; blood falls off slide during staining.	Incorrect ratio of anticoagulant to blood; fill tube completely with blood (7 ml or pediatric draw tube).
Distortion of parasites; same type of distortion can also be seen after blood is refrigerated (not recommended).	Prolonged storage of blood in EDTA may lead to distortion (>1 h) and/or loss of parasites (4–6 h). Trophozoites ( <i>P. vivax</i> ) and gametocytes ( <i>P. falciparum</i> ) tend to round up, thus mimicking <i>P. malariae</i> .
Change in ring form size	Ring forms of <i>P. falciparum</i> continue to enlarge, thus resembling rings of the other species. Typical "small" rings appear larger than usual.
Use of incorrect techniques. EDTA is the anticoagulant used by the hematology laboratory because the cellular components and morphology of the blood cells are preserved. Blood smears for differentials from acceptable specimens should be prepared within 2 h of collection. Blood counts from acceptable venipuncture specimens should be performed within 6 h of collection.  Underfilling the EDTA blood collection tube can lead to erroneously low blood cell counts and hematocrits, morphologic changes to RBCs, and staining alteration.  Excess EDTA can shrink RBCs. Conversely, overfilling the blood collection tube does not allow the tube to be properly mixed and may lead to platelet clumping and clotting.	EDTA prevents coagulation of blood by chelating calcium. Calcium is necessary in the coagulation cascade and its removal inhibits and stops a series of events, both intrinsic and extrinsic, which cause clotting. In some individuals, EDTA may cause inaccurate platelet results. These anomalies, platelet clumping and the formation of platelet satellites, may be the result of changes in the membrane structure occurring when the calcium ion is removed by the chelating agent, allowing the binding of preformed antibodies. Proper mixing of the whole-blood specimen ensures that EDTA is dispersed throughout the sample. Evacuated blood collection tubes with EDTA should be mixed by 8–10 end-to-end inversions immediately following venipuncture collection. Microcollection tubes with EDTA should be mixed by 10 complete end-to-end inversions immediately following collection. They should then be inverted an additional 20 times prior to analysis.

(continued on next page)

**Table 3.6** (continued)

Potential problem	Comments
Loss of Schüffner's dots (stippling) in <i>P. vivax</i> and <i>P. ovale</i>	Schüffner's dots (true stippling) occur in both <i>P. vivax</i> and <i>P. ovale</i> ; in the absence of stippling, identification to the species level may be much more difficult.
Prolonged storage of EDTA-blood (room temperature with stopper removed)	The pH, CO <sub>2</sub> , and temperature changes may reflect conditions within the mosquito. Thus, exflagellation of the male gametocyte may occur while still in the tube of blood prior to thin and thick blood film preparation. Microgametes may be confused with <i>Borrelia</i> or may be ignored as debris. If fertilization occurs between the male and female gametocytes, the crescent-shaped ookinete may resemble a <i>P. falciparum</i> gametocyte.
Release of merozoites from the schizonts into the blood. Normally, merozoites are not found outside of the RBCs, in contrast to <i>Babesia</i> spp. where rings may be seen outside of the RBCs.	Small rings may be seen outside of the RBCs or appear to be appliqué forms, thus suggesting <i>P. falciparum</i> . It is important to differentiate these true rings (both cytoplasmic and nuclear colors) from platelets (uniform color).
Incorrect submission of blood in heparin	EDTA has less impact on parasite morphology than does heparin.

**Table 3.7** Body sites and possible parasites recovered<sup>a</sup>

Site	Parasites	Site	Parasites
Blood RBCs  WBCs  Whole blood/ plasma Bone marrow	<i>Plasmodium</i> spp. <i>Babesia</i> spp. <i>Leishmania donovani</i> <i>Toxoplasma gondii</i> <i>Trypanosoma</i> spp. Microfilariae <i>Leishmania donovani</i> <i>Trypanosoma cruzi</i> <i>Plasmodium</i> spp.	Intestinal tract (continued)	<i>Cyclospora cayetanensis</i> <i>Isospora belli</i> <i>Enterocytozoon bieneusi</i> <i>Encephalitozoon intestinalis</i> Microsporidia <i>Ascaris lumbicoides</i> <i>Enterobius vermicularis</i> Hookworm <i>Strongyloides stercoralis</i> <i>Trichuris trichiura</i> <i>Hymenolepis nana</i> <i>Hymenolepis diminuta</i> <i>Taenia saginata</i> <i>Taenia solium</i> <i>Diphyllobothrium latum</i> <i>Clonorchis sinensis</i> ( <i>Opisthorchis</i> ) <i>Metagonimus yokogawai</i> <i>Heterophyes heterophyes</i>
Central nervous system	<i>Taenia solium</i> (cysticerci) <i>Echinococcus</i> spp. <i>Naegleria fowleri</i> Acanthamoeba and <i>Hartmanella</i> spp. <i>Balamuthia mandrillaris</i> <i>Sappinia diploidea</i> <i>Toxoplasma gondii</i> Microsporidia <i>Trypanosoma</i> spp.	Liver, spleen	<i>Echinococcus</i> spp. <i>Entamoeba histolytica</i> <i>Leishmania donovani</i> Microsporidia
Cutaneous ulcers	<i>Leishmania</i> spp. Acanthamoeba spp. <i>Entamoeba histolytica</i>	Lungs	<i>Cryptosporidium</i> spp. <sup>b</sup> <i>Echinococcus</i> spp. <i>Paragonimus</i> spp. Microsporidia
Eyes	Acanthamoeba spp. <i>Toxoplasma gondii</i> <i>Loa loa</i> Microsporidia	Muscles	<i>Taenia solium</i> (cysticerci) <i>Trichinella</i> spp. <i>Onchocerca volvulus</i> (nodules) <i>Trypanosoma cruzi</i> Microsporidia
Intestinal tract	<i>Entamoeba histolytica</i> <i>Entamoeba dispar</i> <i>Entamoeba coli</i> <i>Entamoeba hartmanni</i> <i>Endolimax nana</i> <i>Iodamoeba bütschlii</i> <i>Blastocystis hominis</i> <i>Giardia lamblia</i> <i>Chilomastix mesnili</i> <i>Dientamoeba fragilis</i> <i>Pentatrichomonas hominis</i> <i>Balantidium coli</i> <i>Cryptosporidium parvum</i> <i>Cryptosporidium hominis</i> <i>Cryptosporidium</i> spp.	Skin	<i>Leishmania</i> spp. <i>Onchocerca volvulus</i> Microfilariae
		Urogenital system	<i>Trichomonas vaginalis</i> <i>Schistosoma</i> spp. Microsporidia Microfilariae

<sup>a</sup> This table does not include every possible parasite that could be found in a particular body site. However, the most likely organisms have been listed. Modified from L. S. Garcia, *Diagnostic Medical Parasitology*, 5th ed., ASM Press, Washington, DC, 2007.

<sup>b</sup> Disseminated in severely immunosuppressed individuals.

## S E C T I O N     4

# Specimen Test Options: Routine Diagnostic Methods and Body Sites

Diagnostic parasitology includes laboratory procedures that are designed to detect organisms within clinical specimens by using morphologic criteria and visual identification, rather than culture, biochemical tests, and/or physical growth characteristics. Many clinical specimens, such as those from the intestinal tract, contain numerous artifacts that complicate the differentiation of parasites from surrounding debris. Final identification is usually based on microscopic examination of stained preparations, often at high magnification such as oil immersion ( $\times 1,000$ ).

Specimen preparation often requires one of the concentration methods, all of which are designed to increase the chances of finding the organism(s). Microscopic examination requires review of the prepared clinical specimen by using multiple magnifications and different time frames; organism identification also depends on the skill of the microbiologist.

Protozoa are quite small and range from  $1.5\text{ }\mu\text{m}$  (microsporidia) to  $\sim 80\text{ }\mu\text{m}$  (*Balantidium coli* [ciliate]). Some are intracellular and require multiple isolation and staining methods for identification. Helminth infections are usually diagnosed by finding eggs, larvae, and/or adult worms in various clinical specimens, primarily those from the intestinal tract. Identification to the species level may require microscopic examination of the specimen. The recovery and identification of blood parasites can require concentration, culture, and microscopy. Confirmation of suspected parasitic infections depends on the proper collection, processing, and examination of clinical specimens; multiple specimens must often be submitted and examined before the suspected organism(s) is detected and confirmed (Table 4.1).

## Ova and Parasite Examination of Stool Specimens

The most common specimen submitted to the diagnostic laboratory is the stool specimen, and the most commonly performed procedure in parasitology is the ova and parasite examination (O&P exam), which consists of three separate protocols: the direct wet mount, the concentration, and the permanent stained smear. The direct wet mount requires fresh stool, is designed to allow detection of motile protozoan trophozoites, and is examined microscopically at low and high dry magnifications ( $\times 100$ , entire 22- by 22-mm coverslip;  $\times 400$ , one-third to one-half of a 22- by 22-mm coverslip). However, due to potential problems with lag time between the time of specimen passage, receipt in the laboratory, and specimen fixation, the direct wet examination has been eliminated from the routine O&P exam in favor of receipt of specimens collected in stool preservatives; if specimens are received in the laboratory in stool collection preservatives, the direct wet preparation is not performed, since no trophozoite motility would be visible.

The second part of the O&P exam is the concentration, which is designed to facilitate recovery of protozoan cysts, coccidian oocysts, microsporidial spores, and helminth eggs and larvae. Both flotation and sedimentation methods are available, the most common procedure being the formalin-ethyl acetate sedimentation method (formerly called the

formalin-ether method). The concentrated specimen is examined as a wet preparation, with or without iodine, using low and high dry magnifications ( $\times 100$  and  $\times 400$ , respectively) as indicated for the direct wet smear examination.

The third part of the O&P exam is the permanent stained smear, which is designed to facilitate identification of intestinal protozoa. Several staining methods are available, the two most common being the Wheatley modification of the Gomori tissue trichrome and the iron hematoxylin staining methods. This part of the O&P exam is critical for the confirmation of suspicious objects seen in the wet examination and for identification of protozoa that might not have been seen in the wet preparation. **The permanent stained smear is the most important procedure performed for the identification of intestinal protozoan infections;** the permanent stained smears are examined using oil immersion objectives ( $\times 600$  for screening,  $\times 1,000$  for final review of  $\geq 300$  oil immersion fields).

Trophozoites (potentially motile forms) of the intestinal protozoa are usually found in liquid specimens; both trophozoites and cysts might be found in soft specimens. The cyst forms are usually found in formed specimens; however, there are always exceptions to these general statements. Coccidian oocysts and microsporidian spores can be found in any type of fecal specimen; for *Cryptosporidium* spp., the more liquid the stool, the more oocysts that are found in the specimen. Helminth eggs may be found in any type of specimen, although the chances of finding eggs in a liquid stool sample are reduced by the dilution factor. Tapeworm proglottids may be found on or beneath the stool on the bottom of the collection container. Adult pinworms and *Ascaris lumbricoides* are occasionally found on the surface or in the stool.

Many laboratories prefer that stool specimens be submitted in some type of preservative. Rapid fixation of the specimen immediately after passage (by the patient) provides an advantage in terms of recovery and identification of intestinal protozoa. **This advantage (preservation of organisms before distortion or disintegration) is thought to outweigh the limited motility information that might be gained by examining fresh specimens as direct wet mounts.**

## Other Diagnostic Methods for Stool Specimens

Several other diagnostic techniques are available for the recovery and identification of parasitic organisms from the intestinal tract. Most laboratories do not routinely offer all of these techniques, but many of the tests are relatively simple and inexpensive to perform. The clinician should be aware of the possibilities and the clinical relevance of information obtained from using such techniques. Occasionally, it is necessary to examine stool specimens for the presence of scolices and proglottids of cestodes and adult nematodes and trematodes to confirm the diagnosis and/or for identification to the species level. A method for the recovery of these stages is also described in this section.

## Culture of Larval-Stage Nematodes

Nematode infections giving rise to larval stages that hatch in soil or in tissues may be diagnosed by using certain fecal culture methods to concentrate the larvae. *Strongyloides stercoralis* larvae are generally the most common larvae found in stool specimens. Depending on the fecal transit time through the intestine and the patient's condition, rhabditiform and rarely filariform larvae may be present. Also, if there is delay in examination of the stool, embryonated eggs as well as larvae of hookworm may be present. Culture of feces for larvae is useful to (i) reveal their presence when they are too scanty to be detected by concentration methods, (ii) distinguish whether the infection is due to *S. stercoralis* or hookworm on the basis of rhabditiform larval morphology by allowing hookworm egg hatching to occur, releasing first-stage larvae, and (iii) allow development of larvae into the filariform stage for further differentiation.

The use of certain fecal culture methods (sometimes referred to as coproculture) is especially helpful to detect light infections by hookworm, *S. stercoralis*, and *Trichostrongylus* spp. and for specific identification of parasites. The rearing of infective-stage nematode larvae also helps in the specific diagnosis of hookworm and trichostrongyle infections because the eggs of many of these species are identical and specific identifications are based on larval morphology. Additionally, such techniques are useful for obtaining a large number of infective-stage larvae for research purposes. Available diagnostic methods include the Harada-Mori filter paper strip culture, the Petri dish filter paper culture, the agar plate method (the most sensitive method for the recovery of *S. stercoralis*), the charcoal culture, and the Baermann concentration.

## Estimation of Worm Burdens through Egg Counts

The only human parasites for which it is reasonably possible to correlate egg production with adult worm burdens are *A. lumbricoides*, *Trichuris trichiura*, and the hookworms (*Necator americanus* and *Ancylostoma duodenale*). The specific instances in which information on approximate worm burdens is useful include determination of the intensity of infection, selection of chemotherapy, and evaluation of the efficacy of the drugs administered. However, with current therapy, the need for monitoring therapy through egg counts is no longer as relevant, and few laboratories perform this test. Egg counts are estimates only; count variations occur regardless of how carefully the procedure is followed. If two or more fecal specimens are being compared, it is best to have the same individual perform the technique on both samples and to do multiple counts.

The direct-smear method of Beaver is the easiest to use and is reasonably accurate when performed by an experienced technologist. A direct smear of 2 mg (enough fresh fecal material to form a low cone on the end of a wooden applicator stick) of stool is prepared. Egg counts on the direct smear are reported as eggs per smear, and the appropriate calculations can be made to determine the number of eggs per gram of stool.

The Stoll count is probably the most widely used dilution egg-counting procedure for the purpose of estimating worm burdens. However, cost containment and clinical relevance issues mean that most laboratories do not offer this procedure.

### **Hatching Test for Schistosome Eggs**

All fecal and urine specimens used for the hatching test must be collected and processed without using preservatives; any rinse steps must be performed with saline (not water, which may cause premature egg hatching). When schistosome eggs are recovered from either urine or stool, they should be carefully examined to determine viability. The presence of living miracidia within the eggs indicates an active infection that may require therapy. The viability of the miracidia can be determined in two ways: (i) the cilia of the flame cells (primitive excretory cells) may be seen on a wet smear by using high dry power and are usually actively moving and (ii) the miracidia may be released from the eggs by a hatching procedure. The eggs usually hatch within several hours when placed in 10 volumes of dechlorinated or spring water (hatching may begin soon after contact with the water). The eggs that are recovered in the urine (24-h specimen collected with no preservatives) are easily obtained from the sediment and can be examined under the microscope to determine viability.

### **Screening Stool Samples for Recovery of a Tapeworm Scolex**

Since the medication used for treatment of tapeworms is usually very effective, screening for tapeworm scolices is rarely requested and no longer clinically relevant. However, stool specimens may have to be examined for the presence of scolices and gravid proglottids of cestodes for proper species identification. This procedure requires mixing a small amount of feces with water and straining the mixture through a series of wire screens (graduated from coarse to fine mesh) to look for scolices and proglottids. Remember to use Standard Precautions and wear gloves when performing this procedure. Appearance of scolices after therapy is an indication of successful treatment. If the scolex has not been passed, it may still be attached to the mucosa; the parasite is capable of producing more segments from the neck region of the scolex, and the infection continues. If this occurs, the patient can be retreated when proglottids begin to reappear in the stool.

After treatment for tapeworm removal, the patient should be instructed to take a saline cathartic and to collect all stool material passed for the next 24 h. The stool material should be immediately placed in 10% formalin, thoroughly broken up, and mixed with the preservative (1-gal [3.8-liter] plastic jars are recommended, half full of 10% formalin).

### **Testing of Other Intestinal Tract Specimens**

Other specimens from the intestinal tract such as duodenal aspirates or drainage material, mucus from the Entero-Test Capsule technique, and

sigmoidoscopy material can also be examined as wet preparations and as permanent stained smears after being processed with either trichrome or iron hematoxylin staining. Although not all laboratories perform these procedures, the procedures are included to give some idea of the possibilities for diagnostic testing.

### **Examination for Pinworm**

A roundworm parasite that has worldwide distribution and is commonly found in children is *Enterobius vermicularis*, known as pinworm or seat-worm. The adult female worm migrates out of the anus, usually at night, and deposits her eggs on the perianal area. The adult female (8 to 13 mm long) is occasionally found on the surface of a stool specimen or on the perianal skin. Since the eggs are usually deposited around the anus, they are not commonly found in feces and must be detected by other diagnostic techniques. Diagnosis of pinworm infection is usually based on the recovery of typical eggs, which are described as thick-shelled, football-shaped eggs with one slightly flattened side. Each egg often contains a fully developed embryo and is infective within a few hours after being deposited. Unfortunately, it takes a minimum of four to six consecutive negative tapes or swabs before the infection can be ruled out.

### **Sigmoidoscopy Material**

Material obtained from sigmoidoscopy can be helpful in the diagnosis of amebiasis that has not been detected by routine fecal examinations; however, a series of at least three routine stool examinations for parasites should be performed on each patient before sigmoidoscopy examination is done. Material from the mucosal surface should be aspirated or scraped and should not be obtained with cotton-tipped swabs. At least six representative areas of the mucosa should be sampled and examined (six samples, six slides). The examination of sigmoidoscopy specimens does not take the place of routine O&P exams.

The specimen should be processed immediately. Three methods of examination can be used. All three are recommended; however, depending on the availability of trained personnel, proper fixation fluids, or the amount of specimen obtained, one or two procedures may be used. If the amount of material limits the examination to one procedure, the use of polyvinyl alcohol (PVA) fixative is highly recommended. If the material is to be examined using any of the new fluorescent antibody or enzyme immunoassay detection kits (*Cryptosporidium* spp. or *Giardia lamblia*), 5 or 10% formalin or sodium acetate-acetic acid-formalin (SAF) fixative is recommended. Many physicians performing sigmoidoscopy procedures do not realize the importance of selecting the proper fixative for material to be examined for parasites. For this reason, it is recommended that a parasitology specimen tray (containing Schaudinn's fixative, PVA, and 5 or 10% formalin) be provided or a trained technologist be available at the time of sigmoidoscopy to prepare the slides. Even the most thorough examination will be meaningless if the specimen has been improperly prepared.

## Duodenal Drainage Material

In infections with *G. lamblia* or *S. stercoralis*, routine stool examinations may not reveal the organisms. Duodenal drainage material can be submitted for examination, a technique that may reveal the parasites. The specimen should be submitted to the laboratory in a tube containing no preservative; the amount may vary from <0.5 ml to several milliliters of fluid. The specimen may be centrifuged (10 min at 500  $\times$  g) and should be examined immediately as a wet mount for motile organisms (iodine may be added later to facilitate identification of any organisms present). If the specimen cannot be completely examined within 2 h after it is taken, any remaining material should be preserved in 5 to 10% formalin. The "falling-leaf" motility often described for *Giardia* trophozoites is rarely seen in these preparations. The organisms may be caught in mucus strands, and the movement of the flagella on the *Giardia* trophozoites may be the only subtle motility seen for these flagellates. *Strongyloides* larvae are usually very motile. It is important to keep the light intensity low.

The duodenal fluid may contain mucus; this is where the organisms are usually found. Therefore, centrifugation of the specimen is important, and the sedimented mucus should be examined. Fluorescent antibody or enzyme immunoassay detection kits (for *Cryptosporidium* or *Giardia*) can also be used with fresh or formalinized material.

If a presumptive diagnosis of giardiasis is obtained on the basis of the wet preparation examination, the coverslip can be removed and the specimen can be fixed with either Schaudinn's fluid or PVA for subsequent staining with either trichrome or iron hematoxylin. If the amount of duodenal material submitted is very small, one can prepare permanent stains rather than using any of the specimen for a wet smear examination. Some workers think that this approach provides a more permanent record, and the potential problems with unstained organisms, very minimal motility, and a lower-power examination can be avoided by using oil immersion examination of the stained specimen at  $\times 1,000$  magnification.

## Duodenal Capsule Technique (Entero-Test)

A simple and convenient method of sampling duodenal contents that eliminates the need for intestinal intubation has been devised. The device consists of a length of nylon yarn coiled inside a gelatin capsule. The yarn protrudes through one end of the capsule; this end of the line is taped to the side of the patient's face. The capsule is then swallowed, the gelatin dissolves in the stomach, and the weighted string is carried by peristalsis into the duodenum. The yarn is attached to the weight by a slipping mechanism; the weight is released and passes out in the stool when the line is retrieved after 4 h. Bile-stained mucus clinging to the yarn is then scraped off (mucus can also be removed by pulling the yarn between thumb and finger) and collected in a small petri dish; disposable gloves are recommended. Usually 4 or 5 drops of material is obtained.

The specimen should be examined immediately as a wet mount for motile organisms (iodine may be added later to facilitate identification

of any organisms present). If the specimen cannot be completely examined within an hour after the yarn has been removed, the material should be preserved in 5 to 10% formalin or PVA-mucus smears should be prepared. The organism motility is like that described above for duodenal drainage specimens. The pH of the terminal end of the yarn should be checked to ensure adequate passage into the duodenum (a very low pH means that it never left the stomach). The terminal end of the yarn should be yellow-green, indicating that it was in the duodenum (the bile duct drains into the intestine at this point).

## Urogenital Tract Specimens

The identification of *Trichomonas vaginalis* is usually based on the examination of wet preparations of vaginal and urethral discharges and prostatic secretions or urine sediment. Multiple specimens may have to be examined to detect the organisms. These specimens are diluted with a drop of saline and examined under low power ( $\times 100$ ) and reduced illumination for the presence of actively motile organisms; as the jerky motility begins to diminish, it may be possible to observe the undulating membrane, particularly under high dry power ( $\times 400$ ). Stained smears are usually not necessary for the identification of this organism. The large number of false-positive and false-negative results reported on the basis of stained smears strongly suggests the value of confirmation by observation of motile organisms from the direct mount, from appropriate culture media, or from direct detection using more sensitive molecular methods.

Examination of urinary sediment may be indicated for specimens from patients with certain filarial infections. Administration of the drug diethylcarbamazine (Hetrazan) has been reported to enhance the recovery of microfilariae from the urine. The triple-concentration technique is recommended for the recovery of microfilariae. The membrane filtration technique can also be used with urine for the recovery of microfilariae. A membrane filter technique for the recovery of *Schistosoma haematobium* eggs has also been useful.

## Sputum

Although not one of the more common specimens, expectorated sputum may be submitted for examination for parasites. Organisms in sputum that may be detected and may cause pneumonia, pneumonitis, or Loefler's syndrome include the migrating larval stages of *Ascaris lumbricoides*, *Strongyloides stercoralis*, and hookworm; the eggs of *Paragonimus* spp.; *Echinococcus granulosus* hooklets; and the protozoa, *Pneumocystis jiroveci* (now classified with the fungi), *Entamoeba histolytica*, *Entamoeba gingivalis*, *Trichomonas tenax*, *Cryptosporidium* spp., and possibly the microsporidia. In a *Paragonimus* spp. infection, the sputum may be viscous and tinged with brownish flecks, which are clusters of eggs ("iron filings"), and may be streaked with blood. Sputum is usually examined as a wet mount (saline or iodine), using low and high dry power ( $\times 100$  and  $\times 400$ ). The spec-

imen is not concentrated before preparation of the wet mount. If the sputum is thick, an equal amount of 3% sodium hydroxide (NaOH) (or undiluted chlorine bleach) can be added; the specimen is thoroughly mixed and then centrifuged. NaOH should not be used if one is looking for *Entamoeba* spp. or *T. tenax*. After centrifugation, the supernatant fluid is discarded and the sediment can be examined as a wet mount with saline or iodine. If examination has to be delayed for any reason, the sputum should be fixed in 5 or 10% formalin to preserve helminth eggs or larvae or in PVA fixative to be stained later for protozoa.

Concentrated stained preparations of induced sputa are commonly used to detect *P. jiroveci* and differentiate trophozoite and cyst forms from other possible causes of pneumonia, particularly in an AIDS patient. Organisms must be differentiated from other fungi such as *Candida* spp. and *Histoplasma capsulatum*. If the clinical evaluation of a patient suggests *P. jiroveci* pneumonia and the induced sputum specimen is negative, a bronchoalveolar lavage specimen should be evaluated by using appropriate stains.

After patients have used appropriate cleansing procedures to reduce oral contamination, induced sputa are collected by pulmonary or respiration therapy staff. The induction protocol is critical for the success of the procedure, and well-trained individuals are mandatory if organisms are to be recovered.

## Aspirates

The examination of aspirated material for the diagnosis of parasitic infections may be extremely valuable, particularly when routine testing methods have failed to demonstrate the organisms. These specimens should be transported to the laboratory immediately after collection. Aspirates include liquid specimens collected from a variety of sites where organisms might be found. Those most commonly processed in the parasitology laboratory include fine-needle aspirates and duodenal aspirates. Fluid specimens collected by bronchoscopy include bronchoalveolar lavage fluid and bronchial washings.

Fine-needle aspirates may be submitted for slide preparation and/or culture. Aspirates of cysts and abscesses for amebae may require concentration by centrifugation, digestion, microscopic examination for motile organisms in direct preparations, and cultures and microscopic evaluation of stained preparations.

Bone marrow aspirates to be tested for *Leishmania* amastigotes, *Trypanosoma cruzi* amastigotes, or *Plasmodium* spp. require staining with any of the blood stains. Examination of these specimens may confirm an infection that has been missed by examination of routine blood films.

## Biopsy Specimens

Biopsy specimens are recommended for the diagnosis of tissue parasites. The following procedures may be used for this purpose in addition to standard histologic preparations: impression smears and teased and

squash preparations of biopsy tissue from skin, muscle, cornea, intestine, liver, lungs, and brain. Tissue to be examined by permanent sections or electron microscopy should be fixed as specified by the laboratories that will process the tissue. In certain cases, a biopsy may be the only means of confirming a suspected parasitic problem. Specimens to be examined as fresh material rather than as tissue sections should be kept moist in saline and submitted to the laboratory immediately.

Detection of parasites in tissue depends in part on specimen collection and having sufficient material to perform the recommended diagnostic procedures. Biopsy specimens are usually quite small and may not be representative of the diseased tissue. The use of multiple tissue samples often improves diagnostic results. To optimize the yield from any tissue specimen, all areas should be examined and as many procedures as possible should be used. Tissues are obtained by invasive procedures, many of which are very expensive and lengthy; consequently, these specimens deserve the most comprehensive procedures possible.

Tissue submitted in a sterile container on a sterile sponge dampened with saline may be used for cultures of protozoa after mounts for direct examination or impression smears for staining have been prepared. If cultures for parasites are to be made, sterile slides should be used for smear and mount preparation.

## Blood

Depending on the life cycle, a number of parasites may be recovered in a blood specimen, either whole blood, buffy coat preparations, or various types of concentrations. Although some organisms may be motile in fresh whole blood, species are usually identified from the examination of permanent stained blood films, both thick and thin films. Blood films can be prepared from fresh whole blood collected with no anticoagulants, anticoagulated blood, or sediment from the various concentration procedures. The recommended stain of choice is Giemsa stain; however, the parasites can also be seen on blood films stained with Wright's or other blood stains. Delafield's hematoxylin stain is often used to stain the microfilarial sheath; in some cases, Giemsa stain does not provide sufficient stain quality to allow differentiation of the microfilariae.

## Thin Blood Films

In any examination of thin blood films for parasitic organisms, the initial screen should be carried out with the low-power objective (10 $\times$ ) of a microscope. Microfilariae may be missed if the entire thin film is not examined. Microfilariae are rarely present in large numbers, and frequently only a few organisms occur in each thin-film preparation. Microfilariae are commonly found at the edges of the thin film or at the feathered end of the film because they are carried to these sites during the process of spreading the blood. The feathered end of the film where the erythrocytes (RBCs) are drawn out into one single, distinctive layer of cells should be examined for the presence of malaria parasites and

trypanosomes. In these areas, the morphology and size of the infected RBCs are most clearly seen.

Depending on the training and experience of the microscopist, examination of the thin film usually takes 15 to 20 min ( $\geq 300$  oil immersion fields) at a magnification of  $\times 1,000$ . Although some people use a  $50\times$  or  $60\times$  oil immersion objective to screen stained blood films, there is some concern that small parasites such as plasmodia, *Babesia* spp., or *Leishmania donovani* may be missed at this lower total magnification ( $\times 500$  or  $\times 600$ ) compared with the  $\times 1,000$  total magnification obtained when the more traditional  $100\times$  oil immersion objective is used. Because people tend to scan blood films at different rates, it is important to examine a minimum number of fields. If something suspicious has been seen in the thick film, considerably more than 300 fields are often examined on the thin film. The request for blood film examination should always be considered a STAT procedure, with all reports (negative as well as positive) being reported by telephone to the physician as soon as possible. If the results are positive, appropriate governmental agencies (local, state, and federal) should be notified within a reasonable time frame in accordance with guidelines and laws.

Both malaria and *Babesia* infections have been missed when automated differential instruments were used, and therapy was delayed. Although these instruments are not designed to detect intracellular blood parasites, the inability of the automated systems to discriminate between uninfected RBCs and those infected with parasites may pose serious diagnostic problems.

### **Thick Blood Films**

In the preparation of a thick blood film, the greatest concentration of blood cells is in the center of the film. The examination should be performed at low magnification to detect microfilariae more readily. Examination of a thick film usually requires 5 min (approximately 100 low and high dry fields). Search for malarial organisms and trypanosomes is best done under oil immersion (total magnification of  $\times 1,000$  [approximately 300 oil immersion fields]). Intact RBCs are frequently seen at the very periphery of the thick film; such cells, if infected, may prove useful in malaria diagnosis since they may demonstrate the characteristic morphology necessary to identify the organisms to the species level.

### **Blood Staining Methods**

For accurate identification of blood parasites, a laboratory should develop proficiency in the use of at least one good staining method. It is better to select one method that provides reproducible results than to use several on a hit-or-miss basis. Blood films should be stained as soon as possible, since prolonged storage may result in stain retention. Failure to stain positive malarial smears within a month may result in failure to demonstrate typical staining characteristics for individual species.

The most common stains are of two types. Wright's stain has the fixative in combination with the staining solution, so that both fixation and

staining occur at the same time; therefore, the thick film must be laked before staining. In Giemsa stain, the fixative and stain are separate; thus, the thin film must be fixed with absolute methanol before being stained. Other blood stains can also be used.

### Buffy Coat Films

*L. donovani*, trypanosomes, and *Histoplasma capsulatum* (a fungus with intracellular elements resembling those of *L. donovani*) are occasionally detected in the peripheral blood. The parasite or fungus is found in the large mononuclear cells in the buffy coat (a layer of white cells resulting from centrifugation of whole citrated blood). The nuclear material stains dark red-purple, and the cytoplasm stains light blue (*L. donovani*). *H. capsulatum* appears as a large dot of nuclear material (dark red-purple) surrounded by a clear halo area. Trypanosomes in the peripheral blood also concentrate with the buffy coat cells.

### QBC Microhematocrit Centrifugation Method

Microhematocrit centrifugation with use of the QBC malaria tube (glass capillary tube and closely fitting plastic insert [QBC malaria blood tubes; Becton Dickinson, Tropical Disease Diagnostics, Sparks, MD]) has been used for the detection of blood parasites. At the end of centrifugation of 50 to 60  $\mu\text{l}$  of capillary or venous blood (5 min in a QBC centrifuge, 14,387  $\times g$ ), parasites or RBCs containing parasites are concentrated into a small, 1- to 2-mm region near the top of the RBC column and are held close to the wall of the tube by the plastic float, thereby making them readily visible by microscopy. Tubes precoated with acridine orange provide a stain which induces fluorescence in the parasites. This method automatically prepares a concentrated smear which represents the distance between the float and the walls of the tube. Once the tube is placed into the plastic holder (Paraviewer) and immersion oil is applied to the top of the hematocrit tube (no coverslip is necessary), the tube is examined with a 40 $\times$  to 60 $\times$  oil immersion objective (the working distance must be 0.3 mm or greater).

### Knott Concentration

The Knott concentration procedure is used primarily to detect the presence of microfilariae in the blood, especially when a light infection is suspected. The disadvantage of the procedure is that the microfilariae are killed by the formalin and are therefore not seen as motile organisms.

### Membrane Filtration Technique

The membrane filtration technique using Nuclepore filters has proved highly efficient in demonstrating filarial infections when microfilaremias are of low density. It has also been successfully used in field surveys.

### Culture Methods

Very few clinical laboratories offer specific culture techniques for parasites. The methods for in vitro culture are often complex, while quality

control is difficult and not really feasible for the routine diagnostic laboratory. In certain institutions, some techniques may be available, particularly where consultative services are provided and for research purposes.

Few parasites can be routinely cultured, and the only procedures in general use are for *Entamoeba histolytica*, *Naegleria fowleri*, *Acanthamoeba* spp., *Trichomonas vaginalis*, *Toxoplasma gondii*, *Trypanosoma cruzi*, and the leishmanias. These procedures are usually available only after consultation with the laboratory and on special request.

Cultures of parasites grown in association with an unknown microbiota are referred to as xenic cultures. A good example of this type of culture would be stool specimens cultured for *E. histolytica*. If the parasites are grown with a single known bacterium, the culture is referred to as monoxenic. An example of this type of culture would be clinical specimens (corneal biopsy) cultured with *Escherichia coli* as a means of recovering species of *Acanthamoeba* and *Naegleria*. If parasites are grown as pure culture without any bacterial associate, the culture is referred to as axenic. An example of this type of culture would be the use of media for the isolation of *Leishmania* spp. or *T. cruzi*.

## Animal Inoculation and Xenodiagnosis

Most routine clinical laboratories do not have the animal care facilities necessary to provide animal inoculation capabilities for the diagnosis of parasitic infections. Host specificity for many animal parasite species is a well-known fact and limits the types of animals available for these procedures. For certain suspect infections, animal inoculation may be requested and can be very helpful in making the diagnosis, although animal inoculation certainly does not take the place of other, more routine procedures.

Xenodiagnosis uses the arthropod host as an indicator of infection. Uninfected reduviid bugs are allowed to feed on the blood of a patient who is suspected of having Chagas' disease (*T. cruzi* infection). After 30 to 60 days, feces from the bugs are examined over a 3-month time frame for the presence of developmental stages of the parasite, which are found in the hindgut of the vector. This type of procedure is used primarily in South America for field work, and the appropriate bugs are raised specifically for this purpose in various laboratories.

## Antibody and Antigen Detection

### Antibody Detection

In certain parasitic infections, the standard diagnostic laboratory procedures may not be sufficient to confirm infection or specimen collection may not be practical or cost-effective. In these circumstances, alternative methods may be helpful; these include antibody, antigen, and nucleic acid detection (Table 4.2). In some cases, serologic methods might be clinically indicated and may be very helpful, particularly if a parasitic infection is suspected and routine results are negative. However, even

with the most sophisticated technology, few serologic tests for parasitic infections can be used to confirm an infection or predict the disease outcome.

Although parasites and their by-products are immunogenic for the host, the host immune response is usually not protective. Any immunity that does develop is usually species specific and even strain or stage specific. Human parasites are generally divided into two groups: (i) those that multiply within the host (e.g., protozoa) and (ii) those that mature within the host but never multiply, e.g., schistosomes and *Ascaris*. In infections caused by protozoa that multiply within the host there is continuous antigenic stimulation of the host's immune system as the infection progresses. In these instances, there is usually a positive correlation between clinical symptoms and serologic test results.

In contrast to the protozoa, helminths often migrate through the body and pass through a number of developmental stages before becoming mature adults. Helminth infections are often difficult to confirm serologically, probably due to a limited antigenic response by the host or failure to use the appropriate antigen in the test system. Most parasitic antigens used in serologic procedures are heterogeneous mixtures that are not well defined. Results of tests performed with such antigens may represent cross-reactions or poor sensitivity.

Interpretation of test results may also present problems, particularly when one is dealing with patients from areas of endemic infection who may have higher baseline titers than do patients from other areas, in whom a low titer may actually be significant. Antibody detection generally indicates exposure to the parasite at some time in the past and may not necessarily reflect a current infection. This is particularly true when testing patients who have lived in an area of endemic infection for some time; their current clinical presentation may have no relationship to a positive antibody titer for a particular parasite. Although antibody levels generally decline over a period of months to years, serologic test results neither confirm nor rule out current infection or cure.

However, a positive serologic titer to a particular parasite in a patient who has had no previous exposure to the organism is clinically relevant and probably indicates recent exposure. The importance of a complete history, including both residence and travel information, is critical for accurate interpretation of serologic results.

Although serologic procedures have been available for many years, they are not routinely offered by most clinical laboratories for a number of reasons (cost, trained personnel, number of test orders, sensitivity, specificity, and interpretation). Standard techniques that have been used include complement fixation, indirect hemagglutination, indirect fluorescent-antibody, soluble-antigen fluorescent-antibody, bentonite flocculation, latex agglutination, double diffusion, counterelectrophoresis, immunoelectrophoresis, radioimmunoassay, and intradermal tests.

The Centers for Disease Control and Prevention (CDC) offer a number of serologic procedures for diagnostic purposes, some of which are not available elsewhere. Because regulations for submission of specimens may vary from state to state, each laboratory should check with its own county

or state department of public health for the appropriate instructions. Additional information on procedures, availability of skin test antigens, and interpretation of test results can be obtained directly from CDC by writing or calling

Serology Unit Parasitology Diseases Branch

Building 4, Room 1009

Mail Stop F13

Centers for Disease Control and Prevention

4770 Buford Highway

Atlanta, GA 30034

Serology: (770) 488-7760

Chagas' Disease and Leishmaniasis: (770) 488-4474

Malaria: (770) 488-7765.

## **Antigen Detection and Nucleic Acid-Based Tests**

Progress has been made in the development and application of molecular methods for diagnostic purposes including the use of purified or recombinant antigens and nucleic acid probes. The detection of parasite-specific antigen is more indicative of current disease. Many of the assays were originally developed with polyclonal antibodies which were targeted to unpurified antigens that markedly decreased the sensitivity and specificity of the tests. Fecal immunoassays are generally simple to perform and allow for a large number of tests to be performed at one time, thereby reducing overall costs. A **major disadvantage of antigen detection (fecal immunoassays)** in stool specimens is that the method can detect only one or two pathogens at a time. A routine O&P exam must be performed to detect other parasitic pathogens. The current commercially available antigen tests (direct and indirect fluorescent-antibody assays and enzyme immunoassay) are more sensitive and more specific than is routine microscopy. Current testing is available for *E. histolytica*, the *E. histolytica/E. dispar* group, *G. lamblia*, and *Cryptosporidium* spp. Diagnostic reagents are also in development for some of the other intestinal protozoa.

Nucleic acid-based diagnostic tests for parasitology are primarily available only in specialized research or reference centers. PCR and other nucleic acid probe tests have been developed for almost all species of parasites. The only nucleic acid-based probe test commercially available is for the detection of *T. vaginalis*. As the costs of these tests decrease and the various steps necessary to perform the tests become automated, there will be increasing demand for commercially available reagents.

## **Intradermal Tests**

In the absence of reliable serologic diagnostic tests, skin tests have been used to provide indirect evidence of infection. However, most skin tests have been used primarily for research and epidemiologic purposes. Some of the more commonly used skin tests are the Casoni (hydatid disease) and Montenegro (*L. donovani*) tests. In many cases, the antigens used are difficult to obtain and are not commercially available. The antigens are

usually crude extracts that have not been standardized and are neither highly sensitive nor specific. They may provoke an immune response that complicates further serologic testing, and there is always the danger of provoking an anaphylactic reaction. In addition, there are ethical questions related to giving patients injections of nonstandardized foreign protein, particularly if the antigens were derived from *in vivo* materials.

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**Table 4.1** Body site, procedures and specimens, recommended methods and relevant parasites, and comments

Body site	Procedures <sup>a</sup> and specimens	Recommended methods and relevant parasites <sup>a</sup>	Comments
Blood	Microscopy <sup>b</sup> : thin and thick blood films; fresh blood (preferred) or EDTA-blood (fill EDTA tube completely with blood, then mix)	Giemsa stain (all blood parasites); hematoxylin-based stain (sheathed microfilariae). For malaria, thick and thin blood films are definitely recommended and should be prepared within 30–60 min of blood collection via venipuncture (other tests may be used as well). Wright-Giemsa stain or DiffQuik (rapid stains) can also be used.	Most drawings and descriptions of blood parasites are based on Giemsa-stained blood films. Although Wright's stain (or Wright-Giemsa combination stain) works, stippling in malaria may not be visible and the organism colors do not match the descriptions. However, with other stains (those listed above, in addition to some of the "rapid" blood stains), the organisms should be detectable on the blood films. The use of blood collected with anticoagulant (rather than fresh) has direct relevance to the morphology of malaria organisms seen in peripheral blood films. If the blood smears are prepared after more than 1 h, stippling may not be visible, even if the correct pH buffers are used. Also, if blood is kept at room temperature (with the stopper removed), the male microgametocyte may exflagellate and fertilize the female macrogametocyte; development continues within the tube of blood (as it would in the mosquito host). The ookinete may actually resemble a <i>P. falciparum</i> gametocyte. The microgamete may resemble spirochetes.
	Concentration methods: EDTA-blood	Buffy coat, fresh blood films for detection of moving microfilariae or trypanosomes. QBC, a screening method for blood parasites (hematocrit tube contains acridine orange), has been used for malaria parasites, <i>Babesia</i> , trypanosomes, and microfilariae. It is usually impossible to identify malaria parasites to the species level; this requires high levels of training.	
	Antigen detection: EDTA-blood for malaria, serum or plasma for circulating antigens (hemolyzed blood can interact in some tests)	Commercial test kits for malaria and some microfilariae	

	<p>PCR: EDTA-blood, ethanol-fixed or unfixed thin and thick blood films, coagulated blood; possible with hemolyzed or frozen blood samples</p> <p>Specific antibody detection: serum or plasma, anticoagulated or coagulated blood (hemolyzed blood can cause problems in some tests)</p>	<p>Sensitivity not higher than thick films for <i>Plasmodium</i> spp., much more sensitive for <i>Leishmania</i> (peripheral blood is used from immunodeficient patients only). Sequencing of PCR product is often used for species or genotype identification.</p> <p>Most commonly used are EIA (many test kits commercially available), EITB (commercially available for some parasites), and IFA.</p>	<p>So far no commercial tests available; high laboratory standards are needed (may work with frozen, coagulated, or hemolyzed blood samples).</p> <p>Many labs are using in-house tests; only a few fully defined antigens are available; sensitivities and specificities of the tests should be documented by the lab.</p>
Bone marrow	<p>Biopsy specimens or aspirates</p> <p>Microscopy: thin and thick films with aspirate collected in EDTA</p> <p>Cultures: sterile material in EDTA or culture medium</p> <p>PCR: aspirate in EDTA</p>	<p>Giemsa stain (all blood parasites)</p> <p>Culture for <i>Leishmania</i> (or <i>Trypanosoma cruzi</i>)</p> <p>PCR for blood parasites including <i>Leishmania</i>, <i>Toxoplasma</i>, and rare other parasites</p>	<p><i>Leishmania</i> amastigotes are recovered in cells of the reticuloendothelial system. If films are not prepared directly after sample collection, infected cells may disintegrate. Sensitivity of microscopy is low; use only in combination with other methods.</p>

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**Table 4.1** Body site, procedures and specimens, recommended methods and relevant parasites, and comments (*continued*)

<b>Body site</b>	<b>Procedures<sup>a</sup> and specimens</b>	<b>Recommended methods and relevant parasites<sup>a</sup></b>	<b>Comments</b>
Central nervous system	<p>Microscopy: spinal fluid and CSF (wet examination, stained smears), brain biopsy specimen (touch or squash preparations, stained)</p> <p>Culture: sterile aspirate or biopsy material (in physiologic NaCl)</p> <p>PCR: aspirate or biopsy material (fresh, frozen, or fixed in ethanol)</p>	<p>Stains: Giemsa (trypanosomes, <i>Toxoplasma</i>); Giemsa, trichrome, or calcofluor (amebae [<i>Naegleria</i> or <i>Sappinia</i> PAM, <i>Acanthamoeba</i> or <i>Balamuthia</i> GAE]); Giemsa, acid-fast, PAS, modified trichrome, silver methenamine (microsporidia) (tissue Gram stains also recommended for microsporidia in routine histologic preparations); H&amp;E, routine histology (larval cestodes, <i>Taenia solium</i> cysticerci, <i>Echinococcus</i> spp.)</p> <p>Free-living amebae (exception: <i>Balamuthia</i> does not grow in the routine agar/bacterial overlay method), microsporidia, and <i>Toxoplasma</i></p> <p>Protozoa and helminths, species and genotype characterization</p>	If CSF is received (with no suspect organism suggested), Giemsa would be the best choice; however, modified trichrome or calcofluor is also recommended as a second stain (amebic cysts, microsporidia). If brain biopsy material is received (particularly from an immunocompromised patient), cultivation is recommended for microsporidia isolation and PCR for identification to the species or genotype level. A small amount of the sample should always be stored frozen for PCR analyses in case the results of the other methods are inconclusive.
Cutaneous ulcers	<p>Microscopy: aspirate, biopsy (smears, touch or squash preparations, histological sections)</p> <p>Cultures (less common)</p> <p>PCR: aspirate, biopsy material (fresh, frozen, or fixed in ethanol)</p>	<p>Giemsa (<i>Leishmania</i>); H&amp;E, routine histology (<i>Acanthamoeba</i> spp., <i>Entamoeba histolytica</i>)</p> <p><i>Leishmania</i>, free-living amebae (often bacterial contaminations)</p> <p><i>Leishmania</i> (species identification), free-living amebae</p>	<p>Most likely causative parasites would be <i>Leishmania</i> spp., which would stain with Giemsa. PAS could be used to differentiate <i>Histoplasma capsulatum</i> from <i>Leishmania</i> in tissue.</p> <p>In the immunocompromised patient, skin ulcers have been documented to have amebae as causative agents.</p>

Eyes	<p>Microscopy: smears; touch or squash preparations; biopsy, scrapings, contact lens, sediment of lens solution</p> <p>Culture: fresh material (see above) in PBS supplemented with antibiotics if possible to suppress bacterial growth</p> <p>PCR: fresh material in physiological NaCl or PBS, ethanol or frozen</p>	<p>Calcofluor, cyst only (amebae [<i>Acanthamoeba</i>]); Giemsa trophozoites, cysts (amebae); modified trichrome (preferred) or silver methenamine stain, PAS, acid-fast stains (microsporidial spores); H&amp;E, routine histology (cysticerci, <i>Loa loa</i>, <i>Toxoplasma</i>)</p> <p>Cultures: free-living amebae, <i>Toxoplasma</i>, microsporidia</p> <p>Free-living amebae, <i>Toxoplasma</i>, microsporidia species and genotype identification</p>	<p>Some free-living amebae (most commonly <i>Acanthamoeba</i>) have been implicated as a cause of keratitis. Although calcofluor stains the cyst walls, it does not stain the trophozoites. Therefore, for suspected cases of amebic keratitis, both stains should be used. H&amp;E (routine histology) can be used to detect and confirm cysticercosis. The adult worm of <i>Loa loa</i>, when removed from the eye, can be stained with a hematoxylin-based stain (Delafield's) or can be stained and examined by routine histology.</p> <p>Microsporidia confirmation to the species or genotype level may be done by PCR and sequence analyses; however, the spores could be found by routine light microscopy with modified trichrome, calcofluor, and/or tissue Gram stains.</p>
Intestinal tract	<p>Stool and other intestinal material</p> <p>Microscopy: stool, sigmoidoscopy material, duodenal contents (all fresh or preserved), direct wet smear, concentration methods</p>	<p>Concentration methods: ethyl acetate sedimentation of SAF-fixed stool samples (most protozoa); flotation or combined sedimentation flotation methods (helminth ova); agar or Baermann concentration (larvae of <i>Strongyloides</i> spp., fresh stool required)</p> <p>Direct wet smear (direct examination of unpreserved fresh material is also used) (motile protozoan trophozoites; helminth eggs and protozoan cysts may also be detected)</p>	<p>Stool fixation with formalin or formalin-containing fixatives preserves parasite morphology, allows prolonged storage (room temperature) and long transportation, and prevents hatching of <i>Schistosoma</i> eggs, but makes <i>Strongyloides</i> larval concentration difficult and impedes further PCR analyses.</p> <p>Taeniid eggs cannot be identified to the species level.</p>

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**Table 4.1** Body site, procedures and specimens, recommended methods and relevant parasites, and comments (*continued*)

Body site	Procedures <sup>a</sup> and specimens	Recommended methods and relevant parasites <sup>a</sup>	Comments
	<p>Anal impression smear</p> <p>Adult worms or tapeworm segments (proglottids)</p> <p>Antigen detection (fresh or frozen material, suitability of fixation is test dependent)</p> <p>PCR: fresh, frozen, or ethanol fixed material</p>	<p>Stains: trichrome or iron hematoxylin (intestinal protozoa); modified trichrome (microsporidia); modified acid-fast (<i>Cryptosporidium</i>, <i>Cyclospora</i>, and <i>Isospora</i>)</p> <p>Adhesive cellulose tape, no stain (<i>Enterobius vermicularis</i>)</p> <p>Carmine stains (rarely used for adult worms or cestode segments). Proglottids can usually be identified to the genus level (<i>Taenia</i>, <i>Diphyllobothrium</i>, <i>Hymenolepis</i>) without using tissue stains</p> <p>Commercial immunoassays, e.g., EIA, FA, cartridge formats (<i>E. histolytica</i>, the <i>E. histolytica/E. dispar</i> group, <i>G. lamblia</i>, <i>Cryptosporidium</i> spp.); in-house tests for <i>T. solium</i> and <i>T. saginata</i></p> <p>No commercial tests available. Primers for genus or species identification of most helminths and protozoa are published.</p>	<p>Microsporidia: confirmation to the species or genotype level requires PCR; however, modified trichrome and/or calcofluor stains can be used to confirm the presence of spores.</p> <p>Four to six consecutive negative tapes are required to rule out infection with pinworm (<i>E. vermicularis</i>).</p> <p>Worm segments can be stained with special stains. However, after dehydration through alcohols and xylenes (or xylene substitutes) without prior staining, the sexual organs and the branched uterine structure are visible, allowing identification of the proglottid to the species level.</p> <p>Coproantigens can be detected in the prepatent period and independently from egg excretion.</p> <p>Due to potential inhibition after DNA extraction from stool samples, concentration or isolation methods may be required prior to DNA extraction. However, new DNA isolation kits facilitate the isolation of high-quality DNA from stool. Sequence analyses may be required for species or genotype identification.</p>

	<p>Biopsy specimens</p> <p>Microscopy: fixed for histology or touch or squash preparations for staining</p> <p>PCR: see above</p>	<p>H&amp;E, routine histology (<i>E. histolytica</i>, <i>Cryptosporidium</i>, <i>Cyclospora</i>, <i>Isospora belli</i>, <i>Giardia</i>, microsporidia); less common findings would include <i>Schistosoma</i> spp., hookworm, or <i>Trichuris</i>.</p>	<p>Special stains may be helpful for the identification of microsporidia: tissue Gram stains, silver stains, PAS, and Giemsa or modified acid-fast stains for the coccidia.</p>
Liver and spleen	<p>Biopsy specimens or aspirates</p> <p>Microscopy: unfixed material in physiological NaCl; fixed for histology</p> <p>Culture: sterile preparation of fresh material</p> <p>Animal inoculation: sterile preparation of fresh material</p> <p>PCR: fresh, frozen, or ethanol fixed</p>	<p>Examination of wet smears for <i>E. histolytica</i> (trophozoites), protoscolices of <i>Echinococcus</i> spp., or eggs of <i>Capillaria hepatica</i>; Giemsa (<i>Leishmania</i>, other protozoa and microsporidia); H&amp;E (routine histology)</p> <p>For <i>Leishmania</i> (not common)</p> <p>Intraperitoneal inoculation of <i>Echinococcus multilocularis</i> cyst material for viability test after long-term chemotherapy</p> <p>Species or genotype identification (e.g., <i>Echinococcus</i> spp.)</p>	<p>There are definite risks associated with punctures (aspirates and/or biopsy) of spleen or liver lesions (<i>Echinococcus</i>). Always keep a small amount of material frozen for PCR.</p>
Respiratory tract	<p>Sputum, induced sputum, nasal and sinus discharge, bronchoalveolar lavage, transbronchial aspirate, tracheobronchial aspirate, brush biopsy specimen, open-lung biopsy specimen</p> <p>Microscopy: unfixed material, treated for smear preparation</p> <p>PCR: fresh, frozen, or fixed in ethanol.</p>	<p>Helminth larvae (<i>Ascaris</i>, <i>Strongyloides</i>), eggs (<i>Paragonimus</i>, <i>Capillaria</i>) or hooklets (<i>Echinococcus</i>) can be recovered in unstained respiratory specimens.</p> <p>Stains: Giemsa for many protozoa including <i>Toxoplasma</i> tachyzoites, modified acid-fast stains (<i>Cryptosporidium</i>); modified trichrome (microsporidia)</p> <p>Routine histology (H&amp;E; silver methenamine stain, PAS, acid-fast stains, tissue Gram stains for helminths, protozoa, and microsporidia)</p>	<p>Immunoassay reagents (FA) are available for the diagnosis of pulmonary cryptosporidiosis. Routine histologic procedures allow the identification of any of the helminths or helminth eggs present in the lungs. Disseminated toxoplasmosis or microsporidiosis is well documented, with organisms being found in many different respiratory specimens.</p>

(continued on next page)

**Table 4.1** Body site, procedures and specimens, recommended methods and relevant parasites, and comments (*continued*)

<b>Body site</b>	<b>Procedures<sup>a</sup> and specimens</b>	<b>Recommended methods and relevant parasites<sup>a</sup></b>	<b>Comments</b>
Muscle	Biopsy material Microscopy: touch and squash preparations, unfixed or fixed for histology and EM  PCR: fresh, frozen, or ethanol fixed	Larvae of <i>Trichinella</i> spp. can be identified unstained (species identification with single larva by PCR). H&E, routine histology ( <i>Trichinella</i> spp., cysticerci); silver methenamine stain, PAS, acid-fast stains, tissue Gram stains, EM (rare microsporidia)  Microsporidia identification to the species level requires subsequent sequencing.	If <i>Trypanosoma cruzi</i> is present in the striated muscle, the organisms could be identified by routine histology preparations. Modified trichrome and/or calcofluor stains can be used to confirm the presence of microsporidial spores.
Skin	Aspirates, skin snips, scrapings, biopsy specimens  Microscopy: wet examination, stained smear (or fixed for histology or EM)  PCR: fresh, frozen, or fixed in ethanol	See cutaneous ulcer (above).  Wet preparations (microfilariae), Giemsa-stained smears or H&E, routine histology ([ <i>Onchocerca volvulus</i> , <i>Dipetalonema streptocerca</i> , <i>Dirofilaria repens</i> , other larvae causing cutaneous larva migrans, zoonotic <i>Strongyloides</i> spp., hookworms], <i>Leishmania</i> , <i>Acanthamoeba</i> spp., <i>Entamoeba histolytica</i> , microsporidia and arthropods [ <i>Sarcopes</i> and other mites])  Primers for most parasite species are available.	Any of the potential parasites present can be identified by routine histology procedures.
Amniotic fluid	PCR (and/or culture): fresh material Animal inoculation (toxoplasmosis)	PCR based on the detection of highly repetitive gene sequences is the method of choice.	Only applicable to confirm suspected prenatal <i>Toxoplasma</i> infections

Urogenital system	<p>Vaginal discharge, saline swab, transport swab (no charcoal), air-dried smear for FA, urethral discharge, prostatic secretions, urine (single unpreserved, 24-h unpreserved, or early-morning specimens)</p> <p>Microscopy: wet smears, smears of urine sediment, stained smears</p> <p>Cultivation: vaginal or urethral discharge or swab preparations</p> <p>PCR: fresh, frozen, or fixed in ethanol</p>	<p>Giemsa, immunoassay reagents (FA, rapid lateral-flow test) (<i>Trichomonas vaginalis</i>); Delafield's hematoxylin (microfilariae); modified trichrome (microsporidia); H&amp;E, routine histology PAS, acid-fast stains, tissue Gram stains (microsporidia); direct examination of urine sediment for <i>Schistosoma haematobium</i> eggs or microfilariae</p> <p>Identification and propagation of <i>T. vaginalis</i> (commercialized plastic envelope culture systems available); moving trophozoites can be detected using microscopy (or in Giemsa-stained smears).</p>	<p>Although <i>T. vaginalis</i> is probably the most common parasite identified, there are others to consider, the most recently implicated organisms being in the microsporidian group. Microfilariae could also be recovered and stained. Fixation of urine with formalin prevents hatching of <i>Schistosoma</i> eggs.</p> <p>Material must be put into culture medium immediately after collection; it should not be cooled or frozen.</p>
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<sup>a</sup> CSF, cerebrospinal fluid; EIA, enzyme immunoassay; EM, electron microscopy; EITB, enzyme-linked immunoelectro transfer blot (Western blot); FA, fluorescent antibody; GAE, granulomatous amebic encephalitis; GI, gastrointestinal; H&E, hematoxylin and eosin; PAM, primary amebic encephalitis; PAS, periodic acid-Schiff stain; PBS, phosphate-buffered saline; QBC, quantitative buffy coat; SAF, sodium acetate-acetic acid-formalin. Although Giemsa stain is mentioned, any blood stain could be used in these circumstances.

<sup>b</sup> Many parasites or parasite stages may be detected in standard histologic sections of tissue material. However, species identification is difficult and additional examinations may be required. Usually, these techniques are not considered first-line methods. Additional methods such as electron microscopy are carried out only by specialized laboratories and are not available for standard diagnostic purposes. Electron microscopy examination for species identification has largely been replaced by PCR assays.

**Table 4.2** Serologic, antigen, and probe tests used in the diagnosis of parasitic infections

Disease	Routine antibody tests <sup>a</sup>	Antigen or probe tests <sup>b</sup>
<b>Protozoa</b>		
Amebiasis	EIA, IHA	EIA, IFA, PCR <sup>c</sup>
Babesiosis	IFA	PCR
Chagas' disease	CF, EIA, IFA	PCR
Cryptosporidiosis		DFA, EIA, IFA, PCR, Rapid
Giardiasis		DFA, EIA, PCR, Rapid
Leishmaniasis	EIA, IFA	PCR, Rapid
Malaria	IFA	PCR, Rapid
Microsporidiosis		IFA <sup>c</sup>
Toxoplasmosis	EIA, IFA, LA	PCR
Trichomoniasis		DFA, LA, DNA probe, Rapid
Trypanosomiasis (African)	CA, IFA	PCR
<b>Helminths</b>		
Cysticercosis	EIA, IB	
Echinococcosis	EIA, IB	
Fascioliasis	EIA, IB	
Filariasis	EIA	Rapid
Paragonimiasis	EIA, IB	
Schistosomiasis	EIA, IB	EIA
Strongyloidiasis	EIA	
Toxocariasis	EIA	
Trichinellosis	BF, EIA	

<sup>a</sup> BF, bentonite flocculation; CA, card agglutination; CF, complement fixation; EIA, enzyme immunoassay; IB, immunoblot; IHA, indirect hemagglutination; IFA, indirect fluorescent antibody.

<sup>b</sup> DFA, direct fluorescent antibody; EIA, enzyme immunoassay; IFA, indirect fluorescent antibody; LA, latex agglutination; Rapid, lateral-flow cartridge.

<sup>c</sup> Reagents are not commercially available (includes all PCR tests and IFA for microsporidia only).

S E C T I O N     5

Specific Test Procedures  
and Algorithms

# Microscopy

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## CALIBRATION OF THE MICROSCOPE

### Description

The identification of protozoa and other parasites depends on several factors, one of which is size. Any laboratory doing diagnostic work in parasitology should have a calibrated microscope available for precise measurements. Measurements are made using a micrometer disk that is placed in the ocular of the microscope; the disk is usually calibrated as a line divided into 50 units. Depending on the objective magnification used, the divisions in the disk represent different measurements. The ocular disk division must be compared with a known calibrated scale, usually a stage micrometer with a scale of 0.1- and 0.01-mm divisions.

### Supplies

1. Ocular micrometer disk (line divided into 50 units) (any laboratory supply distributor: Fisher, Baxter, Scientific Products, VWR, etc.)
2. Stage micrometer with a scale of 0.1- and 0.01-mm divisions (Fisher, Baxter, Scientific Products, VWR, etc.)
3. Immersion oil
4. Lens paper

### Equipment

1. Binocular microscope with 10 $\times$ , 40 $\times$ , and 100 $\times$  objectives. Other objective magnifications may also be used (50 $\times$  oil or 60 $\times$  oil immersion lenses).
2. Oculars should be 10 $\times$ . Some may prefer 5 $\times$ ; however, lower magnification may make final identifications more difficult.
3. Single 10 $\times$  ocular should be used to calibrate all laboratory microscopes (to be used when any organism is being measured).

### Quality Control

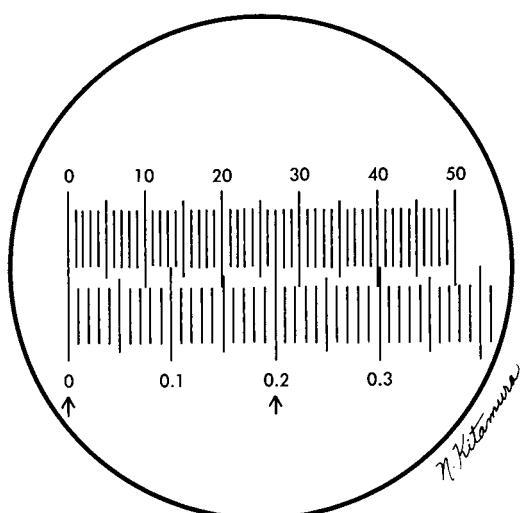
1. If the microscope receives heavy use or is moved throughout the laboratory, it should probably be recalibrated once each year.
2. Often the measurement of red blood cells (RBCs) (approximately 7.5  $\mu\text{m}$ ) is used to check the calibrations of the three magnifications ( $\times 100$ ,  $\times 400$ , and  $\times 1,000$ ).
3. Latex or polystyrene beads of a standardized diameter can be used to check the calculations and measurements (Sigma, J. T. Baker, etc.). Beads of 10 and 90  $\mu\text{m}$  are recommended.
4. All measurements should be recorded in Quality Control records.

### Detailed Procedure

1. Unscrew the eye lens of a 10 $\times$  ocular and place the micrometer disk (engraved side down) within the ocular. Use lens paper (at least dou-

ble thickness) to handle the disk; keep all surfaces free of dust or lint.

2. Place the calibrated micrometer on the stage, and focus on the scale. You should be able to distinguish the difference between the 0.1- and 0.01-mm divisions. Make sure you understand the divisions on the scale before proceeding.
3. Adjust the stage micrometer so that the "0" line on the ocular micrometer is exactly lined up on top of the "0" line on the stage micrometer.
4. After these two "0" lines are lined up, do not move the stage micrometer any farther. Look to the right of the "0" lines for another set of lines that is superimposed. The second set of lines should be as far to the right of the "0" lines as possible; however, the distance varies with the objectives being used (Figure 5.1).
5. Count the number of ocular divisions between the "0" lines and the point where the second set of lines is superimposed. Then, on the stage micrometer, count the number of 0.1-mm divisions between the "0" lines and the second set of superimposed lines.
6. Calculate the portion of a millimeter that is measured by a single small ocular unit.
7. When the high dry and oil immersion objectives are used, the "0" line of the stage micrometer increases in size whereas the ocular "0" line remains the same size. The thin ocular "0" line should be lined up in the center or at one edge of the broad stage micrometer "0" line. Thus, when the second set of superimposed lines is found, the thin ocular line should be lined up in the center or at the corresponding edge of the broad stage micrometer line.



**Figure 5.1** Ocular micrometer, top scale; stage micrometer, bottom scale. Reprinted from *Diagnostic Medical Parasitology*, 5th ed.

## CALIBRATION OF THE MICROSCOPE (*continued*)

### EXAMPLE:

A. 
$$\frac{\text{Stage reading (mm)}}{\text{Ocular reading}} \times \frac{1,000 \mu\text{m}}{1 \text{ mm}} = \text{Ocular units } (\mu\text{m})$$

B. Low power (10 $\times$ ):

$$\frac{0.8 \text{ mm}}{100 \text{ units}} \times \frac{1,000 \mu\text{m}}{1 \text{ mm}} = 8.0 \mu\text{m} \text{ (factor)}$$

C. High dry power (40 $\times$ ):

$$\frac{0.1 \text{ mm}}{50 \text{ units}} \times \frac{1,000 \mu\text{m}}{1 \text{ mm}} = 2.0 \mu\text{m} \text{ (factor)}$$

D. Oil immersion (100 $\times$ ):

$$\frac{0.05 \text{ mm}}{62 \text{ units}} \times \frac{1,000 \mu\text{m}}{1 \text{ mm}} = 0.8 \mu\text{m} \text{ (factor)}$$

**EXAMPLE:** If a helminth egg measures 15 ocular units by 7 ocular units (high dry objective), using the factor of 2.0  $\mu\text{m}$ , the egg measures 30 by 14  $\mu\text{m}$  and is probably *Clonorchis sinensis*.

**EXAMPLE:** If a protozoan cyst measures 27 ocular units (oil immersion objective), using the factor of 0.8  $\mu\text{m}$ , the cyst measures 21.6  $\mu\text{m}$ .

### Reporting

1. For each objective magnification, a factor will be generated (1 ocular unit = certain number of microns).
2. If standardized latex or polystyrene beads or an RBC is measured using various objectives, the size for the object measured should be the same (or very close), regardless of the objective magnification.
3. The multiplication factor for each objective should be posted (either on the base of the microscope or on a nearby wall or bulletin board) for easy reference.
4. Once the number of ocular lines per width and length of the organism is measured, then, depending on the objective magnification, the factor (1 ocular unit = certain number of microns) can be applied to the number of lines to obtain the width and length of the organism.
5. Comparison of these measurements with reference measurements in various books and manuals should confirm the organism identification.

## **Procedure Reminders**

1. The final multiplication factors are only as good as your visual comparison of the ocular "0" and stage micrometer "0" lines.
2. As a rule of thumb, the high dry objective (40 $\times$ ) factor should be approximately 2.5 times the factor obtained from the oil immersion objective (100 $\times$ ). The low-power objective (10 $\times$ ) factor should be approximately 10 times that seen using the oil immersion objective (100 $\times$ ).

## **Procedure Limitations**

1. After each objective has been calibrated, the oculars containing the disk and/or these objectives cannot be interchanged with corresponding objectives or oculars on another microscope.
2. Each microscope used to measure organisms must be calibrated as a unit. The original oculars and objectives that were used to calibrate the microscope must also be used when an organism is measured.
3. The objective containing the ocular micrometer can be stored until needed. This single ocular can be inserted when measurements are taken. However, this particular ocular containing the ocular micrometer disk must have also been used as the ocular during microscope calibration.

## **Ova and Parasite Examination**

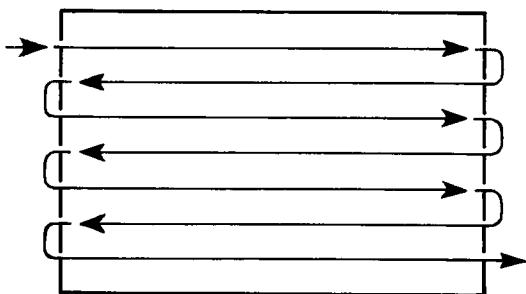
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### **DIRECT WET FECAL SMEAR**

#### **Description**

Normal mixing in the intestinal tract usually ensures an even distribution of organisms. However, depending on the level of infection, examination of the fecal material as a direct smear may or may not reveal organisms. The direct wet smear is prepared by mixing a small amount of stool (about 2 mg) with a drop of 0.85% NaCl; this mixture provides a uniform suspension under a 22- by 22-mm coverslip. Some workers prefer a 1.5- by 3-in. (1 in. = 2.54 cm) slide for the wet preparations rather than the standard 1- by 3-in. slide, which is routinely used for the permanent stained smear. A 2-mg sample of stool forms a low cone on the end of a wooden applicator stick. If more material is used for the direct mount, the suspension is usually too thick for an accurate examination; any sample of <2 mg results in the examination of too thin a suspension, thus decreasing the chances of finding organisms. If present, blood or mucus should always be examined as a direct mount. The entire 22- by 22-mm coverslip should be systematically examined with the low-power objective (10 $\times$ ) under low light intensity; any suspicious objects may then be examined with the high dry objective (40 $\times$ ) (Figure 5.2). Use of an oil immersion objective (100 $\times$ ) on mounts of this kind is not routinely recommended unless the coverslip is sealed to the slide (a no. 1 thickness coverslip is recommended for oil immersion). For a temporary seal, use a cotton-tipped applicator stick dipped in equal parts of heated paraffin

## DIRECT WET FECAL SMEAR (continued)



**Figure 5.2** Method of scanning a direct wet film preparation with the 10 $\times$  objective. (Illustration by Nobuko Kitamura.) Reprinted from *Diagnostic Medical Parasitology*, 5th ed.

and petroleum jelly. Nail polish can also be used to seal the coverslip. Many workers think that the use of the oil immersion objective on this type of preparation is impractical, especially since morphological detail is more readily seen by oil immersion examination of the permanent stained smear. This is particularly true in a busy clinical laboratory situation.

### Reagents

#### Saline (0.85% NaCl)

NaCl .....	0.85 g
Distilled water.....	100.0 ml

1. Dissolve the sodium chloride in distilled water in a flask or bottle, using a magnetic stirrer.
2. Distribute 10 ml into each of 10 screw-cap tubes.
3. Label as 0.85% NaCl with an expiration date of 1 year.
4. Sterilize by autoclaving at 121°C for 15 min.
5. When cool, store at 4°C.

#### D'Antoni's Iodine

Potassium iodide.....	1.0 g
Powdered iodine crystals.....	1.5 g
Distilled water.....	100.0 ml

1. Dissolve the potassium iodide and iodine crystals in distilled water in a flask or bottle, using a magnetic stirrer.
2. The potassium iodide solution should be saturated with iodine, with some excess crystals left on the bottom of the bottle.
3. Store in a brown, glass-stoppered bottle at room temperature and in the dark.
4. This stock solution is ready for immediate use. Label as D'Antoni's iodine with an expiration date of 1 year (the stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle).

- Aliquot some of the iodine into a brown dropper bottle. The working solution should have a strong-tea color and should be discarded when it lightens in color (usually within 10 to 14 days). Note: The stock and working solution formulas are identical, but the stock solution is held in the dark and will retain the strong-tea color while the working solution will fade and have to be periodically replaced.

### Lugol's Iodine

Potassium iodide.....	10.0 g
Powdered iodine crystals.....	5 g
Distilled water.....	100.0 ml

- Follow the directions listed above for D'Antoni's iodine, including the expiration date of 1 year.
- Dilute a portion 1:5 with distilled water for routine use (working solution).
- Place this working solution into a brown dropper bottle. The working solution should have a strong-tea color and should be discarded when it lightens in color (usually within 10 to 14 days).

### Quality Control

- Check the working iodine solution each time it is used or periodically (once a week). The iodine solution should be free of any signs of bacterial or fungal contamination.
- The color of the iodine should be that of strong tea (discard if it is too light).
- Protozoan cysts stained with iodine should contain yellow-gold cytoplasm, brown glycogen material, and paler refractile nuclei. The chromatoidal bodies may not be as clearly visible as they were in a saline mount. Human white blood cells (buffy coat cells) mixed with negative stool can be used as a quality control (QC) specimen. The human cells when mixed with negative stool can be used as a quality control specimen. The human cells will stain with the same color as that seen in the protozoa.
- Protozoan trophozoite cytoplasm should stain pale blue and the nuclei should stain a darker blue with the methylene blue stain. Human leukocyte WBCs mixed with negative stool should stain the same colors as seen with the protozoa.
- The microscope should be calibrated, and the original optics used for the calibration should be in place on the microscope when objects are measured. Although some feel that calibration is not required on a yearly basis, if the microscope receives heavy use, is in a position where it can be bumped, or does not receive routine maintenance, yearly calibration is recommended. The calibration factors for all objectives should be posted on the microscope or close by for easy access.

## DIRECT WET FECAL SMEAR (continued)

6. All QC results should be appropriately recorded; the laboratory should also have an action plan for “out-of-control” results.

### Detailed Procedure

1. Place 1 drop of 0.85% NaCl on the left side of the slide and 1 drop of iodine (working solution) on the right side of the slide. If preferred, two slides can be used instead of one. One drop of Nair's methylene blue can also be placed on a separate slide, although this technique is less commonly used.
2. Take a small amount of fecal specimen (the amount picked up on the end of an applicator stick when introduced into the specimen) and thoroughly emulsify the stool in the saline and iodine preparations (use separate sticks for each).
3. Place a 22-mm coverslip (no. 1) on each suspension.
4. Systematically scan both suspensions with the 10 $\times$  objective. The entire coverslip area should be examined under low power (total magnification,  $\times 100$ ).
5. If something suspicious is seen, the 40 $\times$  objective can be used for more detailed study. At least one-third of the coverslip should be examined under high dry power (total magnification,  $\times 400$ ) even if nothing suspicious has been seen.
6. Another approach is to prepare and examine the saline mount and then add iodine at the side of the coverslip. The iodine will diffuse into the stool-saline mixture, providing some stain for a second examination. Remember, the iodine kills any organisms present; thus, no motility is seen after the iodine is added to the preparation.

### Reporting

Protozoan trophozoites and/or cysts and helminth eggs and larvae may be seen and identified. In a heavy infection with *Cryptosporidium* spp., oocysts may be seen in a direct smear; however, some type of modified acid-fast stain or monoclonal antibody kit is normally used to detect these organisms, particularly when few oocysts are present. Oocysts of *Isospora belli* can also be seen in a direct smear. Spores of the microsporidia are too small, and the shape resembles other debris within the stool; therefore, they are not readily visible in a direct smear.

1. Motile trophozoites and protozoan cysts may or may not be identified to the species level (depending on the clarity of the morphology).  
Examples: *Giardia lamblia* trophozoites  
*Entamoeba coli* cysts
2. Helminth eggs and/or larvae may be identified.  
Examples: *Ascaris lumbricoides* eggs  
*Strongyloides stercoralis* larvae

3. Coccidian oocysts may be identified.  
Example: *Isospora belli* oocysts
4. Artifacts and/or other structures may also be seen and reported as follows (note: these crystals and cells are quantitated; however, the quantity is usually assessed when the permanent stained smear is examined under oil immersion):  
Examples: Moderate Charcot-Leyden crystals  
Few RBCs  
Moderate polymorphonuclear leukocytes (PMNs)

### **Procedure Reminders**

1. In preserved specimens, the formalin replaces the saline and can be used as a direct smear; however, you will not be able to see any organism motility (organisms are killed by 5 or 10% formalin). Consequently, the direct wet smear is usually not performed when the specimen (already preserved) arrives in the laboratory. The technical time is better spent performing the concentration and permanent stained smear.
2. As mentioned above, some workers prefer to make the saline and iodine mounts on separate slides and on 2- by 3-in. slides. Often there is less chance of getting fluids on the microscope stage if separate slides are used (less total fluid on the slide and under the coverslip) or if larger slides are used.
3. The microscope light should be reduced for low-power observations, since most organisms are overlooked with bright light. This is particularly true when the preparation is being examined without the use of iodine. Illumination should be regulated so that some of the cellular elements in the feces show refraction. Most protozoan cysts and some coccidian oocysts are refractile under these light conditions.

### **Procedure Limitations**

1. As mentioned above, because motility is lost when specimens are placed in preservatives, many laboratories are no longer performing the direct wet smear (whose primary purpose is to see motility) but are proceeding directly to the concentration and permanent stained smear procedures as a better, more cost-effective use of personnel time.
2. Most of the time, results obtained from wet smear examinations should be confirmed by permanent stained smears. Some protozoa are very small and difficult to identify to species with just the direct wet smear technique. Confirmation is particularly important in the case of *Entamoeba histolytica*/*E. dispar* versus *E. coli*. Findings from the direct wet smear examination can be reported as "preliminary," and the final report can be submitted after the concentration and permanent stain procedures are completed.

## DIRECT WET FECAL SMEAR (*continued*)

### REVIEW: DIRECT WET FECAL SMEAR

Clinical relevance	To assess the worm burden of the patient, to provide a quick diagnosis of heavily infected specimens, to check organism motility, and to diagnose motile organisms that might not be seen from concentration or permanent stain methods.
Specimen	Any fresh stool specimen that has not been refrigerated. Motile organisms are much more likely to be seen in liquid or very soft stools; formed stool often contains only the nonmotile cyst forms.
Reagents	0.85% NaCl; Lugol's or D'Antoni's iodine.
Examination requirements	Low power examination (100 $\times$ ) of entire 22- by 22-mm coverslip preparation (both saline and iodine); high dry power examination (400 $\times$ ) of at least one-third to one-half of the coverslip area (both saline and iodine).
Results and laboratory reports	Results from the direct smear examination should often be considered presumptive; however, some organisms could be definitively identified ( <i>Giardia lamblia</i> cysts and <i>Entamoeba coli</i> cysts, helminth eggs and larvae, <i>Isospora belli</i> oocysts). The report would be available after the results of both the concentration and permanent stained smear were available.
Procedure reminders and limitations	Once iodine is added to the preparation, the organisms are killed and motility is lost. <b>Specimens that arrive in the laboratory already preserved do not require a direct smear examination; proceed to the concentration and permanent stained smear (consistent with the CAP checklist).</b> Direct smears are normally examined at low (100 $\times$ ) and high dry (400 $\times$ ) power; oil immersion examination (1,000 $\times$ ) is not recommended (organism morphology is not that clear).

## Concentration (Sedimentation and Flotation)

### Description

Fecal concentration is a routine part of the complete ova and parasite examination (O&P exam) for parasites and allows the detection of small numbers of organisms that may be missed by using only a direct wet smear. There are two types of concentration procedures, sedimentation and flotation, both of which are designed to separate protozoan orga-

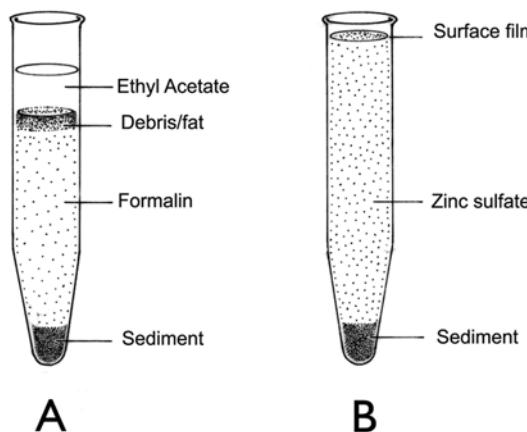
nisms and helminth eggs and larvae from fecal debris by centrifugation and/or differences in specific gravity (Figure 5.3).

**Sedimentation methods** (using centrifugation) lead to the recovery of all protozoa, oocysts, microsporidial spores, eggs, and larvae present; however, since the sediment will be examined, the preparation contains more debris. Although some workers recommend using flotation and sedimentation procedures, this approach is impractical for the majority of laboratories. If one technique is selected for routine use, the sedimentation procedure is recommended as being easier to perform and less subject to technical error.

A **flotation procedure** permits the separation of protozoan cysts, coccidian oocysts, and certain helminth eggs and larvae through the use of a liquid with a high specific gravity. The parasitic elements are recovered in the surface film, and the debris remains in the bottom of the tube. Since the surface film is examined, this technique yields a cleaner preparation than does the sedimentation procedure; however, some helminth eggs (operculated eggs and/or very dense eggs such as unfertilized *Ascaris* eggs) do not concentrate well with the flotation method. **Laboratories that use only flotation procedures may fail to recover all of the parasites present; to ensure detection of all organisms in the sample, both the surface film and the sediment should be carefully examined.** Directions for any flotation technique must be followed exactly to produce reliable results.

### Commercial Devices

There are a number of commercially available fecal concentration devices which may help a laboratory to standardize the concentration technique. Standardization is particularly important when personnel rotate throughout the laboratory and may not be familiar with parasitology techniques. These devices help ensure consistency, thus leading to improved parasite recovery and subsequent identification. Some of the systems are enclosed and provide a clean, odor-free approach to stool processing, features that



**Figure 5.3** Fecal concentration procedures: various layers seen in tubes after centrifugation. (A) Formalin-ether (or ethyl acetate). (B) Zinc sulfate (the surface film should be within 2 to 3 mm of the tube rim). (Illustration by Sharon Belkin.)

## **Concentration (Sedimentation and Flotation) (continued)**

may be important to nonmicrobiology personnel processing such specimens. Both the 15- and 50-ml systems are available. It is important to remember that you want a maximum of .5 to 1.0 ml of sediment in the bottom of the tube. The amount of sediment in the bottom of the tube is often excessive when the 50-ml systems are used. You can remedy this problem by adding less of the fecal specimen to the concentration system prior to centrifugation. Since the sediment is normally mixed thoroughly and 1 drop is transferred to a coverslip for examination, good mixing may not occur if too much sediment is used. There also appears to be layering in the bottom of the tubes; again, adding less material to the concentrator in the beginning should help eliminate this problem.

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### **SEDIMENTATION CONCENTRATION (Formalin-Ethyl Acetate)**

By centrifugation, this concentration procedure leads to the recovery of all protozoa, eggs, and larvae present; however, the preparation contains more debris than is found with the flotation procedure. Ethyl acetate is used to extract debris and fat from the feces and leave the parasites at the bottom of the suspension. The formalin-ethyl acetate sedimentation concentration is recommended because it is the easiest to perform, allows recovery of the broadest range of organisms, and is least subject to technical error.

The specimen must be fresh or formalinized stool (5 or 10% buffered or nonbuffered formalin or sodium acetate-acetic acid-formalin [SAF]). Specimens from the single-vial collections systems can also be used, as can those from polyvinyl alcohol (PVA) preservatives.

#### **Reagents**

##### **5 or 10% Formalin**

Formaldehyde (USP)..... 100 ml (for 10%) or 50 ml (for 5%)

##### **Saline Solution**

0.85% NaCl ..... 900 ml (for 10%) or 950 ml (for 5%)

**Note:** Formaldehyde is normally purchased as a 37 to 40% HCHO solution; however, for dilution it should be considered to be 100%.

Dilute 100 ml of formaldehyde with 900 ml of saline solution. (Distilled water may be used instead of saline solution.)

#### **Quality Control**

1. Check the liquid reagents each time they are used; the formalin and saline should appear clear, without any visible contamination.

2. The microscope should be calibrated (within the last 12 months), and the objectives and oculars used for the calibration procedure should be in place on the microscope when objects are measured. The calibration factors for all objectives should be posted on the microscope or close by for easy access. Although some feel that a microscope does not require calibration every 12 months, if the microscope is moved periodically, can be easily bumped, or does not receive adequate maintenance, it should be rechecked yearly for calibration accuracy.
3. Known positive specimens should be concentrated and organism recovery should be verified at least quarterly, and particularly after the centrifuge has been recalibrated.
4. All QC results should be appropriately recorded; the laboratory should also have an action plan for "out-of-control" results.

### **Detailed Procedure**

1. Transfer 0.5 teaspoon (about 4 g) of fresh stool into 10 ml of 5 or 10% formalin in a shell vial, unwaxed paper cup, or round-bottom tube (the container may be modified to suit individual laboratory preferences). Mix the stool and formalin thoroughly, and let the mixture stand for a minimum of 30 min for fixation. If the specimen is already in 5 or 10% formalin (or SAF), restir the stool-formalin mixture.
2. Depending on the size and viscosity of the specimen, strain a sufficient quantity through wet gauze (no more than two layers of gauze and one layer if the new "pressed" gauze [e.g., Johnson & Johnson nonsterile three-ply gauze, product 7636] is used) into a conical 15-ml centrifuge tube to give the desired amount of sediment (0.5 to 1 ml) for step 3 below. Usually 8 ml of the stool-formalin mixture prepared in step 1 is sufficient. If the specimen is received in vials of preservative (5 or 10% formalin or SAF), unless the specimen has very little stool in the vial, approximately 3 to 4 ml is sufficient. If the specimen contains a lot of mucus, do not strain through gauze but immediately fix in 5 or 10% formalin for 30 min and centrifuge for 10 min at  $500 \times g$ . Then proceed directly to step 10.
3. Add 0.85% NaCl or 5 or 10% formalin (some workers prefer to use formalin for all rinses) almost to the top of the tube, and centrifuge for 10 min at  $500 \times g$ . The amount of sediment obtained should be approximately 0.5 to 1 ml.
4. Decant the supernatant fluid, and resuspend the sediment in saline or formalin; add saline or formalin almost to the top of the tube, and centrifuge again for 10 min at  $500 \times g$ . This second wash may be eliminated if the supernatant fluid after the first wash is light tan or clear. Some prefer to limit the wash to one step (regardless of the clarity or color of the supernatant fluid after centrifugation) to eliminate additional manipulation of the specimen prior to centrif-

## **SEDIMENTATION CONCENTRATION (continued)** **(Formalin-Ethyl Acetate)**

- ugation (some organisms may be lost with each centrifugation and wash step).
5. Decant the supernatant fluid, and resuspend the sediment on the bottom of the tube in 5 or 10% formalin. Fill the tube half full only. If the amount of sediment left in the bottom of the tube is very small or the original specimen contained a lot of mucus, do not add ethyl acetate in step 6; merely add the formalin, spin, decant, and examine the remaining sediment.
  6. Add 4 to 5 ml of ethyl acetate. Stopper the tube, and shake vigorously for at least 30 s. Hold the tube so that the stopper is directed away from your face.
  7. After a 15- to 30-s wait, carefully remove the stopper.
  8. Centrifuge for 10 min at  $500 \times g$ . Four layers should result: a small amount of sediment (containing the parasites) in the bottom of the tube; a layer of formalin; a plug of fecal debris on top of the formalin layer; and a layer of ethyl acetate at the top.
  9. Free the plug of debris by ringing the plug with an applicator stick; decant all of the supernatant fluid. After proper decanting, a drop or two of fluid remaining on the side of the tube may run down into the sediment. Mix this fluid with the sediment.
  10. If the sediment is still somewhat solid, add 1 or 2 drops of saline or formalin to the sediment, mix, place a small amount of material on a slide, add a coverslip (22 by 22 mm, no. 1), and examine.
  11. Systematically scan with the  $10\times$  objective. The entire coverslip area should be examined under low power (total magnification,  $\times 100$ ).
  12. If something suspicious is seen, the  $40\times$  objective can be used for more detailed study. At least one-third of the coverslip should be examined under high dry power (total magnification,  $\times 100$ ) even if nothing suspicious has been seen. As in the direct wet smear, iodine can be added to enhance morphologic detail, and the coverslip can be tapped in an attempt to see objects move and turn over.

### **Reporting**

Protozoan trophozoites and/or cysts and helminth eggs and larvae may be seen and identified. Protozoan trophozoites are less likely to be seen. In a heavy infection with *Cryptosporidium* spp., oocysts may be seen in the concentrate sediment; oocysts of *I. belli* can also be seen. Spores of the microsporidia are too small, and the shape resembles other debris within the stool; therefore, they are not readily visible in the concentration sediment.

1. Protozoan cysts may or may not be identified to the species level (depending on the clarity of the morphology).  
Examples: *Entamoeba coli* cysts

2. Helminth eggs and/or larvae may be identified.  
Examples: *Ascaris lumbricoides* eggs  
*Strongyloides stercoralis* larvae
3. Coccidian oocysts may be identified.  
Example: *Isospora belli* oocysts
4. Artifacts and/or other structures may also be seen and reported as follows (note: these crystals and cells are quantitated; however, the quantity is usually assessed when the permanent stained smear is examined under oil immersion).  
Examples: Moderate Charcot-Leyden crystals  
Few RBCs  
Moderate PMNs

### **Procedure Reminders**

1. The gauze should never be more than one (pressed gauze) or two (woven gauze) layers thick; more gauze may trap mucus (containing *Cryptosporidium* oocysts and/or microsporidial spores).
2. Tap water may be substituted for 0.85% NaCl throughout this procedure, although the addition of water to fresh stool may cause *Blastocystis hominis* cyst (central body) forms to rupture and is not recommended. In addition to the original 5 or 10% formalin fixation, some workers prefer to use 5 or 10% formalin for all rinses throughout the procedure.
3. Ethyl acetate is widely recommended as a substitute for ether. It can be used in the same way in the procedure and is much safer. Hemo-De can also be used and is thought to be safer than ethyl acetate.
  - a. After the plug of debris is rimmed and excess fluid is decanted, while the tube is still upside down the sides of the tube can be swabbed with a cotton-tipped applicator stick to remove excess ethyl acetate. This is particularly important if you are working with plastic centrifuge tubes or plastic commercial concentrators. If the sediment is too dry after the tube has been swabbed, add several drops of saline before preparing the wet smear for examination.
  - b. If there is excess ethyl acetate in the smear of the sediment prepared for examination, bubbles will be present and will obscure the material that you are trying to see.
4. If specimens are received in SAF, begin the procedure at step 2.
5. If specimens are received in PVA, the first two steps of the procedure should be modified as follows.
  - a. Immediately after stirring the stool-PVA mixture with applicator sticks, pour approximately half of the mixture into a tube (container optional) and add 0.85% NaCl (or 5 or 10% formalin) almost to the top of the tube.
  - b. Filter the stool-PVA-saline (or formalin) mixture through wet gauze into a 15-ml centrifuge tube. Follow the standard procedure from here to completion, beginning with step 3.

## **SEDIMENTATION CONCENTRATION (continued)** **(Formalin-Ethyl Acetate)**

6. Too much or too little sediment results in an ineffective concentration.
7. The centrifuge should reach the recommended speed before the centrifugation time is monitored. However, since most laboratories have their centrifuges on automatic timers, the centrifugation time in this protocol takes into account the fact that some time is spent coming up to speed prior to full-speed centrifugation. If the centrifugation time at the proper speed is reduced, some of the organisms (*Cryptosporidium* oocysts or microsporidial spores) may not be recovered in the sediment.

### **Procedure Limitations**

1. Results obtained with wet smears (direct wet smears or concentrated specimens) should always be confirmed by permanent stained smears. Some protozoa are very small and difficult to identify to the species level with just the direct wet smears. Also, special stains are sometimes necessary for organism identification.
2. Confirmation is particularly important in the case of *E. histolytica*/*E. dispar* versus *E. coli*.
3. Certain organisms (*G. lamblia*, hookworm eggs, and occasionally *Trichuris* eggs) may not concentrate as well from PVA-preserved specimens as they do from those preserved in formalin. However, if enough *G. lamblia* organisms are present to concentrate from formalin, the PVA should contain enough for detection on the permanent stained smear. In clinically important infections, the number of helminth eggs present would ensure detection regardless of the type of preservative used. Also, the morphology of *S. stercoralis* larvae is not as clear when PVA is used as when specimens are fixed in formalin.
4. For unknown reasons, *I. belli* oocysts are routinely missed in the sediment when concentrated from PVA-preserved specimens. The oocysts would be found if the same specimen were preserved in formalin rather than PVA.
5. In past publications, recommended centrifugation times have not taken into account potential problems with the recovery of *Cryptosporidium* oocysts. There is evidence (unpublished) to strongly indicate that *Cryptosporidium* oocysts may be missed unless the centrifugation speed is  $500 \times g$  for a minimum of 10 min.
6. Adequate centrifugation time and speed have become very important for recovery of microsporidial spores. In some of the earlier publications, use of uncentrifuged material was recommended. However, we have found that centrifugation for 10 min at  $500 \times g$  definitely increases the number of microsporidial spores available for staining and subsequent examination.

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## **FLOTATION CONCENTRATION (Zinc Sulfate)**

### **Description**

The flotation procedure permits the separation of protozoan cysts and eggs of certain helminths from excess debris through the use of a liquid (zinc sulfate) with a high specific gravity. The parasitic elements are recovered in the surface film, and the debris remains in the bottom of the tube. This technique yields a cleaner preparation than does the sedimentation procedure; however, some helminth eggs (operculated eggs and/or very dense eggs such as unfertilized *Ascaris* eggs) do not concentrate well with the flotation method; a sedimentation technique is recommended to detect these infections.

When the zinc sulfate solution is prepared, the specific gravity should be 1.18 for fresh stool specimens; it must be checked with a hydrometer. This procedure may be used on formalin-preserved specimens if the specific gravity of the zinc sulfate is increased to 1.20; however, this may cause more distortion in the organisms present. To ensure detection of all possible organisms, both the surface film and the sediment must be examined. For most laboratories, this is not a practical approach.

The specimen must be fresh or formalinized stool (5 or 10% buffered or nonbuffered formalin or SAF). PVA-preserved specimens can also be used.

### **Reagents**

#### **Zinc Sulfate (33% aqueous solution)**

Zinc sulfate ..... 330 g  
Distilled water ..... 670 ml

1. Dissolve the zinc sulfate in distilled water in an appropriate flask or beaker, using a magnetic stirrer.
2. Adjust the specific gravity to 1.20 by the addition of more zinc sulfate or distilled water. Use specific gravity of 1.18 when using fresh stool (nonformalinized).
3. Store in a glass-stoppered bottle with an expiration date of 24 months.

### **Quality Control**

1. Check the reagents each time they are used. The formalin, saline, and zinc sulfate should appear clear, without any visible contamination.
2. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope or close by for easy access. As mentioned earlier, some feel recalibration of the microscope is not necessary each year; however, this would depend on the use and maintenance of the equipment.

## FLOTATION CONCENTRATION (*continued*) (Zinc Sulfate)

3. Known positive specimens should be concentrated and organism recovery should be verified at least quarterly and particularly after the centrifuge has been recalibrated.
4. All QC results should be appropriately recorded; the laboratory should also have an action plan for "out-of-control" results.

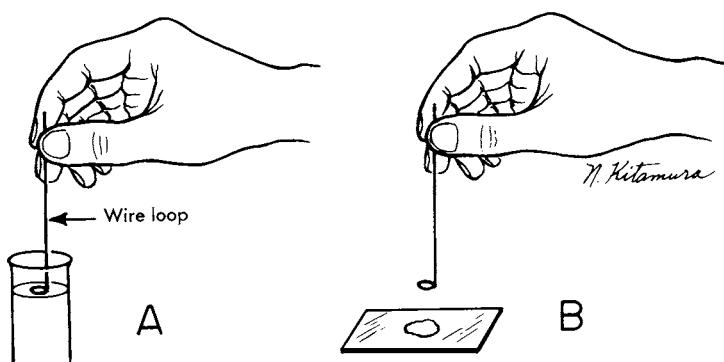
### Detailed Procedure

1. Transfer 0.5 teaspoon (about 4 g) of fresh stool into 10 ml of 5 or 10% formalin in a shell vial, unwaxed paper cup, or round-bottom tube (the container may be modified to suit individual laboratory preferences). Mix the stool and formalin thoroughly, and let the mixture stand for a minimum of 30 min for fixation. If the specimen is already in 5 or 10% formalin (or SAF), restir the stool-formalin mixture.
2. Depending on the size and density of the specimen, strain a sufficient quantity through wet gauze (no more than two layers of gauze or one layer if the new "pressed" gauze [e.g., Johnson & Johnson nonsterile three-ply gauze, product 7636] is used) into a conical 15-ml centrifuge tube to give the desired amount of sediment (0.5 to 1 ml) for step 3 below. Usually 8 ml of the stool-formalin mixture prepared in step 1 is sufficient. If the specimen is received in vials of preservative (5 or 10% formalin or SAF), unless the specimen has very little stool in the vial, approximately 3 to 4 ml is sufficient. If the specimen contains a lot of mucus, do not strain through gauze but immediately fix in 5 or 10% formalin for 30 min and centrifuge for 10 min at  $500 \times g$ . Then proceed directly to step 5.
3. Add 0.85% NaCl almost to the top of the tube, and centrifuge for 10 min at  $500 \times g$ . The amount of sediment obtained should be approximately 0.5 to 1 ml. Too much or too little sediment will result in an ineffective concentration.
4. Decant the supernatant fluid, resuspend the sediment in 0.85% NaCl almost to the top of the tube, and centrifuge for 10 min at  $500 \times g$ . This second wash may be eliminated if the supernatant fluid after the first wash is light tan or clear. Some prefer to limit the wash to one step (regardless of the color and clarity of the supernatant fluid) to eliminate additional manipulation of the specimen prior to centrifugation.
5. Decant the supernatant fluid, and resuspend the sediment on the bottom of the tube in 1 to 2 ml of zinc sulfate. Fill the tube within 2 to 3 mm of the rim with additional zinc sulfate.
6. Centrifuge for 2 min at  $500 \times g$ . Allow the centrifuge to come to a stop without interference or vibration. Two layers should result: a small amount of sediment in the bottom of the tube and a layer of zinc sulfate. The protozoan cysts and some helminth eggs are found in the surface film; some operculated and/or heavy eggs are found in the sediment.

- Without removing the tube from the centrifuge, remove 1 or 2 drops of the surface film with a Pasteur pipette or a freshly flamed (and allowed to cool) wire loop and place them on a slide. Do not use the loop as a “dipper”; simply touch the surface (bend the loop portion of the wire 90° so that the loop is parallel with the surface of the fluid) (Figure 5.4). Make sure that the pipette tip or wire loop is not below the surface film.
- Add a coverslip (22 by 22 mm, no. 1) to the preparation. Iodine may also be added.
- Systematically scan with the 10× objective. The entire coverslip area should be examined under low power (total magnification, ×100).
- If something suspicious is seen, the 40× objective can be used for more detailed study. At least one-third of the coverslip should be examined under high dry power (total magnification, ×400), even if nothing suspicious has been seen. As in the direct wet smear, iodine can be added to enhance morphologic detail, and the coverslip can be tapped gently in an attempt to observe objects moving and turning over.

### Reporting

Protozoan trophozoites and/or cysts and some helminth eggs and larvae may be seen and identified. Heavy helminth eggs and operculated eggs do not float in zinc sulfate; they may be seen in the sediment within the tube. The high specific gravity of the zinc sulfate causes the opercula to pop open; the egg fills with fluid and sinks to the bottom. Protozoan trophozoites are less likely to be seen. In a heavy infection with *Cryptosporidium* spp., oocysts may be seen in the concentrate sediment; oocysts of *I. belli* can also be seen. Spores of the microsporidia are too small, and the shape resembles other debris within the stool; therefore, they are not readily visible in the concentration sediment.



**Figure 5.4** Method used to remove surface film in the zinc sulfate flotation concentration procedure. (A) A wire loop is gently placed on (not under) the surface film. (B) The loop is then placed on a glass slide. (Illustration by Nobuko Kitamura.) Reprinted from *Diagnostic Medical Parasitology*, 5th ed.

## FLOTATION CONCENTRATION (continued) (Zinc Sulfate)

1. Protozoan cysts may or may not be identified to the species level (depending on the clarity of the morphology).  
Example: *Giardia lamblia* cysts
2. Helminth eggs and/or larvae may be identified.  
Example: Hookworm eggs
3. Coccidian oocysts may be identified.  
Example: *Isospora belli* oocysts
4. Artifacts and/or other structures may also be seen and reported as follows (note: these cells are quantitated; however, the quantity is usually assessed when the permanent stained smear is examined).  
Examples: Few macrophages  
Moderate PMNs

### Procedure Reminders

1. The gauze should never be more than one or two layers thick; more gauze may trap mucus (containing *Cryptosporidium* oocysts and/or microsporidial spores). A round-bottom tube is recommended rather than a centrifuge tube.
2. Tap water may be substituted for 0.85% NaCl throughout this procedure, although the addition of water to fresh stool may cause *B. hominis* cyst (central body) forms to rupture and is not recommended. In addition to the original 5 or 10% formalin fixation, some workers prefer to use 5 or 10% formalin for all rinses throughout the procedure.
3. If fresh stool is used (nonformalin preservatives), the zinc sulfate should be prepared with a specific gravity of 1.18. If formalinized specimens are to be concentrated, the zinc sulfate should have a specific gravity of 1.20.
4. If specimens are received in SAF, begin the procedure at step 2.
5. If fresh specimens are received, the standardized procedure requires the stool to be rinsed in distilled water prior to the addition of zinc sulfate in step 4. However, the addition of fresh stool to distilled water may destroy some *B. hominis* cyst (central-body) forms present and is not a recommended approach.
6. Some workers prefer to remove the tubes from the centrifuge prior to sampling the surface film. This is acceptable; however, there is more chance that the surface film will be disturbed prior to sampling.
7. Some workers prefer to add a small amount of zinc sulfate to the tube so that the fluid forms a slightly convex meniscus. A coverslip is then placed on top of the tube so that the undersurface touches the meniscus. The tube is then left undisturbed for 5 min, after which the coverslip is carefully removed and placed on a slide for exami-

- nation. This approach tends to be somewhat messy, particularly if too much zinc sulfate has been added.
8. When using the hydrometer (solution at room temperature), mix the solution well. Float the hydrometer in the solution, giving it a slight twist to ensure that it is completely free from the sides of the container. Read the bottom meniscus, and correct the figure for temperature if necessary. Most hydrometers are calibrated at 20°C. A difference of 3°C between the solution temperature (room temperature) and the hydrometer calibration temperature requires a correction of 0.001 to be added if above and subtracted if below 20°C.

### Procedure Limitations

1. Results obtained with wet smears (direct wet smears or concentrated specimens) should be confirmed by permanent stained smears. Some protozoa are very small and difficult to identify to the species level with just the direct wet smears. Also, special stains are sometimes necessary for organism identification.
2. Confirmation is particularly important in the case of *E. histolytica/E. dispar* versus *E. coli*.
3. Protozoan cysts and thin-shelled helminth eggs are subject to collapse and distortion when left for more than a few minutes in contact with the high-specific-gravity zinc sulfate. **The surface film should be removed for examination within 5 min of the time the centrifuge comes to a stop.** The longer the organisms are in contact with the zinc sulfate, the more distortion will be seen on microscopic examination of the surface film.
4. Since most laboratories have their centrifuges on automatic timers, the centrifugation time in this protocol takes into account the fact that some time will be spent coming up to speed prior to full-speed centrifugation.
5. If zinc sulfate is the only concentration method used, both the surface film and the sediment should be examined to ensure detection of all possible organisms.

### REVIEW: CONCENTRATION

Clinical relevance	To concentrate the parasites present, through either sedimentation or flotation. The concentration is specifically designed to allow recovery of protozoan cysts, coccidian oocysts, microsporidian spores, and helminth eggs and larvae.
Specimen	Any stool specimen that is fresh or preserved in formalin, PVA (mercury- or non-mercury based), SAF, MIF, or the newer single-vial system fixatives

(continued)

## FLOTATION CONCENTRATION (continued) (Zinc Sulfate)

### REVIEW: CONCENTRATION (continued)

Reagents	5 or 10% formalin, ethyl acetate, zinc sulfate (specific gravity of 1.18 for fresh stool or 1.20 for preserved stool); 0.85% NaCl; Lugol's or D'Antoni's iodine
Examination requirements	Low-power examination ( $\times 100$ ) of entire 22- by 22-mm coverslip preparation (iodine is recommended but is optional); high dry power examination ( $\times 400$ ) of at least one-third to one-half of the coverslip area (both saline and iodine)
Results and laboratory reports	Results from the concentration examination should often be considered presumptive; however, some organisms could be definitively identified ( <i>Giardia lamblia</i> cysts and <i>Entamoeba coli</i> cysts, helminth eggs and larvae, <i>Isospora belli</i> oocysts). The final report would be available after the results of the concentration and permanent stained smear were available.
Procedure reminders and limitations	Formalin-ethyl acetate sedimentation concentration is the most commonly used. Zinc sulfate flotation does not detect operculated or heavy eggs; both the surface film and sediment must be examined before a negative result is reported. Smears prepared from concentrated stool are normally examined at low ( $\times 100$ ) and high dry ( $\times 400$ ) power; oil immersion examination ( $\times 1,000$ ) is not recommended (organism morphology is not that clear). The addition of too much iodine may obscure helminth eggs (which mimic debris).

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## PERMANENT STAINED SMEAR

### Description

Detection and correct identification of many intestinal protozoa frequently depend on examination of the permanent stained smear with the oil immersion lens (100 $\times$  objective). Although an experienced microscopist can occasionally identify certain organisms on a wet preparation, most identifications should be considered tentative until confirmed by the permanent stained smear. The smaller protozoan organisms are frequently seen on the stained smear when they are easily missed with only the direct smear and concentration methods. For these reasons, the permanent stain is recommended for every stool sample submitted for a routine parasite examination.

A number of staining techniques are available; selection of a particular method may depend on the degree of difficulty of the procedure and the amount of time necessary to complete the stain. The older classical method is the long Heidenhain iron hematoxylin method; however, for routine diagnostic work most laboratories select one of the shorter procedures, such as the trichrome method or one of the modified methods involving iron hematoxylin.

Most problems encountered in the staining of protozoan trophozoites and cysts in fecal smears occur because the specimen is too old, the smears are too dense, the smears are allowed to dry before fixation, or fixation is inadequate. There is variability in fixation in that immature cysts fix more easily than mature cysts, and *E. coli* cysts require a longer fixation time than do those of other species.

## **Preparation of Material for Staining**

### **Fresh Material**

1. When the specimen arrives, prepare two slides with applicator sticks or brushes and immediately (without drying) place them in Schaudinn's fixative. Allow the slides to fix for a minimum of 30 min; overnight fixation is acceptable. The amount of fecal material smeared on the slide should be thin enough that newsprint can be read through the smear. Smears preserved in liquid Schaudinn's fixative should be placed in 70% alcohol to remove the excess fixative before being placed in iodine-alcohol (used for mercury-based fixatives).
2. If the fresh specimen is liquid, place 3 or 4 drops of PVA fixative on the slide, mix several drops of fecal material with the PVA, spread the mixture, and allow it to dry for several hours in a 37°C incubator or overnight at room temperature. This approach works for liquid specimens or those containing a lot of mucus; it is not recommended for more formed specimens.
3. Proceed with the trichrome staining procedure by placing the slides in iodine-alcohol.

### **PVA-Preserved Material**

1. Stool specimens that are preserved in PVA should be allowed to fix for at least 30 min. Thoroughly mix the contents of the PVA bottle with two applicator sticks.
2. Pour some of the well-mixed PVA-stool mixture onto a paper towel, and allow it to stand for 3 min to absorb out PVA. **Do not eliminate this step.**
3. With an applicator stick (or brush), apply some of the stool material from the paper towel to two slides and allow them to dry for several hours in a 37°C incubator or overnight at room temperature. Note: The PVA-stool mixture should be spread to the edges of the glass slide; this will cause the film to adhere to the slide during staining.

## **PERMANENT STAINED SMEAR (*continued*)**

- It is also important to thoroughly dry the slides to prevent the material from washing off during staining.
4. Place the dry slides into iodine-alcohol. There is no need to give them a 70% alcohol rinse first, because the PVA smears are already dry (unlike the wet smears coming out of the Schaudinn's fixative).

### **SAF-Preserved Material**

1. Mix the SAF-stool mixture thoroughly, and strain it through gauze into a 15-ml centrifuge tube.
2. After centrifugation (1 min at  $500 \times g$ ), decant the supernatant fluid. Although stains for the coccidia are not recommended with PVA-preserved material, remember that the centrifuge speed indicated here is probably not sufficient to recover the oocysts. Recommended centrifugation parameters are 10 min at  $500 \times g$ . The final sediment should be about 0.5 to 1.0 ml. If necessary, adjust by repeating step 1 or by resuspending the sediment in saline (0.85% NaCl) and removing part of the suspension.
3. Prepare a smear from the sediment for later staining by placing 1 drop of Mayer's albumin on the slide, to which is added 1 drop of SAF-preserved fecal sediment. Allow the smear to air dry at room temperature for 30 min prior to staining. The SAF stool smear can also be postfixed in Schaudinn's fixative prior to staining (begin the trichrome stain protocol with the 70% alcohol rinse prior to the iodine-alcohol step).
4. After being dried, the smear can be placed directly into 70% alcohol (step 4) of the staining procedure (the iodine-alcohol step can be eliminated).

### **Stains Used in the Permanent Stained Smear**

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#### **TRICHRome STAIN (Wheatley's Method)**

##### **Description**

The trichrome technique of Wheatley for fecal specimens is a modification of Gomori's original staining procedure for tissue. It is a rapid, simple procedure which produces uniformly well stained smears of the intestinal protozoa, human cells, yeast cells, and artifact material in about 45 min or less.

The specimen usually consists of fresh stool smeared on a microscope slide which is immediately fixed in Schaudinn's fixative or PVA-preserved stool smeared on a slide and allowed to air dry. Although SAF-preserved specimens can be stained with trichrome, there are other stains which are recommended for better overall results.

## **Reagents**

### **Trichrome Stain**

Chromotrope 2R .....	0.6 g
Light green SF .....	0.3 g
Phosphotungstic acid .....	0.7 g
Acetic acid (glacial) .....	1.0 ml
Distilled water.....	100 ml

1. Prepare the stain by adding 1.0 ml of acetic acid to the dry components. Allow the mixture to stand (ripen) for 15 to 30 min at room temperature.
2. Add 100 ml of distilled water. Properly prepared stain is purple.
3. Store in a glass or plastic bottle at room temperature. The shelf life is 24 months.

### **70% Ethanol plus Iodine**

1. Prepare a stock solution by adding iodine crystals to 70% alcohol until a dark solution is obtained (1 to 2 g/100 ml).
2. To use, dilute the stock solution with 70% alcohol until a dark reddish brown (port wine) or strong-tea color is obtained. As long as the color is acceptable, the new working solution does not have to be replaced. The replacement time depends on the number of smears stained and the size of the container (1 week to several weeks).

### **90% Ethanol, Acidified**

90% Ethanol .....	99.5 ml
Acetic acid (glacial) .....	0.5 ml

Prepare by combining.

### **70% Isopropyl or Ethyl Alcohol (100% ethyl alcohol [recommended] or 95% ethyl alcohol [second choice])**

### **Xylene or Xylene Substitute**

### **Quality Control**

1. Stool samples used for QC can be fixed stool specimens known to contain protozoa or PVA-preserved negative stools to which buffy coat cells (PMNs and macrophages) have been added. A QC smear prepared from a positive PVA sample or a PVA sample containing buffy coat cells should be used when new stain is prepared or at least once each week. Cultured protozoa can also be used.
2. A QC slide should be included with each new lot number of stain and once a month, particularly if the staining setup is used infrequently.
3. If the xylene becomes cloudy or there is an accumulation of water in the bottom of the staining dish, discard the old reagents, clean the dishes, dry thoroughly, and replace with fresh 100% ethanol and xylene.

## **TRICROME STAIN (continued) (Wheatley's Method)**

4. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids).
5. Depending on the volume of slides stained, staining solutions should be changed on an as-needed basis.
6. When the smear is thoroughly fixed and the stain is performed correctly, the cytoplasm of protozoan trophozoites is blue-green, sometimes with a tinge of purple. Cysts tend to be slightly more purple. Nuclei and inclusions (chromatoidal bars, RBCs, bacteria, and Charcot-Leyden crystals) are red, sometimes tinged with purple. The background material usually stains green, providing a nice color contrast with the protozoa. This contrast is more distinct than that obtained with the hematoxylin stain, which tends to stain everything with shades of gray-blue. Color variations among background and organisms are normal.
7. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope).
8. Known positive microscope slides, Kodachrome 2-by-2 projection slides, and photographs (reference books) should be available at the workstation.
9. Record all QC results; the laboratory should also have an action plan for "out of control" results.

### **Detailed Procedure for Using Mercury-Based Fixatives**

**Note:** In all staining procedures for fecal and gastrointestinal tract specimens, the term "xylene" is used in the generic sense. Xylene substitutes are recommended for the safety of all personnel performing these procedures.

1. Prepare a slide for staining as described above.
2. Remove the slide from Schaudinn's fixative, and place it in 70% ethanol for 5 min.
3. Place the slide in 70% ethanol plus iodine for 1 min for fresh specimens or 5 to 10 min for PVA air-dried smears.
4. Place the slide in 70% ethanol for 5 min.\*
5. Place the slide in a second container of 70% ethanol for 3 min.\*
6. Place the slide in trichrome stain for 10 min.
7. Place the slide in 90% ethanol plus acetic acid for 1 to 3 s. Immediately drain the rack (see Procedure Notes), and proceed to the next step. Do not allow slides to remain in this solution.
8. Dip the slide several times in 100% ethanol. Use this step as a rinse.
9. Place the slide in two changes of 100% ethanol for 3 min each.\*
10. Place the slide in xylene for 5 to 10 min.\*

11. Place the slide in a second container of xylene for 5 to 10 min.\*
12. Mount with a coverslip (no. 1 thickness), using mounting medium (e.g., Permount).

**Note: An alternative method to using mounting medium is as follows and does not require the use of mounting medium.**

- a. Remove the slide from the last xylene container, place it on a paper towel (flat position), and allow it to air dry. Remember that some of the xylene substitutes may take a bit longer to dry.
  - b. Approximately 5 to 10 min before you want to examine the slide, place a drop of immersion oil on the dry fecal film. Allow the oil to sink into the film for a minimum of 10 to 15 min. If the smear appears to be very refractile on examination, you have not waited long enough for the oil to sink into the film or you need to add a bit more oil to the film.
  - c. Once you are ready to examine the slide, place a no. 1 (22- by 22-mm) coverslip onto the oiled smear, add another drop of immersion oil to the top of the coverslip (as you would normally do for any coverslipped slide), and examine with the oil immersion lens (100 $\times$  objective).
  - d. Make sure that you do not eliminate adding the coverslip; the dry fecal material on the slide often becomes very brittle after dehydration. Without the addition of the protective coverslip, you might scratch the surface of the oil immersion lens. Coverslips are much cheaper than oil immersion objectives!
13. Allow the smear to dry overnight or after 1 h at 37°C.
  14. Examine the smear microscopically with the 100 $\times$  objective. Examine at least 300 oil immersion fields before reporting a negative result.

\* Slides may be held for up to 24 h in these solutions without harming the quality of the smear or stainability of organisms.

#### **Detailed Procedure for Using Non-Mercury-Based Fixatives (Iodine-Alcohol Step and Alcohol Rinse Not Required)**

1. Prepare the slide for staining as described above.
2. Place the slide in 70% ethanol for 5 min.\*
3. Place the slide in trichrome stain for 10 min. Some people prefer to place the dry smear directly into the stain and eliminate step 2.
4. Place the slide in 90% ethanol plus acetic acid for 1 to 3 s. Immediately drain the rack (see Procedure Notes), and proceed to the next step. Do not allow slides to remain in this solution.
5. Dip the slide several times in 100% ethanol. Use this step as a rinse.
6. Place the slide in two changes of 100% ethanol for 3 min each.\*
7. Place the slide in xylene for 5 to 10 min.\*
8. Place the slide in a second container of xylene for 5 to 10 min.\*
9. Mount with a coverslip (no. 1 thickness), using mounting medium (e.g., Permount). An alternative method to using mounting medium is given above.

## **TRICROME STAIN (continued)** **(Wheatley's Method)**

10. Allow the smear to dry overnight or after 1 h at 37°C.
11. Examine the smear microscopically with the 100× objective. Examine at least 300 oil immersion fields before reporting a negative result.

\* Slides may be held for up to 24 h in these solutions without harming the quality of the smear or stainability of organisms.

### **Reporting**

Protozoan trophozoites and cysts are readily seen, although helminth eggs and larvae may not be easily identified because of excess stain retention (wet smears from the concentration procedure[s] are recommended for detection of these organisms). Yeasts (single and budding cells and pseudohyphae) and human cells (macrophages, PMNs, and RBCs) can be identified. The following quantitation chart can be used for examination of permanent stained smears with the oil immersion lens (100× objective; total magnification, ×1,000). With very rare exceptions, coccidia and microsporidia are not seen on a routine trichrome stained fecal smear.

Quantitation of parasites, cells, yeasts,  
and artifacts

<b>Quantity</b>	<b>No./10 oil immersion fields (<math>\times 1,000</math>)</b>
Few	2
Moderate	3–9
Many	10

1. Report the organism and stage (do not use abbreviations).  
Examples: *Entamoeba histolytica*/*E. dispar* trophozoites  
*Giardia lamblia* trophozoites
2. Quantitate the number of *B. hominis* seen (rare, few, moderate, many). Do not quantitate other protozoa.  
Example: Moderate *Blastocystis hominis*
3. Note and quantitate the presence of human cells.  
Example: Moderate WBCs, many RBCs, few macrophages, rare Charcot-Leyden crystals
4. Report and quantitate yeast cells.  
Example: Moderate budding yeast cells and few pseudohyphae
5. Save positive slides for future reference. Label prior to storage (name, patient number, organisms present).

### **Procedure Reminders**

1. The single most important step in the preparation of a well-stained fecal smear is good fixation (rapid fixation after stool passage,

- proper ratio between fixative and stool, adequate mixing). If this has not been done, the protozoa may be distorted or shrunk, may not be stained, or may exhibit an overall pink or red color with poor internal morphology.
2. Slides should always be drained between solutions. Touch the end of the slide to a paper towel for 2 s, to remove excess fluid, before proceeding to the next step. This will maintain the staining solutions for a longer period.
  3. Incomplete removal of mercuric chloride (Schaudinn's fixative and PVA) may cause the smear to contain highly refractive crystals or granules which may prevent finding or identifying any organisms present. Since the 70% ethanol-iodine solution removes the mercury complex, it should be changed at least weekly to maintain the strong-tea color. It is usually sufficient to keep the slides in the iodine-alcohol for a few minutes; too long a time in this solution may also adversely affect the staining of the organisms.
  4. When using non-mercury-based fixatives, the iodine-alcohol step (used for the removal of mercury) and the subsequent alcohol rinse can be eliminated from the procedure. The smears for staining can be prerinsed with 70% alcohol and then placed in the trichrome stain, or they can be placed directly into the trichrome stain as the first step in the staining protocol.
  5. Smears that are predominantly green may be due to inadequate removal of iodine by the 70% ethanol (steps 4 and 5). Lengthening the time of these steps or more frequent changing of the 70% ethanol will help.
  6. To restore weakened trichrome stain, remove the cap and allow the ethanol (carried over on the staining rack from a previous dish) to evaporate. After a few hours, fresh stock stain may be added to restore lost volume. Older, more concentrated stain produces more intense colors and may require slightly longer destaining times (an extra dip). Remember that PVA smears usually require a slightly longer staining time.
  7. Although the trichrome stain is used essentially as a "progressive" stain (that is, no destaining is necessary), the best results are obtained by using the stain " regressively" (destaining the smears briefly in acidified alcohol). Good differentiation is obtained by destaining for a very short time (two dips only, approximately 2 to 3 s); prolonged destaining results in poor differentiation.
  8. It is essential to rinse the smears free of acid to prevent continued destaining. Since 90% alcohol continues to leach trichrome stain from the smears, it is recommended that after the acid-alcohol is used, the slides be quickly rinsed in 100% alcohol and then dehydrated through two additional changes of 100% alcohol.
  9. In the final stages of dehydration (steps 9 to 11), the 100% ethanol and the xylenes (or xylene substitute) should be kept as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from the 100% eth-

## **TRICROME STAIN (continued)**

### **(Wheatley's Method)**

- anol, return the slides to fresh 100% ethanol and replace the xylene with fresh stock.
10. If the smears peel or flake off, the specimen might have been inadequately dried on the slide (for PVA-fixed specimens), the smear may have been too thick, or the slide may have been greasy (fingerprints). However, slides generally do not have to be cleaned with alcohol prior to use.
  11. If the stain appears unsatisfactory upon examination and it is not possible to obtain another slide to stain, the slide may be restained. Place the slide in xylene to remove the coverslip, and reverse the dehydration steps, adding 50% ethanol as the last step. Destain the slide in 10% acetic acid for several hours, and wash it thoroughly first in water and then in 50 and 70% ethanol. Place the slide in the trichrome stain for 8 min, and complete the staining procedure.

### **Procedure Limitations**

1. The permanent stained smear is not recommended for staining helminth eggs or larvae; they are often too dark (excess stain retention) or distorted. However, they are occasionally recognized and identified. The wet smear preparation from the concentrate is the recommended approach for identification of helminth eggs and larvae.
2. The smear should be examined with the oil immersion lens (100 $\times$ ) for the identification of protozoa, human cells, Charcot-Leyden crystals, yeast cells, and artifact material. These cells and other structures are normally quantitated from the examination of the permanent stained smear, not the wet smear preparations (direct wet smear or concentration wet smear).
3. This high-magnification (oil immersion; total magnification,  $\times 1,000$ ) examination is recommended for protozoa, particularly for confirming species identification.
4. With low magnification (10 $\times$  objective), one might see eggs or larvae; however, this is not recommended as a routine approach.
5. In addition to helminth eggs and larvae, *I. belli* oocysts are best seen in wet preparations (concentration wet smears prepared from formalin-preserved, not PVA-preserved, material).
6. *Cryptosporidium* oocysts are generally not recognized on a trichrome-stained smear (acid-fast stains or the immunoassay reagent kits are recommended).

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## **IRON HEMATOXYLIN STAIN**

### **(Spencer-Monroe Method)**

#### **Description**

The iron hematoxylin stain can be used to make a permanent stained slide for detecting and quantitating parasitic organisms. Iron hematoxylin

was the stain used for most of the original morphologic descriptions of intestinal protozoa found in humans. On oil immersion power (magnification,  $\times 1,000$ ), one can examine the diagnostic features used to identify the protozoan parasite. Although there are many modifications of iron hematoxylin techniques, only two methods are outlined here. Both methods can be used with fresh, SAF-preserved, PVA-preserved, or other preserved specimens.

The specimen usually consists of fresh stool smeared on a microscope slide which is immediately fixed in Schaudinn's fixative, PVA-preserved stool smeared on a slide and allowed to air dry, or SAF-preserved stool smeared on an albumin-coated slide and allowed to air dry.

## Reagents

### Solution 1

Hematoxylin (crystal or powder) .....	10 g
Ethanol (absolute) .....	1,000 ml

Place the solution in a stoppered clear flask or bottle, and allow it to ripen in a lighted room for at least 1 week at room temperature.

### Solution 2

Ferrous ammonium sulfate $[Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O]$ .....	10 g
Ferric ammonium sulfate $[FeNH_4(SO_4)_2 \cdot 12H_2O]$ .....	10 g
Hydrochloric acid (concentrated).....	10 ml
Distilled water .....	1,000 ml

## Working Solution

Mix equal volumes of solutions 1 and 2. The working solution should be made fresh every week.

## 70% Ethanol Plus Iodine

1. Prepare a stock solution by adding iodine crystals to 70% alcohol until a dark solution is obtained (1 to 2 g/100 ml).
2. To use, dilute the stock solution with 70% alcohol until a dark reddish brown or strong-tea color is obtained. As long as the color is acceptable, the new working solution does not have to be replaced. Replacement time depends on the number of smears stained and the size of the container (1 week to several weeks).

## 90% Ethanol, Acidified

90% ethanol.....	99.5 ml
Acetic acid (glacial) .....	0.5 ml

Prepare by combining.

## 70% Isopropyl or Ethyl Alcohol (100% ethyl alcohol [recommended] or 95% ethyl alcohol [second choice])

## Xylene or Xylene Substitute

## **IRON HEMATOXYLIN STAIN (*continued*) (Spencer-Monroe Method)**

### **Quality Control**

1. Stool samples used for quality control can be fixed stool specimens known to contain protozoa or PVA-preserved negative stools to which buffy coat cells (PMNs and macrophages) have been added. A QC smear prepared from a positive PVA sample or a PVA sample containing buffy coat cells should be used when new stain is prepared or at least once each week. Cultured protozoa can also be used.
2. A QC slide should be included with each run of stained slides, particularly if the staining setup is used infrequently.
3. If the xylene becomes cloudy or there is an accumulation of water in the bottom of the staining dish, discard the old reagents, clean the dishes, dry them thoroughly, and replace with fresh 100% ethanol and xylene.
4. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids).
5. Depending on the volume of slides stained, staining solutions should be changed on an as-needed basis.
6. When the smear is thoroughly fixed and the stain is performed correctly, the cytoplasm of protozoan trophozoites is blue-gray, sometimes with a tinge of black. Cysts tend to be slightly darker. Nuclei and inclusions (chromatoidal bars, RBCs, bacteria, and Charcot-Leyden crystals) are dark gray-blue, sometimes almost black. The background material usually stains pale gray or blue, providing some color intensity contrast with the protozoa. This contrast is less distinct than that obtained with the trichrome stain, which tends to stain everything with multiple colors (pink, red, purple, green, and blue).
7. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope).
8. Known positive microscope slides, Kodachrome 2-by-2 projection slides, and photographs (reference books) should be available at the workstation.
9. Record all QC results; the laboratory should also have an action plan for “out of control” results.

### **Detailed Procedure for Using Mercury-Based Fixatives**

**Note:** In all staining procedures for fecal and gastrointestinal tract specimens, the term “xylene” is used in the generic sense. Xylene substitutes are recommended for the safety of all personnel performing these procedures.

1. Prepare the slide for staining as described above (for SAF smears, proceed to step 4).

2. Place the slide in 70% ethanol for 5 min.
3. Place the slide in the iodine–70% ethanol (70% ethanol to which is added enough D'Antoni's iodine to obtain a strong-tea color) solution for 2 to 5 min.
4. Place the slide in 70% ethanol for 5 min. Begin the procedure for SAF-fixed slides at this point.\*
5. Wash the slide in running tap water (make sure there is a constant stream of water into the container) for 10 min.
6. Place the slide in iron hematoxylin working solution for 4 to 5 min.
7. Wash the slide in running tap water (make sure there is a constant stream of water into the container) for 10 min.
8. Place the slide in 70% ethanol for 5 min.\*
9. Place the slide in 95% ethanol for 5 min.\*
10. Place the slide in two changes of 100% ethanol for 5 min each.\*
11. Place the slide in two changes of xylene for 5 min each.\*
12. Add Permount to the stained area of the slide, and cover the slide with a coverslip. An alternative method to using mounting medium is described above (Trichrome Stain [Detailed Procedure for Using Mercury-Based Fixatives]).
13. Examine the smear microscopically with the 100 $\times$  objective. Examine at least 300 oil immersion fields before reporting a negative result.

\* Slides may be held for up to 24 h in these solutions without harming the quality of the smear or stainability of organisms.

### **Detailed Procedure for Using Non-Mercury-Based Fixatives**

1. Prepare the slide for staining as described above.
2. Place the slide in 70% ethanol for 5 min.\*
3. Wash the slide in running tap water (make sure there is a constant stream of water into the container) for 10 min.
4. Place the slide in iron hematoxylin working solution for 4 to 5 min.
5. Wash the slide in running tap water (make sure there is a constant stream of water into the container) for 10 min.
6. Place the slide in 70% ethanol for 5 min.\*
7. Place the slide in 95% ethanol for 5 min.\*
8. Place the slide in two changes of 100% ethanol for 5 min each.\*
9. Place the slide in two changes of xylene for 5 min each.\*
10. Add Permount to the stained area of the slide and cover the slide with a coverslip. An alternative method to using mounting medium is described above (Trichrome Stain [Detailed Procedure for Using Non-Mercury-Based Fixatives]).
11. Examine the smear microscopically with the 100 $\times$  objective. Examine at least 300 oil immersion fields before reporting a negative result.

\* Slides may be held for up to 24 h in these solutions without harming the quality of the smear or stainability of organisms.

## **IRON HEMATOXYLIN STAIN (continued)** **(Spencer-Monroe Method)**

### **Reporting**

Protozoan trophozoites and cysts are readily seen, although helminth eggs and larvae may not be easily identified because of excess stain retention (wet smears from the concentration procedure[s] are recommended for detection of these organisms). Yeasts (single and budding cells and pseudohyphae) and human cells (macrophages, PMNs, and RBCs) can be identified.

For the quantitation of parasites, cells, yeasts, and artifacts, refer to the discussion of trichrome stain (above).

### **Procedure Reminders**

1. The single most important step in the preparation of a well-stained fecal smear is good fixation. If this has not been done, the protozoa may be distorted or shrunk, may not be stained, or may exhibit an overall gray or blue-gray color with poor internal morphology.
2. Slides should always be drained between solutions. Touch the end of the slide to a paper towel for 2 s to remove excess fluid before proceeding to the next step. This maintains the staining solutions for a longer period.
3. Incomplete removal of mercuric chloride (Schaudinn's fixative and PVA) may cause the smear to contain highly refractive crystals or granules which may prevent detection or identification of any organisms present. Since the 70% ethanol-iodine solution removes the mercury complex, it should be changed at least weekly to maintain the strong-tea color. It is usually sufficient to keep the slides in the iodine-alcohol for a few minutes; too long a time in this solution may also adversely affect the staining of the organisms.
4. When using non-mercury-based fixatives, the iodine-alcohol step (used for the removal of mercury) and the subsequent alcohol rinse can be eliminated from the procedure. The smears for staining can be prerinced with 70% alcohol and then placed in the trichrome stain, or they can be placed directly into the trichrome stain as the first step in the staining protocol.
5. When large numbers of slides are being stained, the working hematoxylin solution may be diluted; this affects the quality of the stain. If dilution occurs, discard the working solution and prepare a fresh working solution.
6. The shelf life of the stock hematoxylin solutions may be extended by keeping the solutions in the refrigerator at 4°C. Because of crystal formation in the stock solutions, it may be necessary to filter them before preparing a new working solution.
7. In the final stages of dehydration (steps 9 to 11), the 100% ethanol and the xylenes should be kept as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after

- addition of slides from the 100% ethanol, return the slides to fresh 100% ethanol and replace the xylene with fresh stock.
8. If the smears peel or flake off, the specimen might have been inadequately dried on the slide (in the case of PVA-fixed specimens), the smear may have been too thick, or the slide may have been greasy (fingerprints). However, slides generally do not have to be cleaned with alcohol prior to use.
  9. If the stain, upon examination, appears unsatisfactory and it is not possible to obtain another slide to stain, the slide may be restained. Place the slide in xylene to remove the coverslip, and reverse the dehydration steps, adding 50% ethanol as the last step. Destain the slide in 10% acetic acid for several hours, and then wash it thoroughly in water and then in 50 and 70% ethanol. Place the slide in the iron hematoxylin stain for 8 min, and complete the staining procedure.

### **Procedure Limitations**

1. The permanent stained smear is not recommended for staining helminth eggs or larvae; they are often too dark (excess stain retention) or distorted. However, they are occasionally recognized and identified. The wet smear preparation from the concentrate is the recommended approach for identification of helminth eggs and larvae.
2. The smear should be examined with the oil immersion lens (100 $\times$  objective) for the identification of protozoa, human cells, Charcot-Leyden crystals, yeast cells, and artifact material. These cells and other structures are normally quantitated from the examination of the permanent stained smear, not the wet smear preparations (direct wet smear, concentration wet smear).
3. This high-magnification (oil immersion; total magnification,  $\times 1,000$ ) examination is recommended for protozoa, particularly for confirming species identification.
4. If the viewer wants to screen the smear with low magnification (10 $\times$  objective), eggs or larvae might be visible; however, this is not recommended as a routine approach.
5. In addition to helminth eggs and larvae, *I. belli* oocysts are best seen in wet preparations (concentration wet smears prepared from formalin-preserved, not PVA-preserved, material).
6. *Cryptosporidium* oocysts are generally not recognized on an iron hematoxylin-stained smear (acid-fast stains or the immunoassay reagent kits are recommended).

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### **IRON HEMATOXYLIN STAIN (Tompkins-Miller Method)**

#### **Description**

A longer iron hematoxylin method was described by Tompkins and Miller. Since differentiation of overstained slides is critical in most iron hematoxylin staining procedures, Tompkins and Miller have described a

## **IRON HEMATOXYLIN STAIN (*continued*) (Tompkins-Miller Method)**

method in which phosphotungstic acid is used to destain the protozoa and which gives excellent results, even in unskilled hands.

### **Detailed Procedure**

1. Prepare the slide for staining as described above (for SAF or non-mercury-based smears, proceed to step 4).
2. Place the slide in 70% ethanol for 5 min.
3. Place the slide in the iodine-70% ethanol (70% alcohol to which is added enough D'Antoni's iodine to obtain a strong-tea color) solution for 2 to 5 min.
4. Place the slide in 50% ethanol for 5 min. Begin the procedure for SAF- or non-mercury-fixed slides at this point.\*
5. Wash the slide in running tap water (make sure there is a constant stream of water into the container) for 3 min.
6. Place the slide in 4% ferric ammonium sulfate mordant for 5 min.
7. Wash the slide in running tap water (make sure there is a constant stream of water into the container) for 1 min.
8. Place the slide in 0.5% aqueous hematoxylin for 2 min.
9. Wash the slide in tap water (in a container) for 1 min.
10. Place the slide in 2% phosphotungstic acid for 2 to 5 min.
11. Wash the slide in running tap water for 10 min.
12. Place the slide in 70% ethanol (plus a few drops of saturated aqueous lithium carbonate) for 3 min.
13. Place the slide in 95% ethanol for 5 min.\*
14. Place the slide in two changes of 100% ethanol for 5 min each.\*
15. Place the slide in two changes of xylene for 5 min each.\*
16. Add Permount to the stained area of the slide, and cover the slide with a coverslip. An alternative method that does not involve mounting medium is as follows.
  - a. Remove the slide from the last xylene container, place it on a paper towel (flat position), and allow it to air dry. Remember that some of the xylene substitutes may take a bit longer to dry.
  - b. Approximately 5 to 10 min before you want to examine the slide, place a drop of immersion oil on the dry fecal film. Allow the oil to sink into the film for a minimum of 10 to 15 min. If the smear appears to be very refractile on examination, you have not waited long enough for the oil to sink into the film or you need to add a bit more oil to the film.
  - c. Once you are ready to examine the slide, place a no. 1 (22- by 22-mm) coverslip onto the oiled smear, add another drop of immersion oil to the top of the coverslip (as you would normally do for any coverslipped slide), and examine with the oil immersion lens (100 $\times$  objective).
  - d. Make sure that you do not eliminate adding the coverslip; the dry fecal material on the slide often becomes very brittle after

- dehydration. Without the addition of the protective coverslip, you might scratch the surface of your oil immersion lens. Coverslips are much cheaper than oil immersion objectives!
17. Examine the smear microscopically with the 100 $\times$  objective. Examine at least 300 oil immersion fields before reporting a negative result.

\* Slides may be held up to 24 h in these solutions without harming the quality of the smear or stainability of organisms.

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## **MODIFIED IRON HEMATOXYLIN STAIN (Incorporating the Carbol Fuchsin Step)**

### **Description**

The following combination staining method for SAF-preserved fecal specimens was developed to allow the microscopist to screen for acid-fast organisms in addition to other intestinal parasites. For laboratories using iron hematoxylin stains in combination with SAF-fixed material and modified acid-fast stains for *Cryptosporidium*, *Cyclospora*, and *Isospora*, this modification represents an improved approach to current staining methods. This combination stain provides a saving in both time and personnel use.

Any fecal specimen submitted in SAF fixative can be used. Fresh fecal specimens after fixation in SAF for 30 min can also be used. This combination stain approach is not recommended for specimens preserved in Schaudinn's fixative or PVA.

### **Reagents**

#### **Mayer's Albumin**

Add an equal quantity of glycerin to a fresh egg white. Mix gently and thoroughly. Store at 4°C, and indicate an expiration date of 3 months. Mayer's albumin from commercial suppliers can normally be stored at 25°C for 1 year [e.g., product Z69, Hardy Diagnostics, 1430 West McCoy Lane, Santa Maria, CA 93455; (805) 346-2766, (800) 266-2222].

#### **Stock Solution of Hematoxylin Stain**

Hematoxylin powder.....10 g  
Ethanol (95% or 100%) ..... 1,000 ml

1. Mix well until dissolved.
2. Store in a clear glass bottle in a light area. Allow to ripen for 14 days before use.
3. Store at room temperature with an expiration date of 1 year.

#### **Mordant**

Ferrous ammonium sulfate  $[Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O]$  ..... 10 g  
Ferric ammonium sulfate  $[FeNH_4(SO_4)_2 \cdot 12H_2O]$  ..... 10 g  
Hydrochloric acid (concentrated)..... 10 ml  
Distilled water to ..... 1,000 ml

## **MODIFIED IRON HEMATOXYLIN STAIN (*continued*) (Incorporating the Carbol Fuchsin Step)**

### **Working Solution of Hematoxylin Stain**

1. Mix equal quantities of stock solution of stain and mordant.
2. Allow the mixture to cool thoroughly before use (prepare at least 2 h prior to use). The working solution should be made fresh every week.

### **Picric Acid**

Mix equal quantities of distilled water and an aqueous saturated solution of picric acid to make a 50% saturated solution.

### **Acid-Alcohol Decolorizer**

Hydrochloric acid (concentrated).....	30 ml
Alcohol to .....	1,000 ml

### **70% Alcohol and Ammonia**

70% alcohol.....	50 ml
Ammonia.....	0.5–1.0 ml

Add enough ammonia to bring the pH to approximately 8.0.

### **Carbol Fuchsin**

1. To make basic fuchsin (solution A), dissolve 0.3 g of basic fuchsin in 10 ml of 95% ethanol.
2. To make phenol (solution B), dissolve 5 g of phenol crystals in 100 ml of distilled water (gentle heat may be needed).
3. Mix solution A with solution B.
4. Store at room temperature. Solution is stable for 1 year.

### **Detailed Procedure**

1. Prepare the slide.
  - a. Place 1 drop of Mayer's albumin on a labeled slide.
  - b. Mix the sediment from the SAF concentration well with an applicator stick.
  - c. Add approximately 1 drop of the fecal concentrate to the albumin, and spread the mixture over the slide.
2. Allow the slide to air dry at room temperature (the smear appears opaque when dry).
3. Place the slide in 70% alcohol for 5 min.
4. Wash the slide in a container of tap water (not running water) for 2 min.
5. Place the slide in Kinyoun's stain for 5 min.
6. Wash the slide in running tap water (make sure there is a constant stream of water into the container) for 1 min.
7. Place the slide in acid-alcohol decolorizer for 4 min.\*

8. Wash the slide in running tap water (constant stream of water into the container) for 1 min.\*
9. Place the slide in iron hematoxylin working solution for 8 min.
10. Wash the slide in distilled water (in a container) for 1 min.
11. Place the slide in picric acid solution for 3 to 5 min.
12. Wash the slide in running tap water (make sure there is a constant stream of water into the container) for 10 min.
13. Place the slide in 70% alcohol plus ammonia for 3 min.
14. Place the slide in 95% alcohol for 5 min.
15. Place the slide in 100% alcohol for 5 min.
16. Place the slide in two changes of xylene for 5 min.

\* Steps 7 and 8 can also be performed as follows.

- a. Place the slide in acid-alcohol decolorizer for 2 min.
- b. Wash the slide in running tap water (make sure there is a constant stream of water into the container) for 1 min.
- c. Place the slide in acid-alcohol decolorizer for 2 min.
- d. Wash the slide in running tap water (make sure there is a constant stream of water into the container) for 1 min.
- e. Continue the staining sequence with step 9 (iron hematoxylin working solution).

### **Procedure Reminders**

1. The first 70% alcohol step acts with the Mayer's albumin to "glue" the specimen to the glass slide. The specimen may wash off if insufficient albumin is used or if the slides are not completely dry prior to staining.
2. The working hematoxylin stain should be checked each day of use by adding a drop of stain to alkaline tap water. If a blue color does not develop, prepare fresh working stain solution.
3. The picric acid differentiates the hematoxylin stain by removing more stain from fecal debris than from the protozoa and removing more stain from the organism cytoplasm than from the nucleus. When properly stained, the background should be various shades of gray-blue and protozoa should be easily seen, with medium blue cytoplasm and dark blue-black nuclei. Color variations are common.

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### **POLYCHROME IV STAIN**

Polychrome IV stain can be used in place of trichrome for staining fecal smears by the MIF, PVA, or SAF fixative method. Both the stain and staining directions are available commercially [Alpha-Tec Systems, Inc., 12019 N.E. 99th St., Suite 1780, Vancouver, WA 98682; (360) 260-2779]. Polychrome IV stain has been used primarily to stain permanent smears prepared from MIF-preserved fecal specimens.

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### **CHLORAZOL BLACK E STAIN**

Chlorazol black E staining is a method in which both fixation and staining occur in a single solution. This approach is used for fresh specimens

## CHLORAZOL BLACK E STAIN (*continued*)

but is not recommended for PVA-fixed material). This recommendation is based on the fact that the Chlorazol black E staining method does not include an iodine-alcohol step which is used to remove the mercuric chloride compound found in both Schaudinn's fixative and PVA. The optimal staining time must be determined for each batch of fixative-stain. The length of time for which the fixative-stain can be used depends on the number of slides run through the solution within a 30-day period. If the slides appear visibly red, the solution must be changed. Although this stain is not widely used, it is another option to consider. It is available from VWR [(800) 932-5000] and from Medical Chemical Corporation, 19430 Van Ness Avenue, Torrance, CA 90501 [(800) 424-9394].

### REVIEW: PERMANENT STAINED SMEARS

Clinical relevance	To provide contrasting colors for both the background debris and parasites present; designed to allow examination and recognition of detailed organism morphology under oil immersion examination (100 $\times$ objective for a total magnification of $\times 1,000$ ). Designed primarily to allow recovery and identification of the intestinal protozoa.
Specimen	Any stool specimen that is fresh, or preserved in formalin, PVA (mercury- or non-mercury-based), SAF, MIF, or the newer single-vial system fixatives
Reagents	Trichrome, iron hematoxylin, modified iron hematoxylin, polychrome IV, or Chlorazol black E stains and their associated solutions; dehydrating solutions (alcohols and xylenes); mounting fluid optional
Examination requirements	Oil immersion examination of at least 300 fields; additional fields may be required if suspect organisms have been seen in the wet preparations from the concentrated specimen.
Results and laboratory reports	The majority of the suspect protozoa and/or human cells should be confirmed using the permanent stained smear. These reports should be categorized as "final" and would be signed out as such (along with the results from the concentration examination).

*(continued)*

## REVIEW: PERMANENT STAINED SMEARS (continued)

Procedure reminders and limitations	The most commonly used stains include trichrome and iron hematoxylin. Unfortunately, helminth eggs and larvae take up too much stain and usually cannot be identified from the permanent stained smear. Also, coccidian oocysts and microsporidian spores usually require other staining methods for identification. Permanent stained smears are normally examined under oil immersion examination (magnification, $\times 1,000$ ), and low or high dry power is not recommended. Confirmation of the intestinal protozoa (both trophozoites and cysts) is the primary purpose of this technique.
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## Specialized Stains for Coccidia and Microsporidia

### KINYOUN'S ACID-FAST STAIN

#### (Cold Method)

##### Description

*Cryptosporidium* and *Isospora* have been recognized as causes of severe diarrhea in immunocompromised hosts but can also cause diarrhea in immunocompetent hosts. Oocysts in clinical specimens may be difficult to detect without special staining. Modified acid-fast stains are recommended to demonstrate these organisms. Unlike the Ziehl-Neelsen modified acid-fast stain, Kinyoun's stain does not require the heating of reagents for staining. With additional reports of diarrheal outbreaks due to *Cyclospora*, it is also important to remember that these organisms are acid fast and can also be identified using this staining approach. Although the microsporidial spores are also acid fast, their size (1 to 2  $\mu\text{m}$ ) makes identification very difficult without special stains or the use of molecular biology-based reagents.

It is recommended that concentrated sediment of fresh or formalin-preserved stool be used with this staining method. Other types of clinical specimens such as duodenal fluid, bile, and pulmonary sources (induced sputum, bronchial wash, or biopsies) may also be stained.

##### Reagents

###### 50% Ethanol

1. Add 50 ml of absolute ethanol to 50 ml of distilled water.
2. Store at room temperature. The solution is stable for 1 year. Note the expiration date on the label.

###### Kinyoun's Carbol Fuchsin

1. Dissolve 4 g of basic fuchsin in 20 ml of 95% ethanol (solution A).

## KINYOUN'S ACID-FAST STAIN (*continued*) (Cold Method)

2. Dissolve 8 g of phenol crystals in 100 ml of distilled water (solution B).
3. Mix solutions A and B.
4. Store at room temperature. The solution is stable for 1 year. Note the expiration date on the label.

### 1% Sulfuric Acid

1. Add 1 ml of concentrated sulfuric acid to 99 ml of distilled water.
2. Store at room temperature. The solution is stable for 1 year. Note the expiration date on the label.

### Loeffler's Alkaline Methylene Blue

1. Dissolve 0.3 g of methylene blue in 30 ml of 95% ethanol.
2. Add 100 ml of dilute (0.01%) potassium hydroxide.
3. Store at room temperature. The solution is stable for 1 year. Note the expiration date on the label.

### Quality Control

1. A control slide of *Cryptosporidium* from a 10% formalin-preserved specimen is included with each staining batch run. If the *Cryptosporidium* slide stains well, any *Isospora* or *Cyclospora* oocysts present will also take up the stain.
2. *Cryptosporidium* stains pink-red. Oocysts measure 4 to 6  $\mu\text{m}$ , and four sporozoites may be present internally. The background should stain uniformly blue.
3. The specimen is also checked for adherence to the slide (macroscopically).
4. The microscope should be calibrated (within the last 12 months), and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). If the microscopes receive adequate maintenance and are not moved frequently, yearly recalibration may not be necessary.
5. Known positive microscope slides, Kodachrome 2-by-2 projection slides, and photographs (reference books) should be available at the workstation.
6. Record all QC results; the laboratory should also have an action plan for "out of control" results.

### Detailed Procedure

1. Smear 1 to 2 drops of specimen on the slide and allow it to air dry. **Do not make the smears too thick (you should be able to see through the wet material before it dries).** Prepare two smears.

2. Fix with absolute methanol for 1 min.
3. Flood the slide with Kinyoun's carbol fuchsin, and stain it for 5 min.
4. Rinse the slide briefly (3 to 5 s) with 50% ethanol.
5. Rinse the slide thoroughly with water.
6. Decolorize by using 1% sulfuric acid for 2 min or until no more color runs from the slide.
7. Rinse the slide with water (it may take less than 2 min; do not destain too much). Drain.
8. Counterstain with methylene blue for 1 min.
9. Rinse the slide with water. Air dry.
10. Examine with the low or high dry objective. To see internal morphology, use the oil objective (100 $\times$ ).

## Reporting

*Cryptosporidium* and *Isospora* oocysts stain pink to red to deep purple. Some of the four sporozoites may be visible in the *Cryptosporidium* oocysts. Some of the *Isospora* immature oocysts (entire oocyst) stain, while mature oocysts usually appear with the two sporocysts within the oocyst wall stained pink to purple and with a clear area between the stained sporocysts and the oocyst wall. The background stains blue. If *Cyclospora* oocysts are present, they tend to be approximately 10  $\mu\text{m}$ , they resemble *Cryptosporidium* oocysts but are larger, and they have no definite internal morphology; the acid-fast staining tends to be more variable than that seen with *Cryptosporidium* or *Isospora* spp. If the patient has a heavy infection with microsporidia (immunocompromised patient), small (1- to 2- $\mu\text{m}$ ) spores may be seen but may not be recognized as anything other than bacteria or small yeast cells. There is usually a range of color intensity in the organisms present; not every oocyst appears deep pink to purple. The greatest variation in staining is seen with *Cyclospora*.

1. Report the organism and stage (oocyst). Do not use abbreviations.  
Examples: *Cryptosporidium* spp. oocysts  
*Isospora belli* oocysts
2. Call the physician when these organisms are identified.
3. Save positive slides for future reference. Label prior to storage (name, patient number, organisms present).

## Procedure Reminders

1. Routine stool examination stains (trichrome and iron hematoxylin) are not recommended; however, sedimentation concentration (500  $\times g$  for 10 min) is acceptable for the recovery and identification of *Cryptosporidium* spp., particularly after staining with one of the modified acid-fast stains. The routine concentration (formalin-ethyl acetate) can be used to recover *Isospora* oocysts (wet sediment examination and/or modified acid-fast stains), but routine permanent stains (trichrome and iron hematoxylin) are not reliable for this purpose.

## KINYOUN'S ACID-FAST STAIN (*continued*) (Cold Method)

2. PVA-preserved specimens are not acceptable for staining with the modified acid-fast stain. However, specimens preserved in SAF are perfectly acceptable.
3. Avoid the use of wet-gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and prevent them from flowing into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used; another option is to use the commercially available concentration systems that use no gauze but instead use plastic or metal screens.
4. Other organisms that stain positive include acid-fast bacteria, *Nocardia* spp., and the microsporidia (which are very difficult to find and identify even when they appear to be acid fast).
5. **It is very important that smears not be too thick.** Thicker smears may not adequately destain.
6. **Concentration of the specimen is essential to demonstrate organisms (500 × g for 10 min); this approach enhances the sensitivity of the test.** The number of organisms seen in the specimen may vary from numerous to very few.
7. Some specimens require treatment with 10% KOH because of their mucoid consistency. Add 10 drops of 10% KOH to the sediment, and vortex until it is homogeneous. Rinse with 10% formalin, and centrifuge (500 × g for 10 min). Without decanting the supernatant, take 1 drop of the sediment and smear it thinly on a slide.
8. Commercial concentrators and reagents are available.
9. Sulfuric acid at 1.0 to 3.0% is normally used (most laboratories are currently using a 1.0% acid rinse). Concentrations higher than 3% remove too much stain, particularly for *Cyclospora*. The use of acid-alcohol (routinely used in the Ziehl-Neelsen acid-fast staining method for the mycobacteria) decolorizes all organisms; therefore, one must use the modified decolorizer (1 to 3% H<sub>2</sub>SO<sub>4</sub>) for good results.
10. There is some debate whether organisms lose their ability to take up the acid-fast stain after long-term storage in 10% formalin. Some laboratories have reported this diminished staining.
11. Specimens should be centrifuged in capped tubes, and gloves should be worn during all phases of specimen processing.

### Procedure Limitations

1. Light *Cryptosporidium* infections may be missed (small number of oocysts). The immunoassay methods are more sensitive.
2. Multiple specimens must be examined, since the numbers of oocysts present in the stool vary from day to day. A series of three specimens submitted on alternate days is recommended.

3. The identification of both *Cyclospora* and microsporidia is difficult at best. *Cyclospora* may be suspected if the organisms appear to be *Cryptosporidium* but are about twice the size (about 10  $\mu\text{m}$ ). The microsporidial spores are extremely small (1 to 2  $\mu\text{m}$ ) and will probably not be recognized unless they are very numerous and appear to have a somewhat different morphology than do the other bacteria in the preparation.

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## MODIFIED ZIEHL-NEELSEN ACID-FAST STAIN

### (Hot Method)

#### Description

*Cryptosporidium* and *Isospora* have been recognized as causes of severe diarrhea in immunocompromised hosts but can also cause diarrhea in immunocompetent hosts. Oocysts in clinical specimens may be difficult to detect without special staining. Modified acid-fast stains are recommended to demonstrate these organisms. Application of heat to the carbolfuchsin assists in the staining, and the use of a milder decolorizer allows the organisms to retain more of their pink-red color. With continued reports of diarrheal outbreaks due to *Cyclospora*, it is also important to remember that these organisms are acid fast and can be identified by using this staining approach. Although the microsporidial spores are also acid fast, their size (1 to 2  $\mu\text{m}$ ) makes identification very difficult without special stains or the use of molecular-based reagents.

Concentrated sediment of fresh or formalin-preserved stool may be used. Other types of clinical specimens such as duodenal fluid, bile, and pulmonary sources (induced sputum, bronchial washings, or biopsy specimens) may also be stained.

#### Reagents

##### Carbol Fuchsin

1. To make basic fuchsin (solution A), dissolve 0.3 g of basic fuchsin in 10 ml of 95% ethanol.
2. To make phenol (solution B), dissolve 5 g of phenol crystals in 100 ml of distilled water (gentle heat may be needed).
3. Mix solution A with solution B.
4. Store at room temperature. The solution is stable for 1 year. Note the expiration date on the label.

##### 5% Sulfuric Acid

1. Add 5 ml of concentrated sulfuric acid to 95 ml of distilled water.
2. Store at room temperature. The solution is stable for 1 year. Note the expiration date on the label.

##### Methylene Blue

1. Dissolve 0.3 g of methylene blue chloride in 100 ml of distilled water.

## **MODIFIED ZIEHL-NEELSEN ACID-FAST STAIN (*continued*) (Hot Method)**

2. Store at room temperature. The solution is stable for 1 year. Note the expiration date on the label.

### **Quality Control**

QC guidelines are the same as those for the Kinyoun's acid-fast stain indicated above.

### **Detailed Procedure**

1. Smear 1 to 2 drops of specimen on the slide, and allow it to air dry. Do not make the smears too thick (you should be able to see through the wet material before it dries). Prepare two smears.
2. Dry on a heating block (70°C) for 5 min.
3. Place the slide on a staining rack, and flood it with carbol fuchsin.
4. With an alcohol lamp or Bunsen burner, gently heat the slide to steaming by passing the flame under the slide. Discontinue heating once the stain begins to steam. Do not boil.
5. Allow the specimen to stain for 5 min. If the slide dries, add more stain without additional heating.
6. Rinse the slide thoroughly with water. Drain.
7. Decolorize with 5% sulfuric acid for 30 s. (Thicker slides may require a longer destain; however, do not destain too long.)
8. Rinse the slide with water. Drain.
9. Flood the slide with methylene blue for 1 min.
10. Rinse the slide with water, drain, and air dry.
11. Examine with the low or high dry objective. To see internal morphology, use the oil objective (100×).

### **Reporting**

*Cryptosporidium* and *Isospora* oocysts stain pink to red to deep purple. Some of the four sporozoites may be visible in the *Cryptosporidium* oocysts. Some of the *Isospora* immature oocysts (entire oocyst) stain, while mature oocysts usually appear with the two sporocysts within the oocyst wall stained pink to purple and with a clear area between the stained sporocysts and the oocyst wall. The background stains blue. If *Cyclospora* oocysts are present (uncommon), they tend to be approximately 10 µm, they resemble *Cryptosporidium* oocysts but are larger, and they have no definite internal morphology; the acid-fast staining tends to be more variable than that seen with *Cryptosporidium* or *Isospora* spp. If the patient has a heavy infection with microsporidia (immunocompromised patient), small (1- to 2-µm) spores may be seen but may not be recognized as anything other than bacteria or small yeast cells. A range of color intensity is usually seen in the organisms present; not every oocyst appears deep pink to purple. The greatest variation in staining is seen with *Cyclospora*.

1. Report the organism and stage (oocyst). Do not use abbreviations.  
Examples: *Cryptosporidium* spp. oocysts  
*Isospora belli* oocysts
2. Call the physician when these organisms are identified.
3. Save positive slides for future reference. Label prior to storage (name, patient number, organisms present).

### **Procedure Reminders**

1. Routine stool examination stains (trichrome and iron hematoxylin) are not recommended; however, sedimentation concentration ( $500 \times g$  for 10 min) is acceptable for the recovery and identification of *Cryptosporidium* spp. and *Cyclospora*, particularly after staining with one of the modified acid-fast stains. The routine concentration (formalin-ethyl acetate) can be used to recover *Isospora* oocysts (wet sediment examination and/or modified acid-fast stains), but routine permanent stains (trichrome and iron hematoxylin) are not reliable for this purpose.
2. PVA-preserved specimens are not acceptable for staining with the modified acid-fast stain. However, specimens preserved in SAF are perfectly acceptable.
3. Avoid the use of wet-gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and prevent them from flowing into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used. Another option is to use the commercially available concentration systems that use no gauze but instead use metal or plastic screens for filtration.
4. Other organisms that stain positive include acid-fast bacteria, *Nocardia* spp., and the microsporidia (which are very difficult to find and identify even when they appear to be acid fast).
5. **It is very important that smears not be too thick.** Thicker smears may not adequately destain.
6. **Concentration of the specimen is essential to demonstrate organisms ( $500 \times g$  for 10 min); this approach enhances the sensitivity of the test.** The number of organisms seen in the specimen may vary from numerous to very few.
7. Some specimens require treatment with 10% KOH because of their mucoid consistency. Add 10 drops of 10% KOH to the sediment, and vortex until it is homogeneous. Rinse with 10% formalin, and centrifuge ( $500 \times g$  for 10 min). Without decanting the supernatant, take 1 drop of the sediment and smear it thinly on a slide.
8. Commercial concentrators and reagents are available.
9. Do not boil the stain. Gently heat until steam rises from the slide. Do not allow the stain to dry on the slide.
10. Various concentrations of sulfuric acid (0.25 to 10%) may be used; however, the destaining time varies according to the concentration used. Generally, a 1 or 5% solution is used. The use of acid-alcohol

## **MODIFIED ZIEHL-NEELSEN ACID-FAST STAIN (continued) (Hot Method)**

(routinely used in the Ziehl-Neelsen acid-fast staining method for the mycobacteria) decolorizes all organisms; therefore, one must use the modified decolorizer (1.0 to 3% H<sub>2</sub>SO<sub>4</sub>) for good results.

11. There is some debate whether organisms lose their ability to take up the acid-fast stain after long-term storage in 10% formalin. Some laboratories have reported this diminished staining.
12. Specimens should be centrifuged in capped tubes, and gloves should be worn during all phases of specimen processing.

### **Procedure Limitations**

1. Light *Cryptosporidium* or *Cyclospora* infections may be missed (small number of oocysts). The immunoassay methods for *Cryptosporidium* are more sensitive.
2. Multiple specimens must be examined, since the numbers of oocysts present in the stool vary from day to day. A series of three specimens submitted on alternate days is recommended.
3. The identification of both *Cyclospora* and microsporidia may be difficult. *Cyclospora* may be suspected if the organisms appear to be *Cryptosporidium* but are about twice the size (about 10  $\mu\text{m}$ ). The microsporidial spores are extremely small (1 to 2  $\mu\text{m}$ ) and will probably not be recognized unless they are very numerous and appear to have a somewhat different morphology than do the other bacteria in the preparation.

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## **CARBOL FUCHSIN NEGATIVE STAIN FOR CRYPTOSPORIDIUM (W. L. Current)**

1. Mix thoroughly an equal volume (3 to 10  $\mu\text{l}$ ) of fresh or formalin-fixed stool and Kinyoun's carbol fuchsin on a slide.
2. Spread out as a thin film, and allow to air dry at room temperature.
3. Add immersion oil directly to the stained smear, and then cover with a coverslip.
4. Observe with bright-field microscopy ( $\times 400$ ). Everything but the oocysts stains darkly. The oocysts are bright and refractile because they contain water whereas everything else is oil soluble.

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## **RAPID SAFRANIN METHOD FOR CRYPTOSPORIDIUM (D. Baxby)**

1. Smear fresh or formalin-fixed feces on a slide, and allow the film to air dry at room temperature.
2. Fix briefly by one pass through the Bunsen burner flame.
3. Fix for 3 to 5 min with 3% HCl in methanol.
4. Wash the slide with a brief rinse in tap water.

5. Stain with 1% aqueous safranin for 1 min (heat until steam appears) (the authors indicate by personal communication that boiling may be beneficial).
6. Rinse the slide in tap water.
7. Counterstain with 1% methylene blue for 30 s (the authors report that 0.1% aqueous crystal violet was almost as good, but malachite green was unsatisfactory).

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### RAPID SAFRANIN METHOD FOR CYCLOSPORA, USING A MICROWAVE OVEN

(Govinda Visvesvara)

Another rapid safranin method uniformly stains *Cyclospora* oocysts a brilliant reddish orange. In this method, the fecal smears must be heated in a microwave oven before being stained. This stain is fast, reliable, and easy to perform.

1. Using a 10- $\mu$ l microliter aliquot of concentrated stool, prepare the smear by spreading the material thinly across the slide.
2. Allow the smear to dry on a 60°C slide warmer.
3. Cool the slide to room temperature before staining.
4. Place the slide in a Coplin jar containing acidic alcohol (3% [vol/vol] HCl in methanol), and let it stand for 5 min.
5. Wash off the excess acidic alcohol with cold tap water.
6. Place the slide into the Coplin jar containing 1% safranin solution in acidified water (pH 6.5), and microwave on full power (650 W) for 1 min. (Place the staining jar in another container to catch any overflow of stain because of boiling.)
7. Wash off excess stain with cold tap water.
8. Place the slide in a Coplin jar containing 1% methylene blue for 1 min.
9. Rinse gently with cold tap water.
10. Air dry.
11. Coverslip the slide using Cytoseal 60 or other mounting medium; the immersion oil mounting method can also be used.
12. Examine the smear under low power or high dry power objectives. To see additional morphology, use the oil immersion objective (100 $\times$ ).

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### AURAMINE O STAIN FOR COCCIDIA

(Thomas Hänscheid)

Coccidia are acid-fast organisms and also stain well with auramine O (phenolized auramine O). The size and typical appearance of *Cryptosporidium*, *Cyclospora*, and *Isospora* oocysts enable auramine O-stained slides to be examined at low power under the 10 $\times$  objective. The entire sample area can usually be examined in less than 30 s. The low cost of the reagents, the simple staining protocol, and the rapid microscopic exam-

## AURAMINE O STAIN FOR COCCIDIA (continued) (Thomas Hänscheid)

ination also make this staining method suitable for screening unconcentrated fecal specimens.

Concentrated sediment from fresh or non-PVA-preserved stool may be used. Other stool samples may also be used, such as unconcentrated stool submitted for culture in a bacteriology transport medium. However, to increase the sensitivity of the test, small numbers of oocysts are more easily detected in concentrated stools.

### Auramine O Stain

#### 1. Auramine O

Dissolve 0.1 g of auramine O in 10 ml of 95% ethanol.

#### 2. Phenol

Dissolve 3.0 g of phenol crystals in 87 ml of distilled water.

Combine solutions 1 and 2. Store in a dark bottle at room temperature for up to 3 months.

#### 3. Destaining agent: 0.5% acid alcohol

Add 0.5 ml concentrated HCl to 100 ml of 70% ethanol. Store at room temperature for 3 months.

#### 4. Counterstain: 0.5% potassium permanganate

Dissolve 0.5 g of potassium permanganate in 100 ml of distilled water.

### Quality Control

QC guidelines are the same as those for Kinyoun's acid-fast stain and are given on p. 128.

### Procedure

1. Using a 10- to 20- $\mu$ l aliquot of concentrated stool, prepare the smear by spreading the material across the slide.
2. Heat fix the slides either on a 65 to 75°C heat block for at least 2 hr or using the flame of a Bunsen burner. Do not overheat. Another fixation option would be to fix the slide in absolute methanol for 1 min, air dry, and then proceed with staining.
3. Cool the slide to room temperature before staining.
4. Flood the slide with the phenolized auramine O solution.
5. Allow the smear to stain for ca. 15 min. Do not heat.
6. Rinse the slide in water. Drain excess water from the slide.
7. Flood the slide with the destaining solution (0.5% acid-alcohol).
8. Allow the specimen to decolorize for 2 min.
9. Flood the slide with counterstain (potassium permanganate) solution.
10. Stain for 2 min. The timing of this step is critical.
11. Rinse the slide in water. Drain excess water from the slide.
12. Allow the smear to air dry. Do not blot.

13. Examine the smear under a fluorescence microscope with a  $10\times$  objective and fluorescein isothiocyanate FITC optical filters (auramine O: excitation maximum,  $\sim 435$  nm in water; emission maximum,  $\sim 510$  nm). Screen the whole sample area for the presence of fluorescent oocysts. Suspicious objects can be reexamined with a  $20\times$  or  $100\times$  objective.
14. Smears can be restained by any of the carbol fuchsin (modified acid-fast) staining procedures to allow examination by light microscopy.

### **Reporting**

*Cryptosporidium* and *Cyclospora* oocysts fluoresce brightly and have a regular round appearance ("starry-sky" appearance with the  $10\times$  objective). In contrast to the large majority of fluorescent artifacts, the oocysts do not stain homogeneously. Thus, the fluorescence is heterogeneously distributed in the interior of the oocyst. *Isospora* oocysts fluoresce brightly with three patterns: (i) a more or less brightly but heterogeneously stained interior of the whole oocyst, (ii) one brightly staining sporocyst, or (iii) two brightly staining sporocysts within the oocyst wall.

1. Report the organism and stage (oocyst). Do not use abbreviations.  
Examples: *Cryptosporidium* spp. oocysts  
*Isospora belli* oocysts  
*Cyclospora cayetanensis* oocysts
2. Call the physician when these organisms are identified.
3. Save positive slides for future reference. Label prior to storage (name, patient number, organisms present). These slides can be kept at room temperature in the dark, and the fluorescence remains stable for up to 3 to 4 weeks.

### **Procedure Notes**

1. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.
2. For best results, examine the auramine O solution for deposits and remove them by filtration or centrifugation. This problem can also be avoided by preparing smaller volumes more frequently.
3. Slides should be observed as soon as possible after staining. However, they can be kept at room temperature in the dark, and fluorescence remains stable for up to 3 to 4 weeks.

### **Procedure Limitations**

1. Light infections might be missed, particularly if unconcentrated stool is used; it is always recommended that concentrated stool sediment be used for staining ( $500 \times g$  for 10 min).
2. Using the  $40\times$  high dry objective often causes a blurred image (a fluorescent "halo" around the image, hazy contours), which appears to be the effect of interfering fluorescence from the auramine O stain

## AURAMINE O STAIN FOR COCCIDIA (continued)

(Thomas Hänscheid)

located outside the plane of focus. Using the 100 $\times$  oil immersion objective gives higher-quality images. Immersion oils used for light microscopy may be autofluorescent; special low-fluorescence immersion oil should be used.

3. If the fluorescence is not clear or definitive, a suspicious slide can be restained with a modified acid-fast stain and reexamined by light microscopy with the 100 $\times$  oil immersion objective.
4. If protected from sunlight, auramine O slides can be kept on the bench at room temperature for up to 2 to 3 weeks, with only minor loss of fluorescence (photo bleaching).

### REVIEW: MODIFIED ACID-FAST SMEARS AND OTHER STAINS FOR COCCIDIA

Clinical relevance	To provide contrasting colors for both the background debris and parasites present; designed to allow examination and recognition of the acid-fast characteristic of the organisms under high dry examination (40 $\times$ objective for a total magnification of $\times 400$ ). Designed primarily to allow recovery and identification of intestinal coccidian oocysts ( <i>Cryptosporidium</i> , <i>Cyclospora</i> , and <i>Isospora</i> ). Internal morphology (sporozoites) is seen in some <i>Cryptosporidium</i> oocysts under oil immersion (magnification, $\times 1,000$ ).
Specimen	Any stool specimen that is fresh, or preserved in formalin, SAF, or the newer single-vial system fixatives
Reagents	Kinyoun's acid-fast stain, modified Ziehl-Neelsen stain, and their associated solutions; dehydrating solutions (alcohols and xylenes); mounting fluid optional; remember that the decolorizing agents are less intense than the routine acid-alcohol used in routine acid-fast staining (this is what makes these procedures "modified" acid-fast procedures). Safranin and auramine O stains and associated solutions.
Examination requirements	High dry examination of at least 300 fields; additional fields may be required if suspect organisms have been seen but are not clearly acid fast.

(continued)

## REVIEW: MODIFIED ACID-FAST SMEARS AND OTHER STAINS FOR COCCIDIA (continued)

Results and laboratory reports	The identification of <i>Cryptosporidium</i> and <i>Isospora</i> oocysts should be possible; <i>Cyclospora</i> oocysts, which are twice the size of <i>Cryptosporidium</i> oocysts, should be visible but tend to be more acid-fast variable. Although microsporidia are acid fast, their small size makes recognition very difficult. Final laboratory results would depend heavily on the appearance of the QC slides and comparison with patient specimens.
Procedure reminders and limitations	Both the cold and hot modified acid-fast methods are excellent for the staining of coccidian oocysts. There is some feeling that the hot method may result in better stain penetration, but the differences are probably minimal. Procedure limitations are related to specimen handling (proper centrifugation speeds and times, use of no more than two layers of wet gauze for filtration) and a complete understanding of the difficulties in recognizing microsporidial spores. There is also some controversy concerning whether the organisms lose the ability to take up acid-fast stains after long-term storage in 10% formalin. The organisms are more difficult to find in specimens from patients who do not have the typical, watery diarrhea (more formed stool = more artifact material). Both the safranin and auramine O stains are also good options for staining coccidian oocysts.

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## MODIFIED TRICHOME STAIN FOR MICROSPORIDIA (Weber, Green Counterstain)

### Description

A few years ago, the diagnosis of intestinal microsporidiosis (*Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*) depended on the use of invasive procedures and subsequent examination of biopsy specimens, often by electron microscopy methods. Slides prepared from fresh or formalin-fixed stool specimens can be stained using chromotrope-based techniques and can be examined by light microscopy. This staining method is based on the fact that stain penetration of the microsporidial spore is very difficult; thus, more dye is used in the chromotrope 2R than that routinely used to prepare Wheatley's modification of Gomori's trichrome method, and the staining time is much longer (90 min). At least several of these stains are available commercially from a number of suppliers. The specimen can be fresh stool or stool that has been preserved in 5 or 10%

## **MODIFIED TRICHRONE STAIN FOR MICROSPORIDIA (continued) (Weber, Green Counterstain)**

formalin, SAF, or some of the newer single-vial system fixatives. Actually, any specimen other than tissue thought to contain microsporidia could be stained by this method.

### **Reagents**

#### **Stain**

Chromotrope 2R .....	6.0 g*
Fast Green .....	0.15 g
Phosphotungstic acid.....	0.7 g
Acetic acid (glacial) .....	3.0 ml
Distilled water.....	100.0 ml

\*This is 10 times the amount used in the normal trichrome stain formula.

1. Prepare the stain by adding 3.0 ml of acetic acid to the dry ingredients. Allow the mixture to stand (ripen) for 30 min at room temperature.
2. Add 100 ml of distilled water. Properly prepared stain is dark purple.
3. Store in a glass or plastic bottle at room temperature. The shelf life is at least 24 months.

#### **Acid-Alcohol**

90% ethyl alcohol.....	995.5 ml
Acetic acid (glacial) .....	4.5 ml

Prepare by combining the two solutions.

### **Quality Control**

1. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores as the control organisms. Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual organisms because the spores are very small (1 to 1.5  $\mu\text{m}$ ) and difficult to stain.
2. A QC slide should be included with each run of stained slides, particularly if the staining setup is used infrequently.
3. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids should be used).
4. Depending on the volume of slides stained, staining solutions should be changed on an as-needed basis.
5. When the smear is thoroughly fixed and the stain is performed correctly, the spores are seen to be ovoid and refractile, with the spore wall being bright pinkish red. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore. The

- majority of the bacteria and other debris tend to stain green. However, some bacteria and debris stain red.
6. The specimen should also be checked (macroscopically) for adherence to the slide.
  7. The microscope should be calibrated (within the last 12 months), and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this varies from laboratory to laboratory, depending on equipment care and use.
  8. Known positive microscope slides, Kodachrome 2 by 2 projection slides, and photographs (reference books) should be available at the workstation.
  9. Record all QC results; the laboratory should also have an action plan for "out of control" results.

### **Detailed Procedure**

1. Using a 10- $\mu$ l aliquot of unconcentrated, preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material over an area of 45 by 25 mm. **Although this original procedure specifies unconcentrated specimen, organism recovery can be dramatically enhanced by centrifuging the specimen for 10 min at 500  $\times$  g prior to smear preparation.**
2. Allow the smear to air dry.
3. Place the smear in absolute methanol for 5 min.
4. Allow the smear to air dry.
5. Place in trichrome stain for 90 min.
6. Rinse in acid-alcohol for no more than 10 s.
7. Dip slides several times in 95% alcohol. Use this step as a rinse.
8. Place in 95% alcohol for 5 min.
9. Place in 100% alcohol for 10 min.
10. Place in xylene substitute for 10 min.
11. Mount with a coverslip (no. 1 thickness), using mounting medium.
12. Examine smears under oil immersion (1,000 $\times$ ) and read at least 300 fields; the examination time will probably be at least 10 min per slide.

### **Reporting**

Results are reported as for the Ryan stain below (see p. 143).

### **Procedure Reminders**

The procedure is the same as for the Ryan stain below (see p. 144).

### **Procedure Limitations**

The procedure limitations are the same as for the Ryan stain below (see p. 144).

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## **MODIFIED TRICHOME STAIN FOR MICROSPORIDIA**

### **(Ryan, Blue Counterstain)**

#### **Description**

A number of variations to the modified trichrome stain (Weber, green) were tried in an attempt to improve the contrast between the color of the spores and the background staining. Optimal staining was achieved by modifying the composition of the trichrome solution. This stain is also available commercially from a number of suppliers. The specimen can be fresh stool or stool that has been preserved in 5 or 10% formalin, SAF, or some of the newer single-vial system fixatives. Actually, any specimen other than tissue thought to contain microsporidia could be stained by this method.

#### **Reagents**

##### **Stain**

Chromotrope 2R .....	6.0 g*
Aniline blue .....	0.5 g
Phosphotungstic acid .....	0.25 g
Acetic acid (glacial) .....	3.0 ml
Distilled water.....	100.0 ml

\*This is 10 times the amount used in the normal trichrome stain formula.

1. Prepare the stain by adding 3.0 ml of acetic acid to the dry ingredients. Allow the mixture to stand (ripen) for 30 min at room temperature.
2. Add 100 ml of distilled water and adjust the pH to 2.5 with 1.0 M HCl. Correctly prepared stain is dark purple. The staining solution should be protected from light.
3. Store in a glass or plastic bottle at room temperature. The shelf life is at least 24 months.

##### **Acid-Alcohol**

90% ethyl alcohol.....	995.5 ml
Acetic acid (glacial) .....	4.5 ml

Prepare by combining the two solutions.

#### **Quality Control**

1. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores as the control organisms. Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual organisms because the spores are very small (1 to 1.5  $\mu\text{m}$ ) and difficult to stain.
2. A QC slide should be included with each run of stained slides, particularly if the staining setup is used infrequently.

3. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids should be used).
4. Depending on the volume of slides stained, staining solutions should be changed on an as-needed basis.
5. When the smear is thoroughly fixed and the stain is performed correctly, the spores are seen to be ovoid and refractile, with the spore wall being bright pinkish red. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore. The majority of the bacteria and other debris tend to stain blue. However, some bacteria and debris stain red.
6. The specimen should also be checked (macroscopically) for adherence to the slide.
7. The microscope should be calibrated (within the last 12 months), and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this varies from laboratory to laboratory, depending on equipment care and use.
8. Known positive microscope slides, Kodachrome 2 by 2 projection slides, and photographs (reference books) should be available at the workstation.
9. Record all QC results; the laboratory should also have an action plan for "out of control" results.

### **Detailed Procedure**

1. Using a 10- $\mu$ l aliquot of concentrated (10 min at 500  $\times$  g), preserved stool (5 or 10% formalin, SAF, or one of the zinc-based single-vial preservatives), prepare the smear by spreading the material over an area of 45 by 25 mm.
2. Allow the smear to air dry.
3. Place the slide in absolute methanol for 5 or 10 min.
4. Allow the smear to air dry.
5. Place the slide in trichrome stain for 90 min.
6. Rinse in acid-alcohol for no more than 10 s.
7. Dip the slide several times in 95% alcohol. Use this step as a rinse (no more than 10 s).
8. Place the slide in 95% alcohol for 5 min.
9. Place the slide in 95% alcohol for 5 min.
10. Place the slide in 100% alcohol for 10 min.
11. Place the slide in xylene substitute for 10 min.
12. Mount with a coverslip (no. 1 thickness), using mounting medium.
13. Examine the smear under oil immersion (magnification,  $\times$ 1,000), and read at least 300 fields; the examination time will probably be at least 10 min per slide.

### **Reporting**

The microsporidial spore wall should stain pinkish to red, with the interior of the spore being clear or perhaps showing a horizontal or diag-

## **MODIFIED TRICRHOME STAIN FOR MICROSPORIDIA (continued) (Ryan, Blue Counterstain)**

onal stripe which represents the polar tube. The background should appear green or blue, depending on the method. Other bacteria, some yeast cells, and some debris stain pink to red; the shapes and sizes of the various components may be helpful in differentiating the spores from other structures. The results of this staining procedure should be reported only if the positive control smears are acceptable. The use of immunoassay reagents should provide a more specific and sensitive approach to the identification of the microsporidia in fecal specimens.

1. Report the organism. Do not use abbreviations.

Examples: Microsporidia present

Most likely *Enterocytozoon bieneusi* or *Encephalitozoon intestinalis* present (if fecal specimens are used)

*Encephalitozoon intestinalis* present (identification to species is highly likely; this is generally the organism involved in disseminated cases from the gastrointestinal tract to the kidneys, with the organisms being recovered in urine).

### **Procedure Reminders**

1. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.
2. Because of the difficulty in getting stain to penetrate through the spore wall, prepare thin smears and do not reduce the staining time in trichrome. Also, make sure that the slides are not left too long in the decolorizing agent (acid-alcohol). If the control organisms are too light, leave them in the trichrome longer and shorten the time to two dips in the acid-alcohol solution. Also, remember that the 95% alcohol rinse after the acid-alcohol step should be performed quickly to prevent additional destaining from the acid-alcohol reagent.
3. When you purchase the chromotrope 2R, obtain the highest dye content available. Two sources are Harleco (Gibbstown, NJ) and Sigma Chemical Co. (St. Louis, MO) (the dye content is among the highest [85%]). Fast green and aniline blue can be obtained from Allied Chemical and Dye (New York, NY).
4. In the final stages of dehydration, the 100% ethanol and the xylenes (or xylene substitutes) should be kept as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from 100% alcohol, return the slides to 100% alcohol and replace the xylene with fresh stock.

### **Procedure Limitations**

1. Although this staining method stains the microsporidia, the range of stain intensity and the small size of the spores tend to cause

some difficulty in identifying these organisms. Since this procedure results in many other organisms or objects staining in stool specimens, differentiation of the microsporidia from surrounding material is still very difficult. There also tends to be some slight size variation among the spores.

2. If the patient has severe watery diarrhea, there will be less artifact material in the stool to confuse with the microsporidial spores; however, if the stool is semiformed or formed, much more artifact material will be present and the spores will be much harder to detect and identify. Also, remember that the number of spores varies according to the stool consistency (the more diarrheic the stool, the more spores that will be present).
3. The investigators who developed some of these procedures feel that concentration procedures result in an actual loss of microsporidial spores; thus, there is a strong recommendation to use unconcentrated, formalinized stool. However, there are no data indicating what centrifugation speeds, etc., were used in the study.
4. In the UCLA Clinical Microbiology Laboratory, we have generated data (unpublished) to indicate that centrifugation at  $500 \times g$  for 10 min dramatically increases the number of microsporidial spores available for staining (from the concentrate sediment). This is the same method we use for centrifugation of all stool specimens, regardless of the suspected organism.
5. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and allow them to flow into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used. Another option is to use the commercially available concentration systems that use metal or plastic screens for filtration.

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#### **MODIFIED TRICHRONE STAIN FOR MICROSPORIDIA (Evelyn Kokoskin, Hot Method)**

##### **Description**

Changes in temperature from room temperature to 50°C and in the staining time from 90 to 10 min have been recommended as improvements for the modified trichrome staining methods. The procedure is as follows:

1. Using a 10- $\mu$ l aliquot of unconcentrated, preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material over an area of 45 by 25 mm.
2. Allow the smear to air dry.
3. Place the slide in absolute methanol for 5 min.
4. Allow the smear to air dry.
5. Place the slide in trichrome stain for 10 min at 50°C.
6. Rinse in acid-alcohol for no more than 10 s.

## **MODIFIED TRICHOME STAIN FOR MICROSPORIDIA (continued) (Evelyn Kokoskin, Hot Method)**

7. Dip the slide several times in 95% alcohol. Use this step as a rinse (no more than 10 s).
8. Place the slide in 95% alcohol for 5 min.
9. Place the slide in 100% alcohol for 10 min.
10. Place the slide in xylene substitute for 10 min.
11. Mount with a coverslip (no. 1 thickness), using mounting medium.
12. Examine smears under oil immersion (magnification,  $\times 1,000$ ), and read at least 100 fields; the examination time will probably be at least 10 min per slide.

### **REVIEW: MODIFIED TRICHOME-STAINED SMEARS**

Clinical relevance	To provide contrasting colors for both the background debris and parasites present; designed to allow examination and recognition of organism morphology under oil immersion (100 $\times$ objective for a total magnification of $\times 1,000$ ). Designed primarily to allow recovery and identification of microsporidial spores. Internal morphology (horizontal or diagonal "stripes") may be seen in some spores under oil immersion (magnification, $\times 1,000$ ).
Specimen	Any stool specimen that is fresh or preserved in formalin, SAF, or one of the single-vial system fixatives
Reagents	Modified trichrome stain (using high-dye-content chromotrope 2R) and associated solutions; dehydrating solutions (alcohols and xylenes); mounting fluid optional
Examination requirements	Oil immersion examination of at least 300 fields; additional fields may be required if suspect organisms have been seen but are not clearly identified.
Results and laboratory reports	Identification of microsporidial spores may be possible; however their small size makes recognition difficult. Final laboratory results would depend heavily on the appearance of the QC slides and comparison with patient specimens. It is mandatory that some spores be seen, which contain either horizontal or diagonal stripes (polar tubule).

*(continued)*

## **REVIEW: MODIFIED TRICHRONE-STAINED SMEARS (continued)**

Procedure reminders and limitations	Because of the difficulty in getting dye to penetrate the spore wall, this staining approach can be very helpful. Procedure limitations are related to specimen handling (proper centrifugation speeds and times, use of no more than two layers of wet gauze for filtration, and a complete understanding of the difficulties in recognizing microsporidial spores due to their small size [1 to 2.5 $\mu\text{m}$ ]).
Important questions for commercial suppliers	Make sure to ask about specific fixatives and whether the fecal material can be stained with the modified trichrome stains or modified acid-fast stains. Also, ask if the fixatives prevent the use of any of the immunoassay methods now available for several of the intestinal amebae, flagellates, coccidia, and microsporidia.

## **Fecal Immunoassays for Intestinal Protozoa**

Fecal immunoassays are generally simple to perform and allow a large number of tests to be performed at one time, thereby reducing overall costs. A major disadvantage of antigen detection in stool specimens is that the method can detect only one or two pathogens at a time. One still must perform a routine ova and parasite examination to detect other parasitic pathogens. The current commercially available antigen tests have superior sensitivity and specificity compared with routine microscopy.

Current formats include the enzyme-linked immunosorbent assay, the fluorescent-antibody (FA) test, and the rapid membrane flow cartridges. Sensitivity and specificity are comparable among the various formats and kits currently available. Selection of any particular format immunoassay often depends on the workflow options within the laboratory, based primarily on test menu orders received from the physician. The methods are different, but the results are comparable.

### ***Entamoeba histolytica***

Antigen-based fecal immunoassays have several significant advantages over other methods currently used for diagnosis of amebiasis: (i) some of the assays differentiate *E. histolytica* from *E. dispar*, (ii) they have excellent sensitivity and specificity, (iii) they are readily usable by most laboratory personnel, and (iv) they have potential use in situations such as waterborne outbreaks. Because there are distinct genetic differences between *E. dispar* (a nonpathogen) and *E. histolytica* (a true pathogen), commercial kits have been developed to detect their presence and differentiate them in clinical samples. However, current antigen detection tests require the examination of fresh or frozen (not preserved) stool specimens, while many laboratories have switched to stool collection methods using various preservatives.

## Fecal Immunoassays for Intestinal Protozoa (continued)

### ***Cryptosporidium* spp.**

A number of commercially available immunoassay kits are available for detection of *Cryptosporidium* spp. and are more sensitive and specific than routine microscopic examination of modified acid-fast stained smears. Stool specimens may be fresh, frozen, or fixed; however, PVA-fixed specimens are currently unacceptable for use in the fecal immunoassays.

### ***Giardia lamblia***

Detection of *Giardia* in stool specimens by various immunoassay methods has been reported. These tests are reliable and more sensitive and specific than routine O&P exams. Commercial immunoassay kits are readily available. Users will have to evaluate which kit format will be most useful for their own laboratories. Some of the methods may require fresh specimens, and stools fixed in preservatives may not be suitable. Also, some of the kits may not detect both trophozoites and cysts of *G. lamblia* but may be selective for only one life cycle stage.

### **Kits under Development**

Although not currently available commercially, several immunoassays are in various developmental phases. These include antigen detection kits for *Dientamoeba fragilis*, *Blastocystis hominis*, *Cyclospora cayetanensis*, and various species of the microsporidia.

### **Comments on the Performance of Fecal Immunoassays**

Some comments about various immunoassay formats are provided to assist you in evaluating test performance and/or result interpretation. It is very important to read the kit information sheet before use. Currently, fecal immunoassays are available for *G. lamblia*, the *E. histolytica/E. dispar* group, *E. histolytica*, and *Cryptosporidium* spp. Based on the published literature, fecal immunoassays are more sensitive and specific than the routine O&P exam; this is particularly true for *G. lamblia*. However, unlike the O&P exam, which facilitates the recovery of many different parasites, the fecal immunoassays are limited to one or two organisms only. The fecal immunoassays are also more sensitive than the special stains (modified acid-fast stains) for the coccidia (*Cryptosporidium* spp.).

Fresh specimens can be stored at 2 to 8°C and should be tested within 48 h, or they should be frozen at -20 to -70°C (freezing is not acceptable for the FA method; the freeze-thaw cycle damages the organisms). Stool specimens preserved in 10% formalin, MF, or SAF fixatives may be refrigerated at 2 to 8°C or stored at room temperature (20 to 25°C) and should be tested within 2 months. Stool specimens submitted in Cary Blair transport medium (or equivalent) should be refrigerated or frozen and tested within 1 week after collection. Fecal specimens that have been preserved in fixatives containing PVA are not acceptable for testing.

With the FA procedure, the actual organisms (*G. lamblia* and/or *Cryptosporidium* spp.), not antigens, are seen via a color change. To enhance

the sensitivity of the FA procedure, it is recommended that testing be performed on centrifuged ( $500 \times g$  for 10 min) stool specimen.

### **Enzyme Immunoassays (Antigen Detection, No Centrifugation Recommended)**

In enzyme immunoassays, the antigen is found in the top fluid layer of the stool collection vial.

1. Remember to thoroughly rinse the wells according to the instructions; do not eliminate any of the rinse steps. Make sure that each well receives the total number of rinses required.
2. Make sure the stream of buffer goes directly into the wells. Use a wash bottle with a small opening, so you have to squeeze the bottle to get the fluid to squirt directly into the wells.
3. When the directions tell you to "slap" the tray down onto some paper towels to remove the last of the rinse fluid, make sure that you slap it several times. Don't be too gentle; the cups will not fall out of the holder.
4. Before the last reagents are added, the wells should be empty of rinse buffer (not dry, but empty of excess fluid).
5. **Note:** If you shake the specimen vial prior to testing, allow the vial contents to settle out for several minutes. Addition of too much particulate stool to the wells interferes with testing.

### **Fluorescence (Visual Identification of the Organisms)**

1. Since you will be looking for the actual organisms (cysts of *Giardia* and/or oocysts of *Cryptosporidium*), this test should be performed on centrifuged ( $500 \times g$  for 10 min) stool to increase the sensitivity of the test.
2. Remember to thin out the smear; it is important to make sure the slides are thoroughly dry before adding reagents. The slides can be placed in a  $35^{\circ}\text{C}$  incubator for about 30 min to 1 h to make sure they are dry before being processed. If the material on the wells is too thick, it may not dry thoroughly and may fall off of the glass. It is better to let them dry longer rather than for too short a time. A heat block is **not** recommended for this purpose.
3. Gently rinse the reagents from the wells; do not squirt directly into the wells, but allow the rinse fluid to flow over the wells.
4. Remember that not all clinical specimens will provide the 3+ to 4+ fluorescence that is often seen in the positive control. Also, from time to time, you may see fluorescing bacteria and/or some yeasts in certain patient specimens. This is not that common, but the shapes can be distinguished from *Giardia* cysts and *Cryptosporidium* oocysts.
5. The intensity of the fluorescence may vary, depending on the filters. If the fluorescence microscope dual-filter system is used, it demonstrates both the yellow-green fluorescence and the red-orange counterstain, and neither *Giardia* nor *Cryptosporidium* may appear quite as bright as when using the yellow-green filter only. Both approaches are acceptable and may reflect personal laboratory preferences. How-

## Fecal Immunoassays for Intestinal Protozoa (continued)

ever, remember that when the single FITC (yellow-green) filter is used alone, some artifact materials may also appear to fluoresce more brightly, while the artifact material might not be seen when both filters (FITC and counterstain) are used. Artifact material may fluoresce a dull color without the bright outlines seen around the *Giardia* cysts and *Cryptosporidium* oocysts that can be seen when both filter systems are used.

**Both filters = lower fluorescence intensity, less visible artifacts**

**Single FITC filter = brighter fluorescence, more visible artifacts**

6. Make sure to examine the edges of the wells. Sometimes in a light infection, the edges may contain organisms while in the middle of the well the organisms may be a bit more difficult to detect (thick area).

## Lateral-Flow Cartridges (Antigen Detection, No Centrifugation Recommended)

In the lateral-flow cartridge system, the antigen is found in the top fluid layer of the stool collection vial.

1. If the stool is too thick, the addition of reagents will not thin it out enough. If the specimen poured into the well remains too thick, the fluid does not flow up the membrane. If your specimens arrive in fixative and there is no fluid at the top of the vial overlaying the stool, this means that the vial may have been overfilled with stool. These specimens will have to be diluted with the appropriate diluent before being tested.
2. It is always important to see the control line indicated as positive all the way across the membrane, not just at the edges.
3. **A positive test result may be much lighter than the control line; this is normal.**
4. At the cutoff time to read the result, the presence of any color at all visible in the test area should be interpreted as a positive result.
5. Do not read/interpret the results after the time indicated in the directions; you may get a false-positive result.
6. Note: If you shake the specimen vial prior to testing, allow the vial contents to settle out for several minutes. Adding too much particulate stool to the wells interferes with testing.

## Larval Nematode Culture

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### HARADA-MORI FILTER PAPER STRIP CULTURE

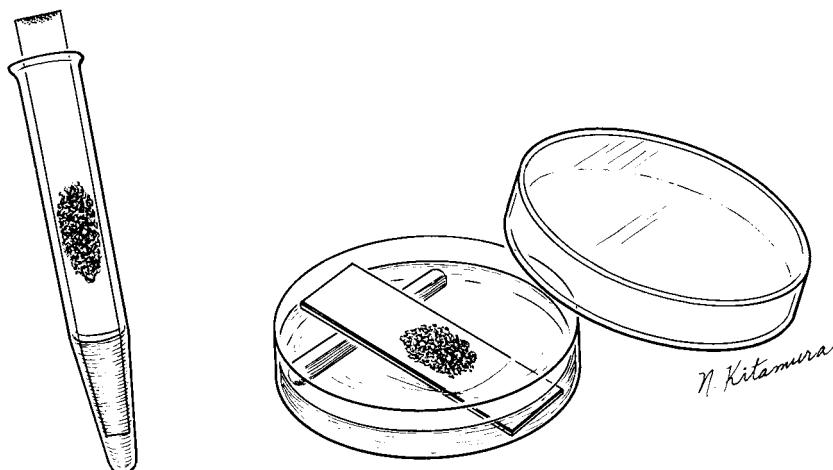
#### Description

To detect light infections with hookworm, *S. stercoralis*, and *Trichostongylus* spp., as well as to facilitate specific identification, the Harada-Mori

filter paper strip culture technique is recommended (Figure 5.5). The technique requires filter paper to which fresh fecal material is added and a test tube into which the filter paper is inserted. Moisture is provided by adding water to the tube, which continuously soaks the filter paper by capillary action. Incubation under suitable conditions favors hatching of ova and/or development of larvae. Fecal specimens to be cultured should not be refrigerated, since some parasites (especially *Necator americanus*) are susceptible to cold and may fail to develop after refrigeration. Also, caution must be exercised in handling the filter paper strip itself, since infective *Strongyloides* larvae may migrate upward as well as downward on the paper strip. Always observe Standard Precautions and wear gloves when performing these procedures.

### Quality Control

1. Follow routine procedures for optimal collection and handling of fresh fecal specimens for parasitologic examination.
2. Examine known positive and negative samples of stools (from laboratory animals), if available, to make sure that the procedure works.
3. Review larval diagrams and descriptions for confirmation of larval identification.
4. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope).
5. Record all QC results.



**Figure 5.5** Culture methods for the recovery of larval-stage nematodes; Harada-Mori tube method and petri dish culture method. (Illustration by Nobuko Kitamura.) Reprinted from *Diagnostic Medical Parasitology*, 5th ed.

## HARADA-MORI FILTER PAPER STRIP CULTURE (continued)

### Detailed Procedure

1. Smear 0.5 to 1 g of feces in the center of a narrow strip of filter paper (3/8 by 5 in. [1 in. = 2.54 cm], slightly tapered at one end).
2. Add 3 to 4 ml of distilled water to a 15-ml conical centrifuge tube; identify the specimen on the tube.
3. Insert the filter paper strip into the tube so that the tapered end is near the bottom of the tube. The water level should be approximately 0.5 in. below the fecal spot. It is not necessary to cap the tube. However, a cork stopper or a cotton plug may be used.
4. Maintain the tube upright in a rack at 25 to 28°C. Add distilled water to maintain the original level (usually evaporation takes place over the first 2 days, and then the culture becomes stabilized).
5. Keep the tube for 10 days, and check it daily by withdrawing a small amount of fluid from the bottom of the tube. Prepare a smear on a glass slide, cover the slide with a coverslip, and examine the smear with the 10× objective.
6. Examine the larvae for motility and typical morphologic features to reveal whether hookworm, *Strongyloides*, or *Trichostrongylus* larvae are present.

### Reporting

Larval nematodes of hookworm, *S. stercoralis*, or *Trichostrongylus* spp. may be recovered. If *Strongyloides* organisms are present, free-living stages and larvae may be found after several days in culture.

1. Report "No larvae detected" if no larvae could be detected at the end of the incubation.
2. Report larvae detected by fecal culture.

Example: *Strongyloides stercoralis* larvae detected by fecal culture

### Procedure Reminders

1. If the larvae are too active to observe under the microscope and morphologic details are difficult to see, the larvae can be heat killed within the tube or after removal to the slide; iodine can also be used to kill larvae.
2. Infective larvae may be found any time after the fourth day or even on the first day in a heavy infection. Since infective larvae may migrate upward as well as downward on the filter paper strip, caution must be exercised in handling the fluid and the paper strip itself to prevent infection. Handle the filter paper with forceps, and wear gloves when handling the cultures.
3. It is important to maintain the original water level to maintain optimum humidity.
4. Fresh stool is required for this procedure; preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method.

## **Procedure Limitations**

1. This technique allows both parasitic and free-living forms of nematodes to develop. If specimens have been contaminated with soil or water containing these forms, it may be necessary to distinguish parasitic from free-living forms. This distinction is possible since parasitic forms are more resistant to slight acidity than are free-living forms. Proceed as follows. Add 0.3 ml of concentrated hydrochloric acid per 10 ml of water containing the larvae (adjust the volume accordingly to achieve a 1:30 dilution of acid). Free-living nematodes are killed, while parasitic species live for about 24 h.
2. Specimens that have been refrigerated or preserved are not suitable for culture. Larvae of certain species are susceptible to cold environments.

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## **BAERMANN CONCENTRATION**

### **Description**

Another method of examining a stool specimen suspected of having small numbers of *Strongyloides* larvae is the use of a modified Baermann apparatus (Figure 5.6). The Baermann technique, which involves using a funnel apparatus, relies on the principle that active larvae migrate from a fresh fecal specimen that has been placed on a wire mesh with several layers of gauze which are in contact with tap water. Larvae migrate through the gauze into the water and settle to the bottom of the funnel, where they can be collected and examined. The main difference between this method and the Harada-Mori and petri dish methods is the greater amount of fresh stool used, possibly providing a better chance of larval recovery in a light infection. Besides being used for patient fecal specimens, this technique can be used to examine soil specimens for the presence of larvae.

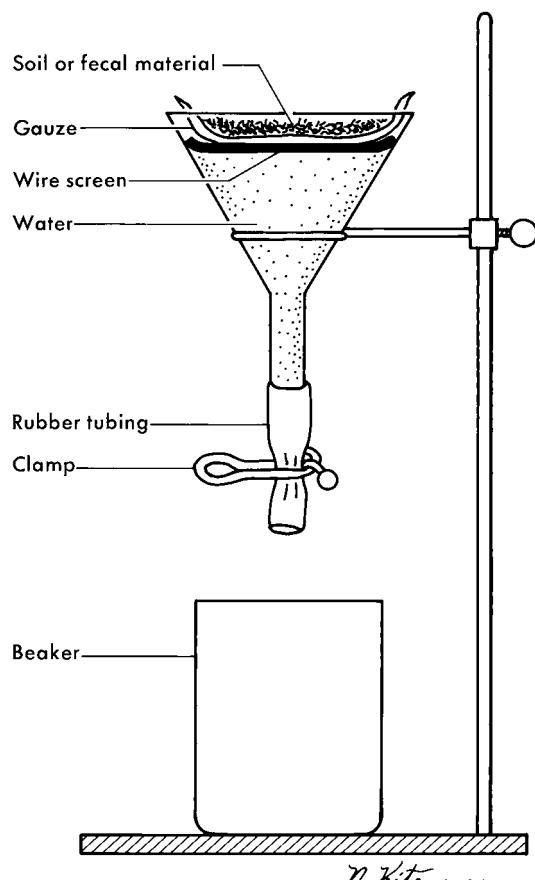
### **Quality Control**

1. Follow routine procedures for optimal collection and handling of fresh specimens for parasitologic examination.
2. Examine known positive and negative samples of stools (from laboratory animals), if available, to make sure that the procedure is precise.
3. Review larval diagrams for confirmation of larval identification.
4. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope).
5. Record all QC results.

### **Detailed Procedure**

1. If possible, use a fresh fecal specimen obtained after administration of a mild saline cathartic, not a stool softener. Soft stool is recommended; however, any fresh fecal specimen is acceptable.

## BAERMANN CONCENTRATION (continued)



**Figure 5.6** Baermann apparatus. (Illustration by Nobuko Kitamura.) Reprinted from *Diagnostic Medical Parasitology*, 5th ed.

N. Kitamura

2. Set up a clamp supporting a 6-in. glass funnel. Attach rubber tubing and a pinch clamp to the bottom of the funnel. Place a collection beaker underneath.
3. Place a wire gauze or nylon filter over the top of the funnel, followed by a pad consisting of two layers of gauze.
4. Close the pinch clamp at the bottom of the tubing, and fill the funnel with tap water until it just soaks the gauze padding.
5. Spread a large amount of fecal material on the gauze padding so that it is covered with water. If the fecal material is very firm, first emulsify it in water.
6. Allow the apparatus to stand for 2 h or longer; then draw off 10 ml of fluid into the beaker by releasing the pinch clamp, centrifuge for 2 min at  $500 \times g$ , and examine the sediment under the microscope (magnification,  $\times 100$  and  $\times 400$ ) for the presence of motile larvae. Make sure that the end of the tubing is well inside the beaker before slowly releasing the pinch clamp. Infective larvae may be present; wear gloves when performing this procedure.

## **Reporting**

Larval nematodes (hookworm, *S. stercoralis*, or *Trichostrongylus* spp.) may be recovered. Both infective and noninfective *Strongyloides* larvae may be recovered, particularly in a heavy infection.

1. Report "No larvae detected" if no larvae could be detected at the end of incubation.
2. Report larvae detected by fecal culture.  
Example: *Strongyloides stercoralis* larvae detected by fecal culture

## **Procedure Reminders**

1. It may be difficult to observe morphological details in rapidly moving larvae; a drop of iodine or formalin or slight heating can be used to kill the larvae.
2. Infective larvae may be found any time after the fourth day and occasionally after the first day in heavy infections. Caution must be exercised in handling the fluid, gauze pad, and beaker to prevent infection. Wear gloves when using this technique.
3. Remember to make sure that the pinch clamp is tight until you want to release some of the water.
4. Preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method; fresh stool specimens must be obtained.

## **Procedure Limitations**

1. This technique allows both parasitic and free-living forms of nematodes to develop. If specimens have been contaminated with soil or water containing these forms, it may be necessary to distinguish parasitic from free-living forms. This distinction is possible since parasitic forms are more resistant to slight acidity than are free-living forms. Proceed as follows. Add 0.3 ml of concentrated hydrochloric acid per 10 ml of water containing the larvae (adjust the volume accordingly to achieve a 1:30 dilution of acid). Free-living nematodes are killed, while parasitic species live for about 24 h.
2. Specimens that have been refrigerated or preserved are not suitable for culture. Larvae of certain species are susceptible to cold environments.
3. Gloves should be worn when this procedure is performed.
4. Release the pinch clamp slowly to prevent splashing; have the end of the tubing close to the bottom of the beaker for the same reason.

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## **AGAR PLATE CULTURE FOR *STRONGYLOIDES STERCORALIS***

### **Description**

Agar plate cultures are also recommended for the recovery of *S. stercoralis* larvae and **tend to be more sensitive than some of the other diagnostic methods**. Stool is placed onto agar plates, and the plates are sealed to prevent accidental infections and held for 2 days at room temperature. As the larvae crawl over the agar, they carry bacteria with them, thus

## **AGAR PLATE CULTURE FOR *STRONGYLOIDES STERCORALIS***

**(continued)**

creating visible tracks over the agar. The plates are examined under the microscope for confirmation of larvae, the surface of the agar is then washed with 10% formalin, and final confirmation of larval identification is made via wet examination of the sediment from the formalin washings (Figure 5.7).

### **Reagents**

#### **Agar**

- 1.5% Agar
- 0.5% Meat extract
- 1.0% Peptone
- 0.5% NaCl

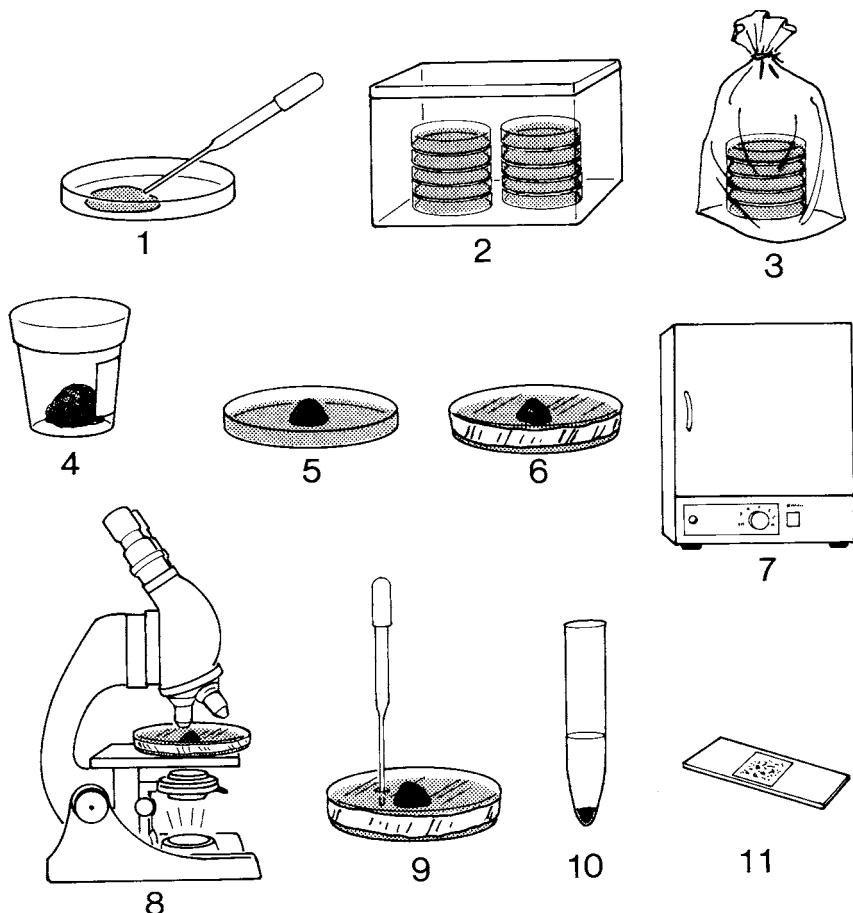
**Note:** Positive tracking on agar plates has been seen on a number of different types of agar. However, the most appropriate agar formula is that seen above.

### **Quality Control**

1. Follow routine procedures for optimal collection and handling of fresh fecal specimens for parasitologic examination.
2. Examine agar plates to ensure that there is no cracking and that the agar pour is sufficient to prevent drying. Also, make sure that there is no excess water on the surface of the plates.
3. Review larval diagrams and descriptions for confirmation of larval identification.
4. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope).
5. Record all QC results (condition of agar plates).

### **Detailed Procedure**

1. Place approximately 2 g of fresh stool (approximately 1 in. in diameter) in the center of the agar plate.
2. Replace the lid, and seal the plate with cellulose tape.
3. Maintain the plate (right side up) at room temperature for 2 days.
4. After 2 days, examine the sealed plate through the plastic lid under the microscope for microscopic colonies that develop as random tracks on the agar and evidence of larvae at the ends of the tracks away from the stool.
5. With the end of hot forceps, make a hole in the top of the plastic Petri dish.



**Figure 5.7** Agar culture method for *Strongyloides stercoralis*. (1) Agar plates are prepared; (2) agar is dried for 4 to 5 days on the bench top; (3) plates are stored in plastic bags; (4) fresh stool is submitted to the laboratory; (5) approximately 2 g of stool is placed onto an agar plate; (6) the plate is sealed with tape; (7) the culture plate is incubated at 26 to 33°C for 2 days; (8) the plate is examined microscopically for the presence of tracks (bacteria carried over the agar by migrating larvae); (9) 10% formalin is placed onto the agar through a hole made in the plastic by hot forceps; (10) material from the agar plate is centrifuged; (11) the material is examined as a wet preparation for rhabditiform or filariform larvae (high dry power; magnification,  $\times 400$ ). (Illustration by Sharon Belkin.) Reprinted from *Diagnostic Medical Parasitology*, 5th ed.

6. Gently add 10 ml of 10% formalin through the hole onto the agar surface, swirl to cover the surface, and rinse the agar plate. Allow the plate to stand for 30 min.
7. Remove the tape and lid of the agar plate. Pour the 10% formalin through a funnel into a centrifuge tube. Do not try and pour the formalin off directly into the centrifuge tube because the tube opening is too small and formalin will be spilled onto the counter.

## **AGAR PLATE CULTURE FOR *STRONGYLOIDES STERCORALIS*** **(continued)**

8. Centrifuge the formalin rinse fluid for 5 min at 500 × g.
9. Prepare a wet smear preparation from sediment, and examine it with the 10× objective (low power) for presence of larvae. If larvae are found, confirm the identification with the 40× objective (high dry power).

### **Reporting**

Larval nematodes of hookworm, *S. stercoralis*, or *Trichostrongylus* spp. may be recovered. If *Strongyloides* organisms are present, free-living stages and larvae may be found after several days on the agar plates.

1. Report "No larvae detected" if no larvae could be detected at the end of the incubation and rinse procedure.
2. Report larvae detected by agar plate culture.

Example: *Strongyloides stercoralis* larvae detected by agar plate culture

### **Procedure Reminders**

1. If the larvae are too difficult to observe under the microscope and morphologic details are difficult to see, the larvae can be formalin-killed within the plate and examined in the formalin concentrated sediment.
2. Infective larvae may be found any time after the first or second day or even on the first day in a heavy infection. Since infective larvae may be present on the agar, caution must be exercised in handling the plates once the cellulose tape is removed. Wear gloves when handling the cultures.
3. It is important to maintain the plates upright at room temperature. Do not incubate or refrigerate them at any time; this also applies to the fresh stool specimen.
4. Fresh stool is required for this procedure; preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method.

### **Procedure Limitations**

1. This technique is successful if any larvae present are viable. If the fresh stool specimen is too old, larvae may not survive and a negative result will be reported.
2. Specimens that have been refrigerated or preserved are not suitable for culture. Larvae of certain species are susceptible to cold environments.

## REVIEW: LARVAL NEMATODE CULTURE

Clinical relevance	Culture of feces for larvae is useful to (i) reveal their presence when they are too scanty to be detected by concentration methods; (ii) distinguish whether the infection is due to <i>S. stercoralis</i> or hookworm on the basis of rhabditiform larval morphology by allowing hookworm egg hatching to occur, thus releasing first-stage larvae; and (iii) allow the development of larvae into the filariform stage for further differentiation.
Specimen	Any stool specimen that is fresh and has not been refrigerated
Reagents	Appropriate tubes, plates, funnels, gauze, and agar formula
Examination requirements	Daily checking of the fluid for the presence of larvae; hold the cultures for 10 days prior to making a final report.
Results and laboratory reports	The failure to recover larvae does not completely rule out the possibility of infection; however, the probability of infection is lower when results are negative.
Procedure reminders and limitations	There is always the prospect of recovering infective larvae; gloves must be worn at all times when performing these procedures and examining fluid. Make sure that the culture systems are kept hydrated; a certain amount of water will evaporate and be lost as a result of culture equilibration, particularly during the first couple of days. The agar plate culture for <i>S. stercoralis</i> is considered to be more sensitive than most other diagnostic methods for this particular parasite.

## Other Methods for Gastrointestinal Tract Specimens

### EXAMINATION FOR PINWORM (Cellulose Tape Preparations)

#### Description

*Enterobius vermicularis*, known as pinworm, is a roundworm parasite that has worldwide distribution and is commonly found in children. The adult female worm migrates out of the anus, usually at night, and deposits her eggs on the perianal area. The adult female (8 to 13 mm long) may be found on the surface of a stool specimen or on the perianal skin.

## **EXAMINATION FOR PINWORM (*continued*) (Cellulose Tape Preparations)**

Since the eggs are usually deposited around the anus, they are not commonly found in feces and must be detected by other diagnostic techniques. Diagnosis of pinworm infection is based on the recovery of typical eggs, which are described as thick-shelled, football-shaped eggs with one slightly flattened side. Each egg often contains a fully developed embryo and is infective within a few hours after being deposited.

The clear-cellulose-tape preparation is the most widely used procedure for the detection of human pinworm infections. Tapes for egg collection are also available commercially. The eggs, and occasionally the adult female worms, stick to the sticky surface of the cellulose tape. These cellulose tape preparations are submitted to the laboratory, where they are examined microscopically. Several commercial collection procedures are also available. Specimens should be obtained in the morning before the patient bathes or goes to the bathroom. At least four to six consecutive negative slides should be observed before the patient is considered free of infection.

### **Quality Control**

The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope). Pictures of *Enterobius* eggs (with measurements) should be available for comparison with the clinical specimen.

### **Detailed Procedure**

1. Place a strip of cellulose tape on a microscope slide, starting 0.5 in. (1 in. = 2.54 cm) from one end and running toward the same end, continuing around this end lengthwise; tear off the strip evenly with the other end. Place a strip of paper, 0.5 by 1 in., between the slide and the tape at the end where the tape is torn flush.
2. To obtain the sample from the perianal area, peel back the tape by gripping the label, and with the tape looped (adhesive side outward) over a wooden tongue depressor held against the slide and extended about 1 in. beyond it, press the tape firmly against the right and left perianal folds.
3. Spread the tape back on the slide, adhesive side down.
4. Write the name and date on the label.

**Note:** Do not use Magic transparent tape; use regular clear cellulose tape. If Magic tape is submitted, a drop of immersion oil can be placed on top of the tape to facilitate clearing.

5. Lift one side of the tape, apply 1 small drop of toluene or xylene, and press the tape down on the glass slide. The preparation will then be cleared, and the eggs will be visible.
6. Examine the slide at low power and under low illumination.

### **Reporting**

Typical pinworm eggs are thick-shelled, football shaped with one flattened side and may contain a partially or fully developed larva. Occasionally adult worms are seen on the scotch tape preparation.

1. Report the organism and stage (do not use abbreviations).  
Example: *Enterobius vermicularis* eggs present
2. Report adult worms.  
Example: *Enterobius vermicularis* adult worm present
3. Report "No *Enterobius vermicularis* eggs or adults seen" for a negative result.

### **Procedure Reminders**

1. Pinworm eggs are usually infectious. The use of glass slides and tapes may expose laboratory personnel to these eggs.
2. Some investigators recommend the use of the Swube (a paddle with a sticky adhesive coat [Becton Dickinson]) as a safer alternative.
3. If opaque tape is submitted by mistake, a drop of immersion oil on top of the tape will clear it enough to proceed with the microscopic examination.

### **Procedure Limitations**

1. The female pinworm deposits eggs on the perianal skin only sporadically. Therefore without multiple tapes (taken consecutively, one each morning), it is not possible to determine if the patient is positive or negative for the infection.
2. Occasionally, a parent will bring in an adult worm collected from the perianal skin or from the surface of the stool. The identification of the adult worm (almost always the female) confirms the infection.

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## **SIGMOIDOSCOPY SPECIMENS**

### **(Direct Wet Smear)**

#### **Description**

The direct smear is used primarily to detect motile parasites that are found in the colon (the organism in question is usually *Entamoeba histolytica*). Specific ulcerated areas should always be sampled; in the absence of specific lesions, the mucosa would be randomly sampled. On low-power (magnification,  $\times 100$ ) examination of the smear, motility of trophozoites and/or human cells might be detected. At high-dry power (magnification,  $\times 430$ ), organisms might be tentatively identified based on size, nuclear/cytoplasm ratio, appearance of the cytoplasm, and motility (sa-

## SIGMOIDOSCOPY SPECIMENS (*continued*) (Direct Wet Smear)

line only). The direct smear can be prepared with either 0.85% NaCl or the addition of iodine (Lugol's or D'Antoni's). Presumptive findings from this procedure must be confirmed by using some type of permanent stained smear. The specimen may consist of mucosal lining, mucus, stool, or a combination of the three. The specimen is taken by the physician and either prepared at bedside for immediate review or submitted to the laboratory for subsequent examination. Prepare direct wet mounts on clean, new 1- by 3-in. glass slides. Depending on the specimen type, the following amount should be used:

1. For mounts of mucus or similar material, place approximately 1 to 2 drops onto the slide.
2. For mounts of stool, place approximately 1 to 2 drops onto the slide.
3. If the material is very wet (watery), add 1 to 2 drops onto the slide.

If the specimen must be transported to the laboratory, the material can be placed in a small amount of 0.85% NaCl (0.5 to 1.0 ml) to keep the specimen from drying out. These specimens should be transported to the laboratory within no more than 30 min from the collection time and should be examined immediately.

### Reagents

#### 0.85% NaCl

Sodium chloride (NaCl)..... 850 mg  
Distilled water..... 100 ml

1. Dissolve the ingredients in distilled water in an appropriate glass flask, using a magnetic stirrer.
2. Store in a glass bottle.
3. Label as 0.85% saline with the preparation date and an expiration date of 6 months. Store at room temperature.

#### Modified D'Antoni's Stock Iodine

Potassium iodide (KI)..... 1.0 g  
Powdered iodine crystals..... 1.5 g  
Distilled water..... 100 ml

1. Dissolve the ingredients in distilled water in an appropriate glass flask, using a magnetic stirrer.
2. The D'Antoni's solution should be saturated with iodine, with some excess crystals left in the bottle. Store the solution in a brown bottle at room temperature. The stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle.
3. Label as D'Antoni's stock iodine with the preparation date and an expiration date of 1 year.

4. Small amounts of stock iodine solution can be aliquoted into brown dropper bottles and used for routine daily use. The expiration date will be 30 to 60 days, depending on the amount of fading of the solution from the normal strong-tea color. The use of a small and/or clear-glass dropper bottle will result in a shorter expiration time, whereas the use of a brown bottle will lengthen the expiration time.

### Lugol's Iodine Solution

Potassium iodide (KI)..... 10.0 g  
Iodine crystals ..... 5.0 g  
Distilled water ..... 100 ml

1. Dissolve the ingredients in distilled water in an appropriate glass flask, using a magnetic stirrer.
2. The Lugol's iodine solution should be saturated with iodine, with some excess crystals left in the bottle. Store the solution in a brown bottle at room temperature. The stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle.
3. Label as Lugol's stock iodine with the preparation date and an expiration date of 1 year.
4. Small amounts of stock iodine solution can be aliquoted into brown dropper bottles and used for routine daily use. The expiration date will be 30 to 60 days, depending on the amount of fading of the solution from the normal strong-tea color. The use of a small and/or clear-glass dropper bottle will result in a shorter expiration time, whereas the use of a brown bottle will lengthen the expiration time.

### Quality Control

1. Check the direct-mount reagents each time they are used.
  - a. The saline should appear clear, without any visible contamination.
  - b. The iodine should be a strong-tea color, and there should be crystals in the bottom of the bottle. Small aliquots of the stock solution should always be a strong-tea color. If not, discard them and aliquot some stock solution into your dropper bottle.
2. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope).
3. Record all QC results.

### Detailed Procedure

1. To 1 or 2 drops of patient material on the slide, add 1 or 2 drops of 0.85% NaCl, mix with the corner of the coverslip or an applicator

## SIGMOIDOSCOPY SPECIMENS (continued) (Direct Wet Smear)

- stick and mount with a no. 1 coverslip (22 by 22 mm). The amount of saline is determined by the specimen (less saline is needed if the material is very liquid).
2. Examine the smear with the low-power objective (10 $\times$ ) under low light. View each field for a few seconds, looking for any organism motility. Any suspicious objects can be examined using the high dry objective (40 $\times$ ) under low light.
  3. Prepare a wet mount using Lugol's or D'Antoni's iodine (working solution) rather than saline. Another option would be to add a small drop of iodine at the side of the coverslip on the saline wet preparation. The iodine will diffuse into the saline suspension under the coverslip. However, if the specimen is thick or contains mucus, capillary action pulling the iodine under the coverslip and into the saline may not occur and a separate iodine mount may be required. Addition of the iodine gives the material some color (organisms may be easier to see); however, motility is lost.

### Reporting

1. The organism and stage (trophozoite, cyst, oocyst, etc.) should be reported (do not use abbreviations); however, confirmation of species may require some type of permanent stained smear.  
Example: *Cryptosporidium* oocysts
2. The presence of human cells should be noted and quantitated.  
Example: Moderate WBCs, many RBCs, few macrophages
3. The physician should be called if pathogenic organisms are identified.
4. If the results are negative, this should be reported as a presumptive report (based on wet examination only) prior to the examination of the permanent stained smear.

Quantitation of parasites, cells, yeast, and artifacts

Quantity	Protozoa, cells, yeasts, artifacts		Helminths
	PVA smears (no. per 10 oil immersion fields) ( $\times 1,000$ )	Wet preparations (no. per 10 40 $\times$ fields) ( $\times 400$ )	Wet preparations (no. per 22-mm coverslip)
Few	$\leq 2$	$\leq 2$	$\leq 2$
Moderate	3–9	3–9	3–9
Many	$\geq 10$	$\geq 10$	$\geq 10$

This is a general chart for the quantitation of parasites, cells, yeast, and artifacts found in specimens from the intestinal tract. In general, protozoa are not quantitated on the laboratory slip (*Blastocystis hominis* is an ex-

ception); however, human cells, yeast, and artifacts like Charcot-Leyden crystals are normally reported and quantitated.

### **Procedure Reminders**

1. Remember that the iodine working solution should be a strong-tea color; if it is not, discard it and prepare a new working solution.
2. Final identification of some of the intestinal protozoa may be difficult (small size, confusion between organisms and human cells); a permanent stained smear must be used as a confirmatory method and examined at  $\times 1,000$  to see morphologic details.
3. In saline, human cells and/or protozoan trophozoites may exhibit some motility.
4. In iodine, human cells and/or protozoan trophozoites may be seen (but exhibit no motility).
5. Presumptive findings (either positive or negative) must be confirmed using a permanent stained smear.
6. Protozoan trophozoites may be confused with human cells (macrophages), so any identification should be reported as "presumptive" until the permanent stained smears have been examined.
7. The presumptive identification and quantitation of the human cells (macrophages, PMNs, eosinophils, and RBCs) could be obtained from the wet preparations. However, this information should also be considered "presumptive" until the permanent stained smears have been examined.

### **Procedure Limitations**

1. Multiple areas of the mucosa should be examined (six smears are often recommended); this technique should not take the place of the routine O&P exam.
2. Wet preparations are normally not examined by using oil immersion power (magnification,  $\times 1,000$ ). Consequently, permanent stained smears should be used to confirm morphology and organism (or human cell) identification.
3. If the specimen amount is limited, then the wet preparation should not be performed and the specimen that is available should be processed by the permanent stained smear method to maximize the amount and clinical relevance of the information obtained.

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## **SIGMOIDOSCOPY SPECIMENS**

### **(Permanent Stained Smear)**

#### **Description**

Most of the material obtained at sigmoidoscopy can be smeared (gently) onto a slide and immediately immersed in Schaudinn's fixative. These slides can then be stained with trichrome stain and examined for specific cell morphology, either protozoan or otherwise. The procedure and staining times are identical to those for routine fecal smears. Specific ulcerated areas are always sampled; in the absence of specific lesions, the mucosa

## **SIGMOIDOSCOPY SPECIMENS (continued)**

### **(Permanent Stained Smear)**

would be randomly sampled. On oil immersion power (magnification,  $\times 1,000$ ) examination of the smear, protozoan trophozoites and/or cysts might be detected. Coccidian oocysts, helminth eggs or larvae, and/or human cells are also detected by this procedure. The permanent smear can be stained with trichrome or iron hematoxylin stains. These permanent stained smears usually confirm the identity of structures that might have been seen on the wet specimen examinations. The specimen may consist of mucosal lining, mucus, stool, and/or a combination of the three.

1. Prepare smears on clean, new 1- by 3-in. glass slides.
2. For mounts of mucus or similar material, place approximately 1 to 2 drops on the slide.
3. For mounts of stool, place approximately 1 drop on the slide.
4. If the material is very wet (watery), you can add 1 to 2 drops to the slide.

#### **Reagents**

1. Schaudinn's fixative (see Section 3)
2. PVA fixative (see Section 3)

#### **Quality Control**

1. Check the fixatives weekly or when a new lot number is used. Fresh stool containing protozoa or negative stool seeded with human buffy coat cells can be used to evaluate the efficacy of the fixatives. Cultured protozoa can also be used.
2. The Schaudinn's fixative should appear clear, without floating debris or crystals. It is acceptable to have some crystal sediment on the bottom of the Coplin jar or dish.
3. The PVA should be clear (slight milky or smoky color is acceptable). There may be a slight precipitate on the bottom of the container (acceptable). The fluid should easily move in the bottle when it is inverted, and the viscosity of many of the available formulations actually approaches that of water.

#### **Detailed Procedure**

1. Gently smear 1 or 2 drops of patient material onto the slide, and immediately immerse the slide in Schaudinn's fixative. The fixation and staining times are identical to those for routine fecal smears. Refer to the trichrome stain procedure above.
2. If the material is bloody, contains a lot of mucus, or is a "wet" specimen, gently mix 1 or 2 drops of patient material with 3 to 4 drops of PVA fixative directly on the slide. The smear should be allowed to air dry for at least 2 h before being stained. The fixation and staining times are identical to those for routine fecal smears.

3. Examine the stained smear using the oil immersion lens (97 to 100 $\times$  objective) under maximum light. At least 300 oil immersion fields of the smear should be examined.

### **Reporting**

1. With either the trichrome or iron hematoxylin stains, the protozoan trophozoites and cysts can easily be seen. Oocysts are not clearly delineated; if suspect organisms are seen, additional procedures should be used for confirmation (see modified acid-fast stains for coccidia, above).
2. Helminth eggs or larvae may not be easily identified on the permanent stained smear, and wet mount examinations may have to be performed.
3. Human cells are readily identified (macrophages, PMNs, RBCs, etc.). Yeast cells (single cells, budding, presence of pseudohyphae) are also readily identified.
4. The organism and stage should be reported (do not use abbreviations).  
Example: *Entamoeba histolytica* trophozoites (visible ingested RBCs)
5. The presence of human cells should be noted and quantitated.  
Example: Moderate WBCs, many RBCs, few macrophages, etc.
6. Yeast cells should also be reported and quantitated.  
Example: Moderate budding yeast cells and few pseudohyphae. Refer to the quantitation table (in the section on sigmoidoscopy specimens [direct wet preparation], above).

### **Procedure Reminders**

1. Sigmoidoscopy specimens are submitted to help differentiate between inflammatory bowel disease and amebiasis. It is critical that the specimens be preserved immediately after being taken. Any delay could result in the disintegration of amebic trophozoites or distortion of human cells.
2. It is critical that permanent stained smears of this material be carefully examined using the oil immersion lens (magnification,  $\times 1,000$ ).

### **Procedure Limitations**

1. The more areas of the mucosa sampled, the more likely it is that the organisms might be found (*Entamoeba histolytica*). If only one or two smears are submitted for examination, the physician must be informed (the recommendation is to submit six smears from representative areas of the mucosa).
2. The examination of smears prepared at sigmoidoscopy does not take the place of routine O&P exams, but serves as a supplemental procedure. Stools for routine examinations should also be submitted (a minimum of three specimens collected every other day or within no more than 10 days).

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## DUODENAL ASPIRATES

### Description

Some organisms may be more difficult to recover in the stool, particularly those normally found in the duodenum. An alternative approach to routine stool examinations would be to sample the duodenal contents. Samples are obtained through the use of nasogastric intubation or the Entero-Test capsule (string test). Fluid from the duodenum is examined for the presence of *Strongyloides stercoralis*, *Giardia lamblia* trophozoites, *Cryptosporidium* spp., and *Isospora belli* oocysts. The specimen can be examined as a wet preparation or as a permanent stained smear. In rare instances, *Clonorchis sinensis* eggs may be recovered. Duodenal fluid must be transported STAT in a securely covered container placed in a plastic bag. A screw-cap urine container or plastic centrifuge tube with no preservatives is practical for this purpose.

### Reagents

1. Schaudinn's fixative (see Section 3)
2. PVA fixative (see Section 3)
3. 10% Formalin (see Section 3)

### Quality Control

1. Check the fixatives weekly or when a new lot number is used. Fresh stool containing protozoa or negative stool seeded with human buffy coat cells can be used to evaluate the efficacy of the fixatives. Cultured protozoa can also be used.
2. The Schaudinn's fixative should appear clear, without floating debris or crystals. It is acceptable to have some crystal sediment on the bottom of the Coplin jar or dish.
3. The PVA should be clear (a slightly milky or smoky color is acceptable). There may be a slight precipitate on the bottom of the container (this is acceptable). The fluid should easily move in the bottle when it is inverted, and the viscosity of many of the available formulations actually approaches that of water.
4. The 10% formalin should appear clear, without any visible contamination.
5. Record all QC results.

### Detailed Procedure

1. Gloves must be worn when handling this specimen; infectious *Strongyloides* larvae can penetrate intact skin.
2. Examine the specimen within 1 h after it is taken. Note the amount of yellow color present, which indicates bile staining and confirms that it is actually from the duodenum.
3. Centrifugation ( $500 \times g$  for 10 min) may be necessary to concentrate the mucus and any organisms present. Centrifugation should be routinely performed if the volume of fluid is  $\geq 2$  ml.

4. Place 1 drop of fluid on a clean slide, and cover with a 22- by 22-mm coverslip. If the specimen is very viscous, a drop of saline may be added.
5. Examine the entire coverslip under low power (magnification,  $\times 100$ ) for larvae or motile trophozoites, looking especially carefully around the mucus, where *Giardia* may be entangled.
6. Examine the mucus present under high dry power (magnification,  $\times 400$ ), since *Giardia* may be detectable only by the flutter of the flagella rather than by the motility of the organism.
7. Immediately after reading, place the slide in a Coplin jar containing Schaudinn's solution so that permanent stained smears can be prepared. **Do not dry the slide**, or the coverslip will float off and sink to the bottom. If you have enough material, gently smear 1 or 2 drops of specimen on the slide and immediately immerse the slide in Schaudinn's fixative. The fixation and staining times are identical to those for routine fecal smears.
8. If the material contains a lot of mucus or is a "wet" specimen, gently mix 1 or 2 drops of the specimen with 3 or 4 drops of PVA fixative directly on the slide. The smear should be allowed to air dry for at least 2 h before being stained. The fixation and staining times are identical to those for routine fecal smears.
9. Place 1 drop of the duodenal fluid on one or more slides to be stained for *Cryptosporidium* and *Isospora*, then repeat the wet mount procedure (steps 4 to 7) until all the remaining mucus (after centrifugation) or sediment is gone.
10. Stain the *Cryptosporidium/Isospora* slide(s) with modified acid-fast stain, and examine as usual.
11. Examine the permanent stained smear, using the oil immersion lens (97 to 100 $\times$  objective) under maximum light. At least 300 oil immersion fields on each smear should be examined.
12. If *Strongyloides* larvae are found, the rest of the specimen can be preserved in 10% formalin for teaching purposes.

## Reporting

1. With either the trichrome or iron hematoxylin stain, the protozoan trophozoites and cysts can easily be seen. Oocysts are not clearly delineated; if suspect organisms are seen, additional procedures should be used for confirmation.
2. Oocysts of *Cryptosporidium* and *Isospora* are visible on permanent stained smears (modified acid-fast procedures).
3. Helminth eggs or larvae may not be easily identified on the permanent stained smear but are visible in the wet preparations.
4. Report the organism and stage (trophozoite, cyst, oocyst, etc.); do not use abbreviations. However, confirmation of species may require some type of permanent stained smear.  
Examples: *Giardia lamblia* trophozoites or *Strongyloides stercoralis* larvae
5. Call the physician if pathogenic organisms are identified.

## **DUODENAL ASPIRATES (continued)**

6. Quantitate *Clonorchis sinensis* eggs if they are recovered.
7. If the results are negative on the wet smear examination, report them as a presumptive (based on the wet examination only) prior to examination of the permanent stained smear.

### **Procedure Reminders**

1. If you receive more than 2 ml of specimen, you will have to centrifuge the specimen ( $500 \times g$  for 10 min) and examine the mucus or material in the bottom of the tube.
2. Modified acid-fast methods (or monoclonal antibody direct-detection methods) should be used for the identification of *Cryptosporidium* spp. *Isospora belli* could be identified on the basis of the wet examination or from smears stained by the modified acid-fast methods.

### **Procedure Limitations**

1. Many of the parasites will be caught up in the mucus; therefore, it is very important to centrifuge the specimen, concentrating this material for examination. Centrifugation is mandatory if there is more than 2 ml of specimen.
2. Although duodenal aspirate specimens are normally examined as wet preparations, it is important to remember that some of the organisms may be missed without the use of additional permanent stains (*Cryptosporidium*).

## **Methods for Urogenital Tract Specimens**

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### **RECEIPT OF DRY SMEARS**

If dry smears prepared from urogenital specimens are received in the laboratory, several options can be used to salvage the smears, as follows.

1. If you have a fluorescence microscope (blue-light excitation as for fluorescent antibody work and/or auramine acid-fast staining) and can obtain the low-pH acridine orange stain, you can perform a very sensitive acridine orange stain (twice the sensitivity of the wet preparation) for *Trichomonas* on air-dried smears made when and where the specimen is collected.
2. You can treat the dry smear like a thin blood film. Fix the smear with absolute methanol, allow the slide to dry, and then stain it with a blood stain.
3. In addition to salvaging the smear, these approaches are more sensitive than a simple direct saline mount (see below). These approaches also avoid the problem of delays in transport to the laboratory.

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## DIRECT SALINE MOUNT

### Description

*Trichomonas vaginalis* infections are diagnosed primarily by detecting live motile flagellates from direct saline (wet) mounts. Microscope slides containing patient specimens can be examined under low and high power for the presence of actively moving organisms. The specimens may include vaginal discharge, urethral discharge, penile discharge, urethral mucosa scrapings, and first-voided urine with or without prostatic massage. The specimens may be collected with a platinum loop, cotton or Dacron swab, or speculum. They can be placed in a small amount (<1.0 ml) of 0.85% NaCl in a test tube or placed on a microscopic slide and diluted with a drop of 0.85% NaCl. If the specimen cannot be examined immediately, the swab may be placed in Amies transport medium, which keeps the organisms viable for approximately 24 h. Urine specimens should be collected in a clean-catch urine collection container. The urine should be centrifuged at 500  $\times$  g, and the sediment should be examined for *Trichomonas*. All specimens should be held at room temperature because refrigerator temperatures inhibit motility and have a deleterious effect on the organisms. Returning the specimen to room temperature does not reverse these deleterious morphologic changes. Specimens older than 24 h old should be rejected.

### Reagents

#### 0.85% NaCl

Sodium chloride (NaCl) ..... 850 mg  
Distilled water ..... 100 ml

1. Dissolve the NaCl in distilled water in an appropriate glass flask, using a magnetic stirrer.
2. Store in a glass bottle.
3. Label as 0.85% NaCl with the preparation date and an expiration date of 6 months. Store at room temperature.

#### Modified D'Antoni's Stock Iodine

Potassium iodide (KI) ..... 1.0 g  
Powdered iodine crystals ..... 1.5 g  
Distilled water ..... 100 ml

1. Dissolve in distilled water in an appropriate glass flask, using a magnetic stirrer.
2. The D'Antoni's iodine solution should be saturated with iodine, with some excess crystals left in the bottle. Store in a brown bottle at room temperature. The stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle.

## DIRECT SALINE MOUNT (*continued*)

3. Label as D'Antoni's stock iodine with the preparation date and an expiration date of 1 year.
4. Small amounts of stock iodine solution can be aliquoted into brown dropper bottles and used for routine daily use. The expiration date is 30 to 60 days, depending on the amount of fading of the solution from the normal strong-tea color. The use of a small and/or clear-glass dropper bottle results in a shorter expiration time. The use of a brown bottle lengthens the expiration time.

### Quality Control

1. Check the direct-mount reagents each time they are used.
  - a. The saline should appear clear, without any visible contamination.
  - b. The iodine should be a strong-tea color, and there should be crystals in the bottom of the bottle. Small aliquots of the stock solution should always be a strong-tea color. If not, discard them and aliquot some stock solution into the dropper bottle.
2. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope).
3. Record all QC results.

### Detailed Procedure

1. Apply the specimen to a small area of a clean microscope slide.
2. Immediately before the specimen dries, add 1 or 2 drops of saline with a pipette. If urine sediment is used, the addition of saline may not be necessary.
3. Mix the saline and specimen with the pipette tip or the corner of the coverslip.
4. Cover the specimen with the no. 1 coverslip.
5. Examine the wet mount with the low-power objective (10 $\times$ ) under low light.
6. Examine the entire coverslip for motile flagellates. Suspicious objects can be examined with the high-power objective (40 $\times$ ).
7. The organism is usually slightly larger than a polymorphonuclear leukocyte and flagellar movement should be detected.

### Reporting

1. If motile flagellates are seen (axostyle and undulating membrane), trophozoites of *T. vaginalis* are present.
2. If the nonmotile organisms are visible after staining with D'Antoni's iodine (axostyle), trophozoites of *T. vaginalis* are present.

3. Report the presence of the organism. The organism stage is not necessary since there is no known cyst stage for the trichomonads. The organisms do not have to be quantitated.  
Example: *Trichomonas vaginalis* present
4. If no flagellated organisms are seen, report the specimen as negative for *T. vaginalis*.  
Example: No *Trichomonas vaginalis* seen

### **Procedure Reminders**

1. It is very important that specimens to be examined for *T. vaginalis* should be delivered to the laboratory within 1 h after collection.
2. The organisms will lose their motility, particularly when they begin to dry out.
3. If a dry smear is delivered to the laboratory, it can be salvaged by being fixed as for a thin blood film (absolute methanol) and stained with Giemsa for at least 20 min at a 1:20 dilution (see the Giemsa staining procedure in the section on blood film preparation). The stained organisms may be difficult to see, but the specimen may provide some clinically relevant information if you can actually see and identify the organisms as *T. vaginalis*.
4. Calgiswabs are not recommended (because of tight adherence of the specimen to the swab), and the specimen should be rejected if submitted on this type of swab.
5. When the specimen is examined microscopically, always confirm that no fecal contamination is present (artifacts, vegetable debris, etc.). This type of contamination is rare and would probably be limited to a urine specimen.

### **Procedure Limitations**

1. If the specimen is left at room temperature or held at refrigerator temperature for a prolonged period (usually >1 h), the organisms round up, lose their motility, and eventually die. Motility is occasionally enhanced by warming the specimen to 37°C, but this does not revive dying organisms.
2. Wet mounts have been reported to detect *T. vaginalis* in 75 to 85% of infected patients. Alternative diagnostic methods include culture, monoclonal antigen detection kits, permanent stained slides, and collection of a second sample for examination.
3. If the patient had a *Pentatrichomonas hominis* intestinal infection and the urogenital specimen becomes contaminated with fecal material, a false-positive *T. vaginalis* result may be reported because *P. hominis* and *T. vaginalis* are similar in shape. This false-positive report can result in risk management issues (sexual abuse if patient is a child).

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### **PERMANENT STAINED SMEAR**

#### **Description**

*Trichomonas vaginalis* infections are diagnosed primarily from direct saline (wet) mounts by detecting live motile flagellates. Permanent stained

## **PERMANENT STAINED SMEAR (continued)**

smears can be made from patient specimens for specific identification of the organism. Although a number of stains can be used, Giemsa and Papanicolaou stains are the ones most frequently used to diagnose *T. vaginalis* infections. Acceptable specimens include vaginal discharge, urethral discharge, penile discharge, urethral mucosa scrapings, and first-voided urine with or without prostatic massage. The specimens may be collected with a platinum loop, cotton or Dacron swab, or speculum. They can be placed in a small amount (<1.0 ml) of 0.85% NaCl in a test tube or smeared directly on a microscope slide. A drop of 0.85% NaCl may be used to dilute the direct smear when it is placed on the slide. Slides prepared in this manner should be air dried before they are transported to the laboratory. Specimens collected with a cotton or Dacron swab may also be placed in Amies transport medium if the specimen cannot be immediately processed. Organisms remain viable for approximately 24 h in Amies transport medium. Urine specimens should be collected in a clean-catch urine collection container. The urine should be centrifuged at  $500 \times g$  for 5 min, and the sediment should be examined for *Trichomonas*. All specimens should be held at room temperature because refrigerator temperatures have a deleterious effect on the organisms. Returning the specimen to room temperature does not reverse these deleterious morphologic changes. Specimens older than 24 h old should be rejected.

### **Reagents**

#### **0.85% NaCl**

Sodium chloride (NaCl) ..... 850 mg  
Distilled water ..... 100 ml

1. Dissolve the NaCl in distilled water in an appropriate glass flask, using a magnetic stirrer.
2. Store in a glass bottle.
3. Label as 0.85% NaCl with a preparation date and an expiration date of 6 months. Store at room temperature.

### **Giemsa Stain**

For preparation of Giemsa stain and phosphate buffer solutions, see Section 3.

### **Absolute Methanol**

### **Quality Control**

1. Check the direct mount reagents each time they are used.
  - a. The saline should appear clear, without any visible contamination.
  - b. A peripheral blood film may be used to quality control the Giemsa stain. For staining characteristics, see Section 3.

- c. Check the phosphate buffer each time it is used. The buffer should appear clear, with no signs of visible contamination or precipitates. The pH should be between 6.8 and 7.2.
2. The Giemsa-stained control slide should be reviewed before searching the patient's specimen for the organism. If there was potential fecal contamination of the specimen, one may have to differentiate *T. vaginalis* from *P. hominis*.
3. The microscope would be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope).
4. Record all QC results.

### Detailed Procedure

1. Apply the specimen to a small area of a clean microscope slide.
2. Immediately before the specimen dries, add 1 or 2 drops of saline with a pipette. If urine sediment is used, the addition of saline may not be necessary.
3. Mix the saline and specimen with the pipette tip.
4. Air dry the slide.
5. Fix in absolute methanol for 1 min (Coplin jar or slide staining rack).
6. Place the slide in the Giemsa solution, and stain for the desired time depending on the stain dilution used (20 min at 1:20 dilution).
7. Rinse the slide with tap water to remove excess stain solution (use gently running water or a Coplin jar).
8. Air dry the slide; do not apply a coverslip.
9. Examine the slide with the oil objective (100 $\times$ ).
10. Examine the entire smear for flagellates.
11. The organism is usually slightly larger than a PMN. It is 7 to 23  $\mu\text{m}$  long and 5 to 15  $\mu\text{m}$  wide. Differential characteristics to be observed include anterior flagella, undulating membrane, axostyle, and nucleus.

### Reporting

1. If motile flagellates are seen (axostyle and undulating membrane), trophozoites of *T. vaginalis* are present.
2. If nonmotile organisms are visible after staining with D'Antoni's iodine (axostyle), trophozoites of *T. vaginalis* are present.
3. Report the organism. The organism stage is not necessary since there is no known cyst stage for the trichomonads. The organisms do not have to be quantitated.  
Example: *Trichomonas vaginalis* present
4. If no flagellated organisms are seen, report the specimen as negative for *T. vaginalis*.  
Example: No *Trichomonas vaginalis* seen

## **PERMANENT STAINED SMEAR (continued)**

### **Procedure Reminders**

1. It is very important that specimens to be examined for *T. vaginalis* should be delivered to the laboratory within 1 h after collection.
2. The organisms will lose their motility, particularly when they begin to dry out.
3. If a dry smear is delivered to the laboratory, you can salvage it by fixing it as you would a thin blood film (absolute methanol) and staining it with Giemsa for at least 20 min at a 1:20 dilution. The stained organisms may be difficult to see but may provide some clinically relevant information if you can actually see and identify them as *T. vaginalis*.
4. Calgiswabs are not recommended (because of tight adherence of the specimen to the swab), but a specimen should not be rejected if submitted on this type of swab.
5. When the specimen is examined microscopically, always confirm that no fecal contamination is present (artifacts, vegetable debris, etc.). This type of contamination is rare and would probably be limited to a urine specimen.

### **Procedure Limitations**

1. If the specimen is left at room temperature or held at refrigerator temperature for a prolonged period (usually >1 h), the organisms will round up and eventually die.
2. If the patient had a *Pentatrichomonas hominis* intestinal infection and the urogenital specimen becomes contaminated with fecal material, a false-positive *T. vaginalis* result may be reported because *P. hominis* and *T. vaginalis* are similar in shape. The position of the undulating membrane will allow differentiation speciation between *T. vaginalis* and *P. hominis*.

Diagnostic differential characteristics

Characteristic	<i>T. vaginalis</i>	<i>P. hominis</i>
Size and shape	7–23 $\mu\text{m}$ long 5–15 $\mu\text{m}$ wide Pear shaped	5–15 $\mu\text{m}$ long 7–10 $\mu\text{m}$ wide Pear shaped
Flagella	Four anterior	Three to five anterior
Undulating membrane	Extends half the length of the organism	Extends the entire length of the organism, with a free trailing flagellum
Axostyle	Present	Present
Nucleus	Anterior end, oval	Anterior end, oval

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## **URINE CONCENTRATION (Centrifugation)**

### **Description**

Helminthic larval stages and eggs and some protozoa infecting humans may be found in the urine whether or not they cause pathologic sequelae

in the urinary tract. Infections diagnosed or organisms which can be detected in the urine include *Trichomonas vaginalis*, *Schistosoma haematobium*, and filariasis. Microfilariae may be detected in the urine in heavily infected patients or in patients recently treated with diethylcarbamazine. The urine specimen should be collected as follows:

1. For *Trichomonas vaginalis*, collection of first-voided urine, particularly after prostatic massage in male patients, is useful for the diagnosis.
2. For *Schistosoma haematobium*, collection of a midday urine specimen or a 24-h collection in a container without preservatives is recommended. Peak egg excretion occurs between noon and 3 p.m. In patients with hematuria, eggs may be found trapped in the blood and mucus in the terminal portion (last portion voided) of the urine specimen.
3. For filariasis, microfilariae may be detected in urine of patients with chyluria, those who have very heavy filarial infections, and those treated with diethylcarbamazine.

## Reagents

### 0.85% NaCl

Sodium chloride (NaCl)..... 850 mg  
Distilled water..... 100 ml

1. Dissolve the NaCl in distilled water in an appropriate glass flask, using a magnetic stirrer.
2. Store in a glass bottle.
3. Label as 0.85% NaCl with the preparation date and an expiration date of 6 months. Store at room temperature.

## Quality Control

1. Check the direct-mount reagents each time they are used. The saline should appear clear, without any visible contamination.
2. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope).
3. Record all QC results.

## Detailed Procedure

1. If a 24-h urine specimen was collected, allow it to sediment for 2 h and decant the major portion of the supernatant. There may be 100 to 200 ml of sediment left. If a first-voided urine specimen is received, use the entire specimen.
2. Place the remaining urine specimen (sediment) in centrifuge tubes.
3. Centrifuge the specimen at  $500 \times g$  for 5 min.
4. Decant the supernatant fluid.
5. With a pipette, mix and aspirate the sediment.

## URINE CONCENTRATION (continued) (Centrifugation)

6. Place 1 drop of the sediment on a microscope slide.
7. Place a coverslip on top of the sediment.
8. Observe the specimen under the coverslip at magnifications of  $\times 100$  and  $\times 400$ . Examine the entire coverslip at  $\times 100$  and at least half the coverslip at  $\times 400$ .

### Reporting

1. If motile flagellates are seen (axostyle and undulating membrane), trophozoites of *T. vaginalis* are present.
2. If live microfilariae are seen, species identification could be confirmed or accomplished with permanent stains.
3. If eggs of *S. haematobium* are seen, observe the eggs for live miracidia. If flame cell activity (motile cilia) is detected inside the miracidium larva, the miracidium is viable. A hatching test may also be used to determine if the eggs are viable.
4. For *T. vaginalis*, report the organism. The stage is not necessary since there is no known cyst stage for the trichomonads. The organisms do not have to be quantitated.

Example: *Trichomonas vaginalis* present

5. For filariae, report the presence of microfilariae. Genus and species should be reported if possible. The organisms do not have to be quantitated.

Example: *Wuchereria bancrofti* microfilariae present

6. For *S. haematobium*, if eggs are present, report the genus and species and whether the eggs are viable or nonviable.

Examples: *Schistosoma haematobium* eggs present (some viable, some nonviable)

*Schistosoma haematobium* eggs present (nonviable, egg-shells only)

### Procedure Reminders

1. Specimens to be examined for *T. vaginalis* should be delivered to the laboratory as soon as possible after collection. Hold all specimens at room temperature because refrigerator temperatures have a deleterious affect on *T. vaginalis*.
2. Species identification of the microfilariae may not be possible if unstained preparations (urine sediment) are used; permanent stains may be necessary for further identification.
3. It is very important that all urine specimens (24-h and single voided specimens) be collected with no preservatives. It is clinically important to determine whether the eggs are viable. This can be accomplished by examining eggs in the wet preparations at a magnification of  $\times 400$ .

4. Reject specimens more than 24 h old for *T. vaginalis*, and filariae, and midday urine specimens for schistosomes.
5. Reject all 24-h urine specimens more than 48 h old.
6. When the specimen is examined microscopically, always confirm that no fecal contamination is present (artifacts, vegetable debris, etc.). This type of contamination is rare and would probably be limited to a urine specimen.

### **Procedure Limitations**

1. If the urine specimen is left at room temperature or held at a low temperature for a prolonged period, *T. vaginalis* may round up, become nonmotile, and eventually die.
2. If the patient had a *P. hominis* intestinal infection and the urogenital specimen becomes contaminated with fecal material, a false-positive *T. vaginalis* result may be reported because *P. hominis* and *T. vaginalis* are similar in shape.
3. Microfilariae can be identified to the species level only by making a permanent stained slide from the specimen.

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### **URINE CONCENTRATION (Nuclepore Membrane Filter)**

#### **Description**

Microfilariae may be detected in the urine of heavily infected patients or in patients recently treated with diethylcarbamazine. Eggs of *Schistosoma haematobium* can also be recovered in urine specimens. Microfilariae and *S. haematobium* eggs can be easily concentrated by passing the specimen through a membrane filter and then observing the filter through a microscope. Specimens should be collected as indicated below:

1. For filariasis, microfilariae may be detected in urine of patients with chyluria, those who have very heavy filarial infections, and those treated with diethylcarbamazine. Specimens should be collected as first-voided specimens or a 24-h collection in a container without preservatives.
2. For *S. haematobium*, collection of a midday urine specimen or a 24-h collection in a container without preservatives is recommended. Peak egg excretion occurs between noon and 3 p.m. In patients with hematuria, eggs may be found trapped in the blood and mucus in the terminal portion (last voided portion) of the urine specimen.

#### **Reagents**

##### **0.85% NaCl**

Sodium chloride (NaCl)..... 850 mg  
Distilled water ..... 100 ml

1. Dissolve the NaCl in distilled water in an appropriate glass flask, using a magnetic stirrer.

## **URINE CONCENTRATION (continued)** **(Nuclepore Membrane Filter)**

2. Store in a glass bottle.
3. Label as 0.85% NaCl with the preparation date and an expiration date of 6 months. Store at room temperature.

### **Quality Control**

1. Check the direct-mount reagents each time they are used. The saline should appear clear, without any visible contamination.
2. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope).
3. Record all QC results.

### **Detailed Procedure**

1. If a 24-h urine sample was collected for *S. haematobium* diagnosis, allow the specimen to sediment for 2 h and decant the major portion of the supernatant. There may be 100 to 200 ml of sediment left.
2. Thoroughly mix the urine specimen.
3. Draw up 10 ml of urine into the syringe.
4. Attach the filter holder containing the filter to the syringe. For *S. haematobium*, use the 8- $\mu\text{m}$  filters; for *Wuchereria bancrofti*, *Brugia malayi*, and *Loa loa*, use the 5- $\mu\text{m}$  filters; and for *Mansonella* species, use the 3- $\mu\text{m}$  filters.
5. Express the urine through the filter.
6. The membrane may be washed with physiological saline by removing the filter holder, drawing up 10 ml of saline into the syringe, reattaching the filter holder, and expressing the saline through the filter.
7. Repeat step 6, but fill the syringe with air rather than saline and express the air through the filter.
8. Remove the filter holder from the syringe.
9. Disassemble the filter holder to expose the filter.
10. Remove the filter from the holder with forceps.
11. Place the filter upside down on a microscope slide.
12. With a Pasteur pipette, add 1 drop of saline to moisten the filter.
13. Examine the filter for microfilariae and eggs under  $\times 100$  magnification.

### **Reporting**

1. If live microfilariae are seen, species identification could be confirmed or accomplished with permanent stains.
2. If eggs of *S. haematobium* are seen, observe them for live miracidia. If flame cell activity (motile cilia) is detected inside the miracidium

- larva, the miracidium is viable. A hatching test may also be used to determine if the eggs are viable.
3. For filariae, report the presence of microfilariae. Genus and species should be reported if possible. The organisms do not have to be quantitated.

Example: *Wuchereria bancrofti* microfilariae present

4. For *S. haematobium*, if eggs are present, report the genus and species and whether the eggs are viable or nonviable.
- Examples: *Schistosoma haematobium* eggs present (some viable, some nonviable)  
*Schistosoma haematobium* eggs present (nonviable, egg-shells only)

### Procedure Reminders

- Species identification of the microfilariae may not be possible from unstained preparations (urine sediment), and permanent stains may be necessary for further identification. They measure 3 to 10  $\mu\text{m}$  wide by 160 to 330  $\mu\text{m}$  long. Depending on the species, a sheath may or may not be present.
- It is very important that all urine specimens (24-h and single voided specimen) be collected with no preservatives. It is clinically important to determine whether the eggs are viable. This can be accomplished by examining eggs in the wet preparations at a magnification of  $\times 400$ .
- Reject specimens more than 24 h old for filariae and midday urine for schistosomes.
- Reject all 24-h urine specimens more than 48 h old.
- If you accidentally put the filter right side up, don't add more than 1 drop of saline (the organisms may accidentally float off the filter and onto the glass slide).

### Procedure Limitations

- Microfilariae can be identified to the species level only by making a permanent stained slide from the specimen. For a method using the membrane filter, refer to *Diagnostic Medical Parasitology*, 5th ed.
- A hatching test may also be used to determine if the eggs are viable. For the hatching method, refer to *Diagnostic Medical Parasitology*, 5th ed.
- Infrequently, eggs of other *Schistosoma* species may be recovered in the urine.

Species	Egg	
	Size	Shape
<i>S. haematobium</i>	112–170 by 40–70 $\mu\text{m}$	Elongate, terminal spine
<i>S. japonicum</i>	55–85 by 40–60 $\mu\text{m}$	Oval, minute lateral spine
<i>S. mansoni</i>	114–180 by 45–73 $\mu\text{m}$	Elongate, prominent lateral spine

## Preparation of Blood Films

Although some parasites (microfilariae and trypanosomes) can be detected in fresh blood on the basis of their characteristic shape and motility, specific identification of the organisms is best done with a permanent stain. Two types of blood films are recommended for detection of all blood-borne parasites. Thick films allow a larger amount of blood to be examined and provide an increased possibility of detecting light infections. However, species identification from the thick film, particularly for malaria parasites, can be difficult and can usually be made only by experienced workers. The morphologic characteristics of blood parasites are best seen in thin films, in which the RBC morphology is preserved and the size relationship between infected and uninfected RBCs can be determined after staining. Examination of the thin film is often valuable in determining the *Plasmodium* species present.

Accurate examination of thick and thin blood films and identification of parasites depend on the use of clean, grease-free slides. Old (unscratched) slides should be cleaned first with detergent and then with 70% ethyl alcohol. New, unused slides are coated with a greasy substance that allows them to be pulled apart; therefore, these slides must be cleaned with alcohol prior to use for preparation of blood films.

Blood films should be ordered and prepared immediately. Blood collection should not be delayed for any evidence of periodic fevers, since periodicity is often not yet evident in travelers who present to the clinic or emergency room. When malaria is a possible diagnosis, after the first set of negative smears, samples should be taken at intervals of 6 to 8 h for at least 36 h. If the finger stick method is used, the blood should flow freely; blood that has to be "milked" from the finger is diluted with tissue fluids, decreasing the number of parasites per field. If the blood specimen from a venipuncture is sent directly to the laboratory, the following approach can be used. Request a tube of fresh blood (EDTA anticoagulant is preferred over heparin), and prepare the smears.

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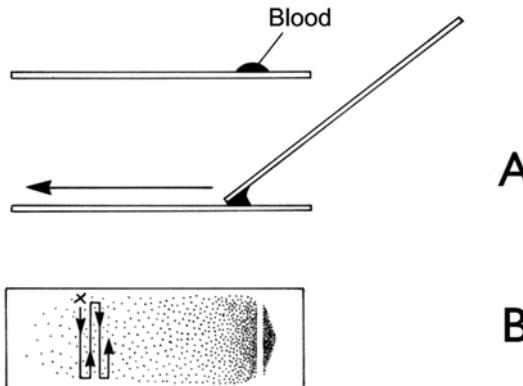
### THIN BLOOD FILMS

#### Description

The thin film is prepared like that for a differential blood cell count and provides an area for examination where the RBCs are neither overlapping nor distorted (Figure 5.8). Here the morphologies of parasites and infected RBCs are most typical. The specimen usually consists of fresh whole blood collected by finger puncture or whole blood containing EDTA (0.020 g per 10 ml of blood) collected by venipuncture that is less than 1 h postcollection. Occasionally a buffy coat (for leishmaniasis) or the sediment from a special concentration procedure (triple centrifugation for trypanosomes) is spread into a thin film.

#### Reagents

Absolute Methanol (for Giemsa stain)



**Figure 5.8** Method of thin blood film preparation. (A) Position of spreader slide; (B) well-prepared thin film. Arrows indicate the area of the slide used to observe accurate cell morphology. (Illustration by Nobuko Kitamura.)

Reprinted from *Diagnostic Medical Parasitology*, 5th ed.

## Quality Control

1. Visually, the thin film should be rounded, feathered, and progressively thinner toward the middle of the slide.
2. There should not be any clear areas or smudges in the film itself (indicating that grease or fingerprints were on the glass).

## Detailed Procedure

1. Wear gloves when performing this procedure.
2. The procedure depends on the source of the specimen.
  - a. For blood from finger puncture, after wiping off the first drop of blood, touch a clean 1- by 3-in. glass microscope slide, about 0.5 in. from the end, to a small drop of blood (10 to 15  $\mu$ l) standing on the finger, remove the slide from the finger, turn it blood side up, and place it on a horizontal surface.
  - b. For blood from venipuncture, place a clean 1- by 3-in. glass microscope slide on a horizontal surface. Place a small drop (10 to 15  $\mu$ l) of specimen on the center of the slide about 0.5 in. from the end.
3. Holding a second clean glass slide at a 40° angle, touch the angled end to the midlength area of the specimen slide.
4. Pull the angled slide back into the blood, and allow the blood to almost fill the end area of the angled slide.
5. Continuing contact with the blood under the lower edge, quickly and steadily move the angled slide toward the opposite end of the specimen slide until the blood is used up.
6. The result will be a thin film that is rounded, feathered, and progressively thinner toward the center of the slide.
7. Label the slide appropriately, and allow it to air dry for at least 10 min while protected from dust.
8. If the film will be stained with Giemsa stain, after the film is completely dry, fix it by dipping the slide into absolute methanol and allow the film to air dry in a vertical position. If the film will be

## THIN BLOOD FILMS (*continued*)

stained with Wright's stain, it does not have to be fixed because Wright's stain contains the fixative and stain in one solution.

### Procedure Reminders

1. A diamond marking pen is recommended.
2. An indelible ink pen can be used.
3. Pencil can be used if the information is actually written in the thick part of the smear (where the original drop of blood was placed).
4. Do not use wax pencils; the material may fall off during the staining procedure.
5. Make sure the films are protected from dust (while drying).
6. The last few drops of blood remaining in the needle after a venipuncture can also be used to prepare thin blood films. However, if you are preparing thick films, remember that the blood has not been in contact with the anticoagulant and so you will have to follow directions given in the protocol for thick blood films (from finger puncture). This approach carries the risk of a needlestick injury.

### Procedure Limitations

1. A light infection may be missed in a thin film, whereas the increased volume of blood present on a thick film may allow it to be detected, even with a low parasitemia.
2. If the smears are prepared from anticoagulated blood which is more than 1 h old, the morphology of both parasites and infected RBCs may not be typical.
3. *Plasmodium vivax* and *P. ovale* should be able to be identified, even with the absence of Schüffner's dots (stippling).

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## THICK BLOOD FILMS

### Description

The thick film contains more blood than the thin film, and therefore this method is more likely to demonstrate a low parasitemia. The RBCs are lysed during staining, making the preparation more or less transparent and leaving only parasites, platelets, and WBCs for examination. The specimen usually consists of fresh whole blood collected by finger puncture or whole blood containing EDTA (0.02 g per 10 ml of blood) collected by venipuncture that is less than 1 h postcollection. Heparin (2 mg per 10 ml of blood) or sodium citrate (0.05 g per 10 ml of blood) may be used as an anticoagulant if trypanosomes or microfilariae are suspected. The sediment from a concentration procedure for trypanosomes or microfilariae is frequently spread into a thick film that is stained, examined, and kept as a permanent record.

## **Quality Control**

1. Visually, the thick smear should be round to oval, approximately 2.0 cm across.
2. One should be able to barely read newsprint through the wet or dry film.
3. The film itself should not have any clear areas or smudges (indicating that grease or fingerprints were on the glass).

## **Detailed Procedure**

1. Wear gloves when performing this procedure.
2. The procedure depends on the source of the specimen.
  - a. For blood from finger puncture, after wiping off the first drop of blood, touch a clean 1- by 3-in. glass microscope slide to a large drop of blood standing on the finger, and rotate the slide on the finger until the circle of blood is nearly the size of a dime or nickel (1.8 to 2.0 cm). Without breaking contact with the blood, rotate the slide back to the center of the circle. Remove the slide from the finger, quickly turn it blood side up, and place it on a horizontal surface. The blood should spread out evenly over the surface of the circle and be sufficiently thin that fine print ("newsprint" size) can be just barely read through it. If not, take the corner of a second clean slide or an applicator stick and expand the size of the circle until the print is just readable. The final thickness of the film is important. If it is too thick, it might flake off while drying or wash off while staining. If it is too thin, the amount of blood available for examination is insufficient to detect a low parasitemia. Continue stirring for 30 s to prevent the formation of fibrin strands.
  - b. For blood from venipuncture, place a clean 1- by 3-in. glass microscope slide on a horizontal surface. Place a drop (30 to 40  $\mu\text{l}$ ) of blood onto the center of the side about 0.5 in. from the end. Using either the corner of another clean glass slide or an applicator stick, spread the blood into a circle about the size of a dime or nickel (1.8 to 2.0 cm). Immediately place the thick film over some small print and be sure that the print can be just barely read through it. If not, expand the size of the film until the print can be read. Three or four small drops of blood may be used in place of the larger drop, and the small ones can be pooled into a thick film by using the corner of a clean slide or an applicator stick. Be sure that small print can be read through it.
3. Allow the film to air dry in a horizontal position and protected from dust for several hours (6 to 8 h) or overnight. Do not attempt to speed the drying process by applying any type of heat, because heat fixes the RBCs and they subsequently do not lyse in the staining process.
4. **Do not fix the thick film.** If thin and thick films are made on the same slide, do not allow the methanol or its vapors to contact the thick film when fixing the thin film.

## THICK BLOOD FILMS (*continued*)

5. Label the slide appropriately.
6. If staining with Giemsa stain is delayed for more than 3 days or the film is to be stained with Wright's stain, lyse the RBCs in the thick film by placing it in buffered water (pH 7.0 to 7.2) for 10 min, remove it from the water, and place it in a vertical position (thick film down) to air dry.

### Procedure Reminders

1. A diamond marking pen is recommended.
2. An indelible ink pen can be used.
3. Do not use wax pencils; the material may fall off during the staining procedure.
4. Make sure the films are protected from dust (while drying).
5. The last few drops of blood remaining in the needle after a venipuncture can also be used to prepare thick blood films. However, when you are preparing thick films, remember that the blood has not been in contact with the anticoagulant and so you will have to follow directions given in the protocol for thick blood films (from finger puncture). Remember the possibility of a needlestick injury.

### Procedure Limitations

1. If the smears are prepared from anticoagulated blood that is more than 1 h old, the morphology of the parasites may not be typical and the film may wash off the slide during the staining procedure.
2. Identification to species, particularly between *P. ovale* and *P. vivax* and between the ring forms of *P. falciparum* and *Babesia* spp., may be impossible without examining the stained thin blood film. Also, *T. cruzi* trypanastigotes are frequently distorted in thick films.
3. Excess stain deposition on the film may be confusing and make the detection of organisms difficult.

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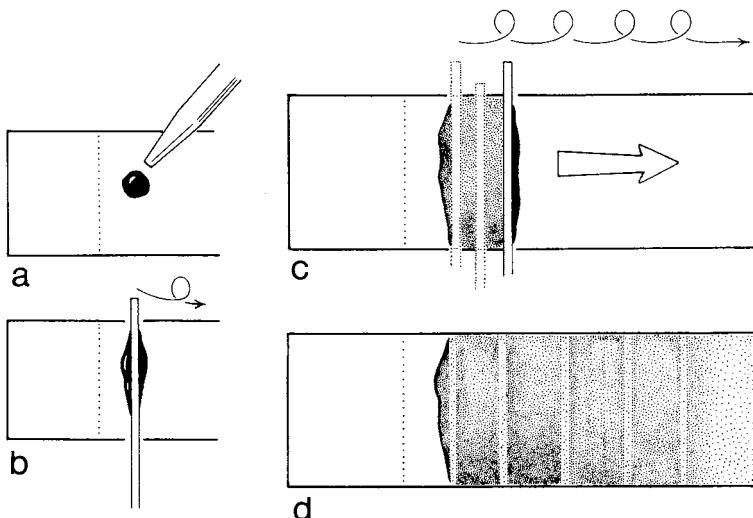
## COMBINATION THICK-THIN BLOOD FILMS

### Description

To prepare a slide containing blood that can be stained for either a thick or thin blood film, the following method was developed (Figure 5.9). The specimen usually consists of fresh whole blood collected by finger puncture or whole blood containing EDTA (0.02 g per 10 ml of blood) collected by venipuncture that is less than 1 h postcollection.

### Quality Control

1. Visually, the smear should be alternate, with thick and thin portions throughout the length of the glass slide.
2. One should be able to barely read newsprint through the wet or dry film.



**Figure 5.9** Method of thick-thin combination blood film preparation. (a) Position of a drop of EDTA-blood; (b) position of the applicator stick in contact with blood and glass slide; (c) rotation of the applicator stick; and (d) completed thick-thin combination blood film prior to staining. (Illustration by Sharon Belkin.) Reprinted from *Diagnostic Medical Parasitology*, 5th ed.

3. The film itself should not have any clear areas or smudges (indicating that grease or fingerprints were on the glass).

### Detailed Procedure

1. Wear gloves when performing this procedure.
2. The procedure depends on the source of the specimen.
  - a. Finger puncture blood is not recommended; the procedure is best performed with blood that contains EDTA anticoagulant. The procedure does not lend itself to "stirring" to prevent fibrin strands.
  - b. For blood from venipuncture, place a clean 1- by 3-in. glass microscope slide on a horizontal surface. Place 1 drop (30 to 40  $\mu\text{l}$ ) of blood at one end of the side about 0.5 in. from the end. Using an applicator stick lying across the glass slide and keeping the applicator stick in contact with the blood and glass, rotate the stick in a circular motion while moving it down the glass slide to the opposite end. The appearance of the smear should be alternate thick and thin areas of blood that cover the entire slide. Immediately place the film over some small print and be sure that the print can be just barely read through it.
3. Allow the film to air dry in a horizontal position and protected from dust for at least 30 min to 1 h. Do not attempt to speed the drying process by applying any type of heat, because heat fixes the RBCs and they subsequently will not lyse in the staining process.

## **COMBINATION THICK-THIN BLOOD FILMS (continued)**

4. This slide can be stained as either a thick or thin blood film.
5. Label the slide appropriately.
6. If staining with Giemsa is delayed for more than 3 days or the film is to be stained with Wright's stain, lyse the RBCs in the thick film by placing it in buffered water (pH 7.0 to 7.2) for 10 min, then remove it from the water and place it in a vertical position (thick film down) to air dry. Other blood stains are also possible.
7. Although thick blood films are not fixed with absolute methanol, after the thick films are thoroughly dry, they can be dipped twice in acetone and allowed to dry before being stained. This extra step does not interfere with RBC lysis that occurs either prior to or during staining. The acetone "quick dip" makes the thick film less likely to fall off during staining and provides a cleaner background for microscopic examination.

### **Procedure Reminders**

1. A diamond marking pen is recommended.
2. An indelible ink pen can be used.
3. Do not use wax pencils; the material may fall off during the staining procedure.
4. Make sure that the films are protected from dust while drying.
5. Anticoagulated blood is recommended for this procedure (EDTA).

### **Procedure Limitations**

1. If the smears are prepared from anticoagulated blood that is over 1 h old, the morphology of the parasites may not be typical and the film may wash off the slide during the staining procedure.
2. Identification to the species level, particularly between *P. ovale* and *P. vivax* and between the ring forms of *P. falciparum* and *Babesia* spp., may be impossible without examining the stained thin blood film. Also, *Trypanosome cruzi* trypomastigotes are frequently distorted in thick films.
3. Excess stain deposition on the film may be confusing and make the detection of organisms difficult.

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### **BUFFY COAT BLOOD FILMS**

In suspected infections with malaria (negative thick and thin blood films), trypanosomiasis, filariasis, and leishmaniasis, concentration procedures are designed to increase the number of organisms recovered from blood specimens. The buffy coat (a layer of WBCs resulting from centrifugation of whole anticoagulated blood) containing WBCs and platelets, and the layer of RBCs just below the buffy coat layer, can be used to prepare thick and thin blood films. The sensitivity of this approach is much higher than that of the routine thick film (see below under Blood Concentration for the complete protocol).

*L. donovani*, trypanosomes, and *Histoplasma capsulatum* (a fungus with intracellular elements resembling those of *L. donovani*) are occasionally detected in the peripheral blood. The parasites or fungi are found in the large mononuclear cells in the buffy coat. Depending on the stain used, the nuclear material and cytoplasm stain with colors similar to the WBCs. *H. capsulatum* appears as a large dot of nuclear material (dark stain) surrounded by a clear halo area. Trypanosomes in the peripheral blood also concentrate with the buffy coat cells.

After centrifugation and removal of the relevant layers, some of the material can be examined as a wet mount; trypomastigotes and microfilariae can be seen as motile objects in the wet mount. After staining, *L. donovani* amastigotes can be found in the monocytes and *Plasmodium* parasites can be seen in the thick and thin films.

## Blood Stains

For accurate identification of blood parasites, a laboratory should develop proficiency in the use of at least one good staining method. It is better to select one method that provides reproducible results than to use several on a hit-or-miss basis. Blood films should be stained as soon as possible, since prolonged storage may result in stain retention. Failure to stain positive malarial smears within a month may result in failure to demonstrate typical staining characteristics for individual species.

Although in the past the recommended stain of choice has been Giemsa stain, the parasites can also be seen on blood films stained with Wright's stain, a Wright-Giemsa combination stain, or one of the more rapid stains. Delafield's hematoxylin stain is often used to stain the microfilarial sheath; in some cases, Giemsa stain does not provide sufficient stain quality to allow differentiation of the microfilariae.

The most common stains are of two types. Wright's stain has the fixative in combination with the staining solution, so that both fixation and staining occur at the same time; therefore, the thick film must be laked (i.e., the RBCs must be ruptured) before staining. Giemsa stain has the fixative and stain separate; therefore, the thin film must be fixed with absolute methanol before staining. Rapid blood stains are also good options. The WBCs on the blood film will serve as the QC "organisms"—if they look good, any parasites will also exhibit good morphology and will stain the same colors as the WBCs.

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## GIEMSA STAIN

### Description

Giemsa stain is used to differentiate nuclear and/or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites. The most dependable stain for blood parasites, particularly in thick films, is obtained with Giemsa stain containing Azure B. Liquid stock is available commercially or can be made from dry stain powder. Either must be diluted for use

## **GIEMSA STAIN (*continued*)**

with water buffered to pH 6.8 or 7.0 to 7.2, depending on the specific technique used. Either should be tested for proper staining reaction before use. The stock is stable for years but must be protected from moisture because the staining reaction is oxidative; therefore, the oxygen in water initiates the reaction and ruins the stock stain. The aqueous working dilution of stain is good only for 1 day.

Although not essential, the addition of Triton X-100, a nonionic surface-active agent, to the buffered water used to dilute the stain enhances the staining properties of Giemsa stain and helps to eliminate possible transfer of parasites from one slide to another. For routine staining of thin films and combination thin and thick films, a 0.01% (vol/vol) final concentration of Triton X-100 is best. For staining thick films for microfilariae, use a 0.1% (vol/vol) concentration. The specimen may consist of a thin blood film that has been fixed in absolute methanol and allowed to dry, a thick blood film that has been allowed to dry thoroughly and is not fixed, or a combination of a fixed thin film and an adequately dried thick film (not fixed) on the same slide.

### **Reagents**

#### **Stock Giemsa Stain**

#### **Stock Solution of Triton X-100 (10% aqueous solution)**

#### **Buffered Water, pH 7.0 to 7.2 (for diluting stain and washing films)**

#### **Buffered Water, pH 6.8 (called for by some commercial stains for diluting stain and washing films)**

#### **Triton-Buffered Water Solutions (optional)**

1. For combination thin and thick blood films, after determining the pH of the buffered water, add 1 ml of the stock 10% aqueous dilution of Triton X-100 to 1 liter of buffered water (pH 7.0 to 7.2; 0.01% final concentration).
2. Label appropriately, and store in a tightly stoppered bottle. The solution can be used as long as the pH is within limits listed for the procedure.

#### **Methyl Alcohol, Absolute**

### **Quality Control**

1. The stock buffer solutions and buffered water should appear clear, without any visible contamination.
2. Check the Giemsa stain reagents, including the pH of the buffered water, before each use. If Triton X-100 has been added to the buffered water, do not use a colorimetric method to determine the pH because Triton X-100 interferes with the color indicators. Use a pH meter to test buffered water that contains Triton X-100. The buffered water is usable as long as the pH is within the limits listed for the procedure.

3. Prepare and stain films from "normal" blood, and microscopically evaluate the staining reactions of the RBCs, platelets, and WBCs.
  - a. Macroscopically, blood films appear purplish. If they are blue, the buffered water was too alkaline; if they are pink to red, the buffered water was too acidic.
  - b. Microscopically, the RBCs appear pinkish gray, the platelets appear deep pink, and the WBCs have purple-blue nuclei and lighter cytoplasm. Eosinophilic granules are bright purple-red, and neutrophilic granules are purple. Basophilic stippling within uninfected RBCs is blue.
  - c. Slight variations may appear in the colors described above, depending on the batch of stain used and the character of the blood itself, but if the various morphological structures are distinct the stain is satisfactory.
4. The microscope should be calibrated (within the last 12 months), and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope).
5. Record the QC results on appropriate sheets.

## Detailed Procedure

### Thin Blood Films (Only)

1. Fix the air-dried film in absolute methyl alcohol by dipping the film briefly (two dips) in a Coplin jar containing methyl alcohol.
2. Remove the film and let it air dry.
3. Stain the film with diluted Giemsa stain (1:20 [vol/vol]) for 20 min. (Add 2 ml of stock Giemsa stain to 40 ml of buffered water containing 0.01% Triton X-100 in a Coplin jar.)
4. Wash by briefly dipping the slide in and out of a Coplin jar of buffered water (one or two dips). Note: Excessive washing will decolorize the film.
5. Let the film air dry in a vertical position.

### Thick Blood Films (Only)

1. Allow the film to air dry thoroughly for several hours or overnight. Do not dry films in an incubator ( $>30$  min) or by heat because this will fix the blood and interfere with the lysing of the RBCs. Note: If a rapid diagnosis of malaria is needed, thick films can be made slightly thinner than usual, allowed to dry for 1 h, and then stained.
2. Do not fix.
3. Stain with diluted Giemsa stain (1:50 [vol/vol]) for 50 min. Add 1 ml of stock Giemsa stain to 50 ml of buffered water containing 0.01% Triton X-100 (if staining microfilariae, use 0.1% Triton X-100) in a Coplin jar.
4. Wash by placing the film in buffered water for 3 to 5 min.
5. Let the film air dry in a vertical position.

## GIEMSA STAIN (*continued*)

### Combination Thin and Thick Blood Films

1. Stain some of these slides as thin blood films (see above).
2. Stain some of these slides as thick blood films (see above).

### Reporting

1. If *Plasmodium* spp. are present, the cytoplasm of the organisms stains blue and the nuclear material stains red to purple-red.
2. Schüffner's stippling and other inclusions in the RBCs infected by *Plasmodium* spp. stain red.
3. Nuclear and cytoplasmic colors that are seen in the malarial parasites also apply to the trypanosomes and any intracellular leishmaniae that might be present.
4. The sheath of microfilariae may or may not stain with Giemsa stain, while the body usually appears blue to purple.
5. Any parasite, including the stage(s) seen, should be reported (do not use abbreviations).

Examples: *Plasmodium falciparum* rings and gametocytes, or rings only

*Plasmodium vivax* rings, trophozoites, schizonts, and gametocytes

*Wuchereria bancrofti* microfilariae

*Trypanosoma brucei gambiense/rhodesiense* trypomastigotes

*Trypanosoma cruzi* trypomastigotes

*Leishmania donovani* amastigotes

6. Any laboratory providing malaria diagnoses should be able to identify *Plasmodium vivax* and *P. ovale*, even in the absence of Schüffner's stippling.

### Procedure Reminders

1. It is important that if blood films are to be prepared from venipuncture blood (use of anticoagulant), they must be prepared within 1 h of collection. Otherwise, certain morphologic characteristics of both parasites and infected RBCs may be atypical. Also, thick blood films may wash off the slide during the staining procedure.
2. It is important to use the correct pH for all buffered water and staining solutions. Solutions with an incorrect pH prevent certain morphologic characteristics from being visible (stippling) and do not give typical nuclear and cytoplasmic colors on the stained film.
3. A QC slide can be stained each time patient blood films are stained. If several patient specimens are stained on the same day (using the same reagents), only one control slide needs to be stained and examined. Also, the patient slide serves as its own control.

### Procedure Limitations

1. Finding no parasites in one set of blood films does not rule out a parasitic infection.

2. A minimum of 300 oil immersion (magnification,  $\times 1,000$ ) fields should be examined before reporting "no parasites found."
3. The entire smear should be examined under low power ( $\times 100$ ) for the presence of microfilariae. Remember that the sheath may not be visible (*W. bancrofti*).

## Blood Concentration

Blood concentration procedures increase the number of organisms recovered from blood specimens submitted for diagnosis of trypanosomiasis, filariasis, and leishmaniasis. A concentration procedure should be performed routinely on all blood specimens submitted for examination for trypanosomes or microfilariae when the suspected organisms are not found in thick blood films or so few organisms are present that more are needed to make a positive identification of species.

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## BUFFY COAT CONCENTRATION

### Description

*Leishmania donovani* amastigotes are difficult to detect in blood specimens but are occasionally found within monocytes by fractional centrifugation of comparatively large amounts of blood. The procedure may also be used to recover trypanosomes and microfilariae, both of which are found in the plasma. The specimen of choice is whole blood, collected using EDTA, heparin, or sodium citrate anticoagulant.

### Reagents

Methyl Alcohol, Absolute

Giemsa Stain (see above)

### Quality Control

1. Check the calibration of the centrifuge.
2. Perform the procedure on "normal" blood. The film should be composed almost exclusively of WBCs, which stain characteristically with Giemsa or other blood stains. If parasites are present, they should also stain like the WBCs.
3. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope).
4. Record QC results on appropriate sheets.

### Detailed Procedure

1. Wear gloves when performing this procedure.
2. Centrifuge the anticoagulated blood specimen in a sealed cup at 100  $\times g$  for 15 min.

## BUFFY COAT CONCENTRATION (*continued*)

3. Remove the thin creamy layer (buffy coat) between the RBCs and plasma with a capillary pipette, or transfer the buffy coat and plasma to another tube and centrifuge in a sealed cup at  $300 \times g$  for 15 min.
4. Examine the buffy coat directly for motile trypomastigotes and microfilariae.
  - a. Place 0.5 drop of saline on a clean microscope slide.
  - b. Remove 1 drop of sediment, and mix it in the saline.
  - c. Add a coverslip, and examine for organism motility with the low-power ( $10\times$ ) and high dry ( $40\times$ ) objectives.
5. Prepare thin films, dry, fix, and stain with a blood stain.

### Reporting

1. If present, *Leishmania donovani* amastigotes are found within the monocytes on a Giemsa-stained film. Nuclear material stains dark purple-red, the cytoplasm is light blue, and the kinetoplast may or may not be visible as a dark bluish purple structure.
2. Trypomastigotes are found extracellularly (they are motile in the wet smear). Morphologic detail is seen in the Giemsa-stained film. The stain reaction is like that of *Leishmania*; the kinetoplast is visible.
3. Microfilariae may be found in the wet smear. Morphologic detail is seen in a Giemsa- or hematoxylin-stained film. The stain reaction is typical for each stain.
4. Report the presence of organisms from the wet smear.  
Examples: Trypomastigotes present  
Microfilariae present
5. Report the genus and species of organisms from the Giemsa-stained film.  
Examples: *Trypanosoma cruzi* trypomastigotes present  
*Leishmania donovani* amastigotes present

### Procedure Reminders

1. If you need to add anticoagulant to blood, mix 9 ml of blood and 1 ml of 5% sodium citrate in a glass centrifuge tube. Then proceed with the centrifugation.
2. This procedure can be performed in a microhematocrit tube if the tube is carefully scored and broken at the buffy coat interface and the WBCs are prepared and stained as a thin blood film.
3. Also, the tube can be examined microscopically (high dry magnification) at the buffy coat layer for motile trypomastigotes and microfilariae, before the tube is scored and broken.

### Procedure Limitations

1. When examined as a wet smear, the intracellular leishmaniae are very difficult to see.

2. Although trypomastigote and microfilarial motility may be visible on the wet smear, specific identification may be difficult.

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## KNOTT CONCENTRATION

### Description

This technique is used to recover low numbers of microfilariae from blood. A solution is used to lyse the red blood cells in a large blood sample, and the organisms are concentrated from the supernatant fluid by centrifugation. The disadvantage of this technique is that the microfilariae are killed and immobilized and are therefore not readily revealed by their motility. The specimen of choice is whole blood, collected using EDTA, heparin, or sodium citrate anticoagulant.

### Reagents

#### 2% Aqueous Formalin

Formaldehyde, liquid..... 2 ml  
Distilled water ..... 98 ml

Mix thoroughly. Store in a stoppered bottle. Label appropriately. The shelf life is 24 months.

### Quality Control

1. Check the calibration of the centrifuge.
2. If possible, check the procedure with human or canine blood containing microfilariae, with or without a sheath (often not practical).
3. If positive blood is not available, follow the procedure carefully in testing the specimen submitted for diagnosis. Examine the sediment thoroughly using low- and high-power magnification.
4. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope).
5. Record QC results on appropriate sheets.

### Detailed Procedure

1. Wear gloves when performing this procedure.
2. Place 1 ml of fresh whole blood or anticoagulated blood in a centrifuge tube containing 10 ml of 2% formalin. Mix thoroughly.
3. Centrifuge for 5 min at  $300 \times g$ .
4. Pour off the supernatant fluid without disturbing the sediment.
5. Using a capillary pipette, transfer a portion of the sediment to a slide.
6. Apply a coverslip, and examine microscopically under low-power ( $\times 100$ ) and high-power ( $\times 400$ ) magnification.
7. If microfilariae are present, prepare a thick film from the remainder of the sediment, air dry, fix in absolute methanol for 5 min, air dry again, and stain with Giemsa or Delafield's hematoxylin.

## **KNOTT CONCENTRATION (continued)**

### **Reporting**

1. If present in the sample, microfilariae are concentrated and appear nonmotile in the wet smear.
2. After staining with Giemsa or Delafield's hematoxylin, the microfilariae exhibit diagnostic morphology and typical staining characteristics.
3. Report the presence of organisms from the wet smear.  
Example: Microfilariae present
4. Report the genus and species of organisms from the Giemsa- or hematoxylin-stained film.  
Example: *Wuchereria bancrofti* microfilariae present

### **Procedure Reminders**

1. If you need to add anticoagulant to blood, mix 9 ml of blood and 1 ml of 5% sodium citrate in a glass centrifuge tube. Then proceed with the centrifugation.
2. Morphologic details may not be visible prior to Giemsa or hematoxylin staining.

### **Procedure Limitations**

1. Motility is not visible after formalin fixation.
2. Species determination may be difficult for most laboratorians without additional staining.
3. The blood-formalin mixture can be sent to a reference laboratory for staining and identification of microfilariae.

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## **MEMBRANE FILTRATION CONCENTRATION**

### **Description**

Membrane filtration methods have been developed for recovering microfilariae in light infections. They have an advantage over simple centrifugation methods in that large samples of blood (20 ml or more) can be used if necessary. The technique described here is one of the most efficient for the clinical laboratory when other procedures used to recover microfilariae are unsatisfactory. Membrane filtration recovers most species of microfilariae; however, because of their small size, *Mansonella perstans* and *M. ozzardi* may not be recovered. Membranes with smaller pores ( $3 \mu\text{m}$ ) have been suggested to recover these two species. The specimen of choice is whole blood, collected using EDTA, heparin, or sodium citrate anticoagulant.

### **Reagents**

Distilled Water

Methyl Alcohol, Absolute

Giemsa Stain (see above)

Toluene

## **Quality Control**

1. If possible, check the procedure using human or canine blood containing microfilariae (not practical).
2. If positive blood is not available, follow the procedure carefully in testing the specimen submitted for diagnosis. Examine sediment thoroughly under low- and high-power magnification.
3. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope).
4. Record QC results on appropriate sheets.

## **Detailed Procedure**

1. Wear gloves when performing this procedure.
2. Draw 1 ml of fresh whole blood or anticoagulated blood into a 15-ml syringe containing 10 ml of distilled water.
3. Gently shake the mixture for 2 to 3 min to ensure that all blood cells are lysed.
4. Place a 25-mm Nuclepore filter, 5- $\mu\text{m}$  porosity, over a moist 25-mm filter paper pad and place in a Swinney filter adapter.
5. Attach the Swinney filter adapter to the syringe containing the lysed blood.
6. With gentle but steady pressure on the piston, push the lysed blood through the filter.
7. Without disturbing the filter, remove the Swinney adapter from the syringe and draw approximately 10 ml of distilled water into the syringe. Replace the adapter, and gently push the water through the filter to wash the debris from the filter.
8. Remove the adapter again, draw the piston of the syringe to about half the length of the barrel, replace the adapter, and push the air in the barrel through the filter to expel excess water.
9. To prepare the filter for staining, remove the adapter, draw the piston about half the length of the barrel, and then draw 3 ml of absolute methanol into the syringe. Holding the syringe vertically, replace the adapter and push the methanol followed by the air through the filter to fix the microfilariae and expel the excess methanol, respectively.
10. To stain, remove the filter from the adapter, place it on a slide, and allow it to air dry thoroughly. Stain with Giemsa stain as for a thick film.
11. To cover the stained filter, dip the slide in toluene before mounting with neutral mounting medium and a coverslip. This will lessen the formation of bubbles in or under the filter.

## **Reporting**

1. If present in the sample, microfilariae are concentrated and appear on the wet membrane.

## **MEMBRANE FILTRATION CONCENTRATION (continued)**

2. The microfilariae stain characteristically with Giemsa or Delafield's hematoxylin. The sheath, if present, may or may not stain with Giemsa.
3. Report the presence of organisms from the wet Nuclepore membrane.  
Example: Microfilariae present
4. Report the genus and species of organisms from the Giemsa- or hematoxylin-stained membrane.  
Example: *Wuchereria bancrofti* microfilariae present

### **Procedure Reminders**

1. Gently shake the water-blood mixture to ensure total lysis of blood cells. Some parasitologists prefer to use an aqueous solution of 10% Teepol (Shell Oil Co.) to lyse the blood cells.
2. Motile microfilariae may be seen on the membrane filter; however, low light intensity is needed.
3. The membrane filter must be supported by the moistened filter pad to prevent rupture when the water is expelled through the membrane.
4. If you need to add anticoagulant to the blood, mix 9 ml of blood and 1 ml of 5% sodium citrate in a glass centrifuge tube. Then proceed with centrifugation.

### **Procedure Limitations**

1. Giemsa or hematoxylin staining may be necessary to identify the organisms to the species level.
2. Species identification of microfilariae on filters may be difficult.

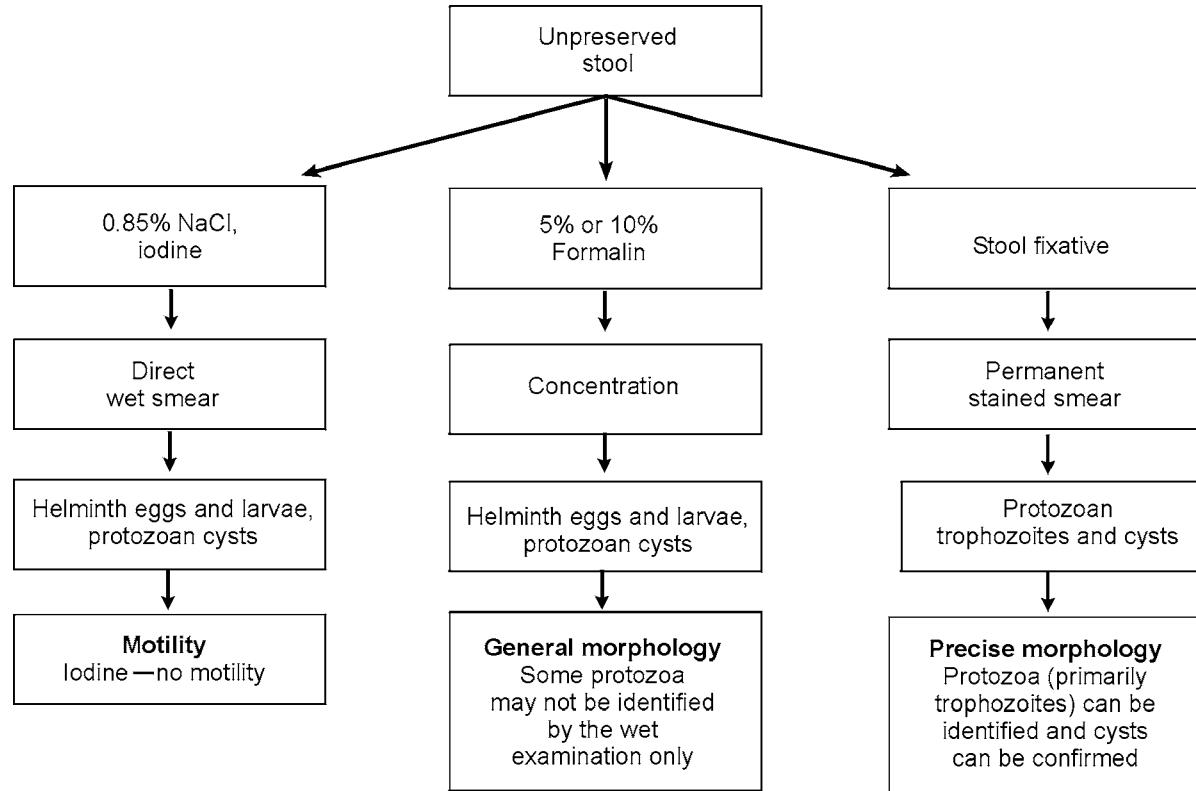
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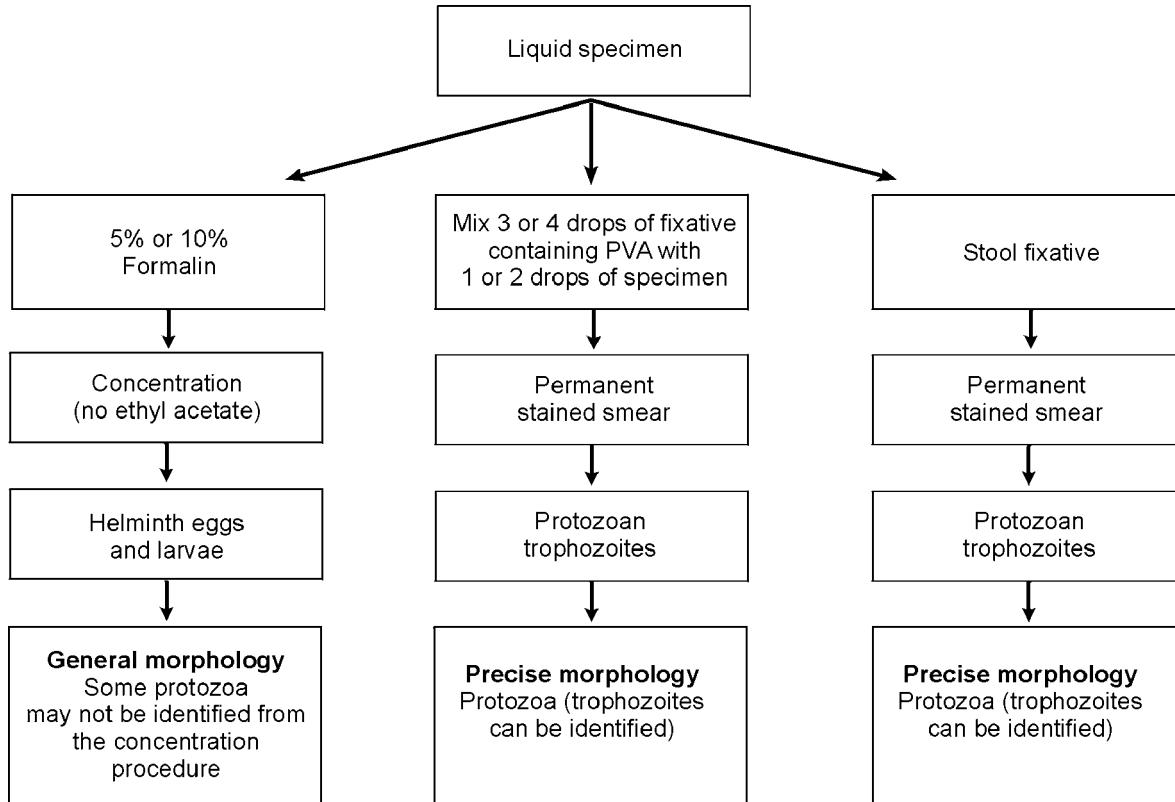
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**Algorithm 5.1** Procedure for processing fresh stool for the O&P examination

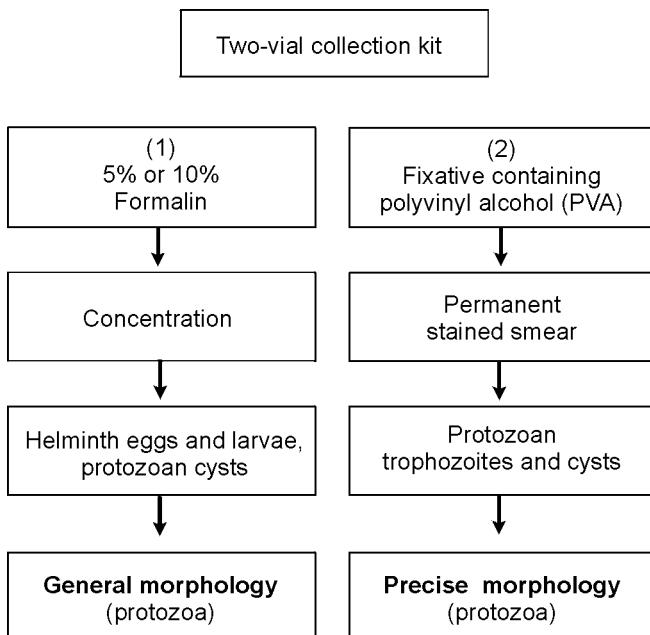


**Notes:** Special stains will be necessary for *Cryptosporidium* and *Cyclospora* (modified acid-fast stains) and the microsporidia (modified trichrome stain, or the nonspecific calcofluor). If the iron hematoxylin permanent staining method (containing the carbol fuchsin step) is used for the O&P examination, the coccidia will stain pink. Also, fecal immunoassay kits are now available for *Giardia lamblia*, *Cryptosporidium* spp., *Entamoeba histolytica*, and the *Entamoeba histolytica/E. dispar* group.

**Algorithm 5.2** Procedure for processing liquid specimens for the O&P examination


**Notes:** PVA/fixative and specimen will be mixed on the slide, allowed to air dry, and then stained. This approach is appropriate for liquid specimens or those containing a lot of mucus only; semiformed or formed specimens will not be adequately fixed using this method.

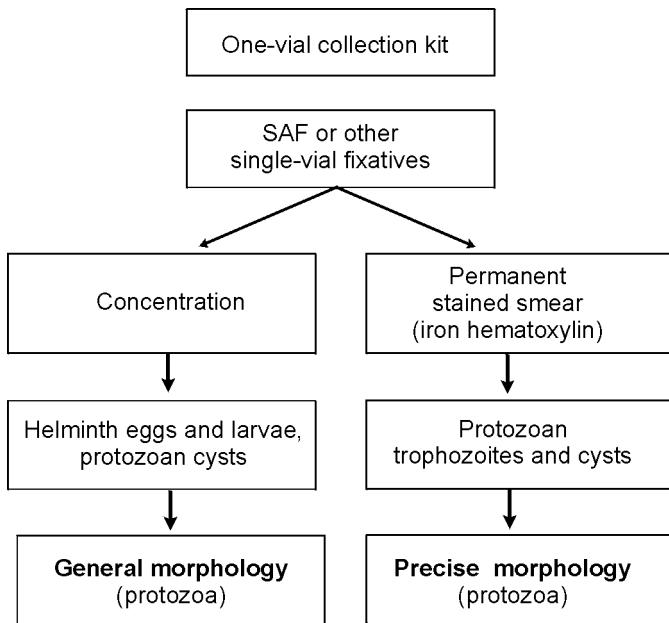
**Algorithm 5.3** Procedure for processing preserved stool for the O&P examination—two-vial collection kit



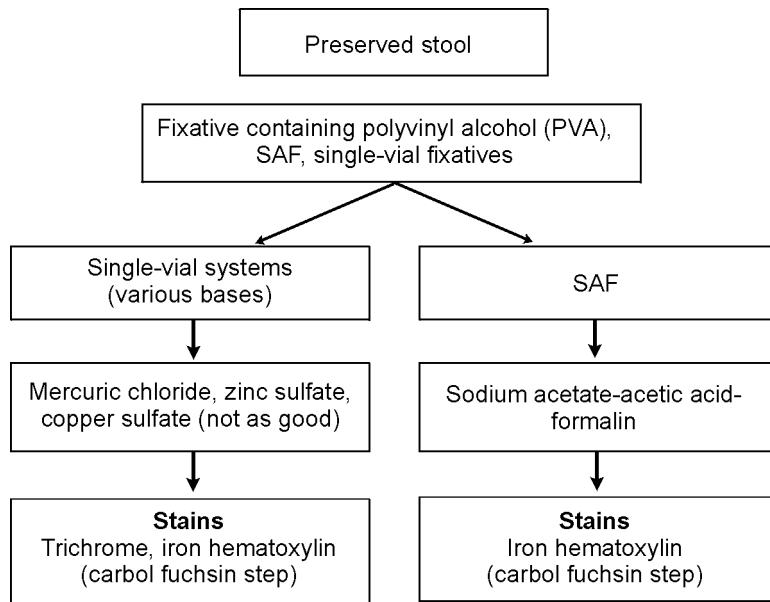
**Notes:** The following fixatives are listed and compared with those containing the mercuric chloride base and plastic powder (used to glue the stool onto the slide) polyvinyl alcohol (PVA).

1. Mercuric chloride base (Schaudinn's) containing PVA (gold standard) (trichrome, iron hematoxylin)
2. Zinc sulfate base containing PVA (good substitute) (trichrome, iron hematoxylin)
3. Copper sulfate base containing PVA (fair substitute) (trichrome, iron hematoxylin)
4. SAF (no PVA) (good substitute) (single-vial system) (iron hematoxylin best, trichrome OK)
5. Unifix (no PVA) (good substitute) (single-vial system) (trichrome probably best)
6. Ecofix (no PVA) (acceptable substitute) (good when used with Ecostain, trichrome, OK)
7. There are other single-vial systems available—check the literature for published results

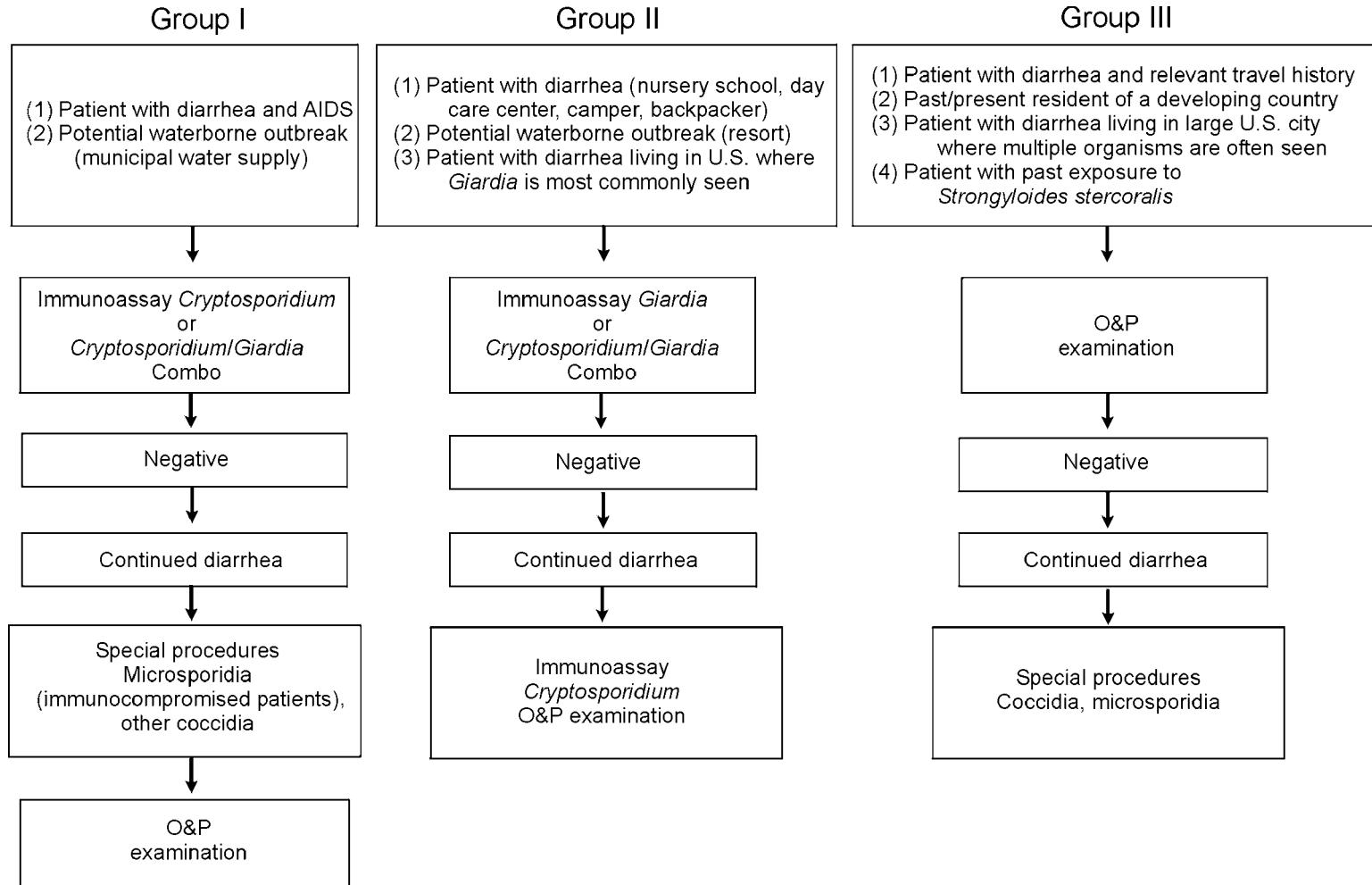
**Algorithm 5.4** Procedure for processing SAF-preserved stool for the O&P examination



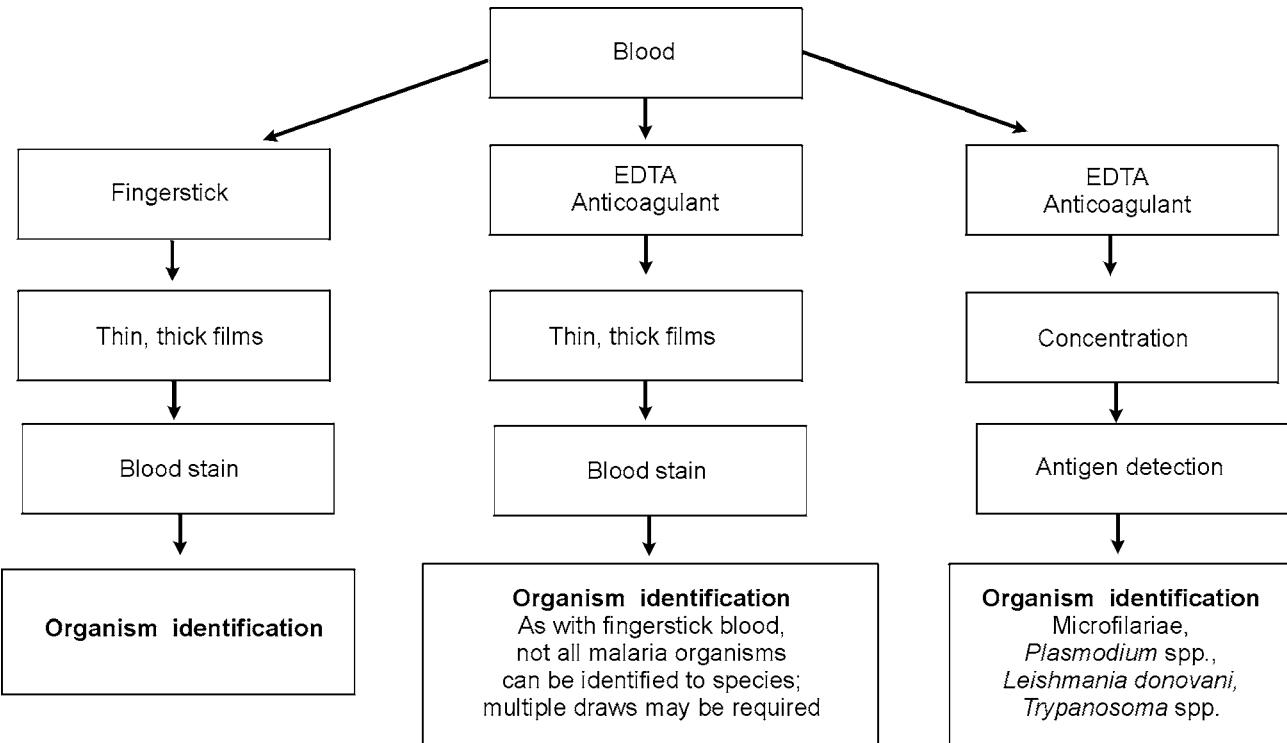
**Notes:** SAF and some of the other single-vial systems can also be used with EIA, FA, and cartridge rapid fecal immunoassays for the detection of *Giardia* and *Cryptosporidium*. Centrifuged material can also be used for the modified acid-fast and/or modified trichrome stains.

**Algorithm 5.5** Use of various fixatives and their recommended stains

**Notes:** 5% or 10% formalin, SAF, Unifix, Ecofix, and some of the other single-vial systems can also be used with EIA, FA, and rapid cartridge fecal immunoassay kits, as well as modified acid-fast (coccidia) and modified trichrome (microsporidia) stains. Check with the manufacturer and/or published results. Although both the concentration and permanent stained smear can be performed using these fixatives, the concentration performed from PVA-containing fixatives is an uncommon approach.

**Algorithm 5.6** Ordering algorithm for laboratory examination for intestinal parasites


**Notes:** The first group of patients for which the *Cryptosporidium* fecal immunoassay is recommended as the first test order includes (1) patients with diarrhea and AIDS (or other compromised states) and (2) patients with diarrhea from a potential municipal waterborne outbreak. Group 2 for which the first test order would be the *Giardia* fecal immunoassay includes (1) patients with diarrhea from a nursery school, day care center, a camper, or backpacker, (2) patients with diarrhea from a potential resort waterborne outbreak, and (3) patients with diarrhea who live in areas within the United States where *Giardia* is the most common organism seen. Group 3 for which the first test order would be the O&P examination includes (1) patients with diarrhea and relevant travel history, (2) patients with diarrhea who are a past/present resident of a developing country, (3) patients with diarrhea who live in large metropolitan areas within the United States where multiple parasites are often seen, and (4) patients who may have a history of past exposure to *Strongyloides stercoralis*. In group 3, it is also very important to remember that there may be symptomatic patients who are immunocompromised (either from underlying disease or those who may/will be intentionally immunocompromised from therapy, etc.) who may have a history of possible exposure to *Strongyloides stercoralis*. Remember, this past exposure could occur as long as 40+ years ago, after the patient has left the endemic area. With the internal autoinfection cycle, the patient can carry this infection without symptoms until becoming debilitated/immunocompromised. In these cases, it would be very important to perform the O&P examination, as well as the agar plate culture for *Strongyloides* infection. In the event of disseminated disease with strongyloidiasis, this type of hyperinfection can be fatal if not recognized and treated.

**Algorithm 5.7** Procedure for processing blood specimens for examination

**Notes:** Special stains may be necessary to identify some of the microfilariae to the species level (Delafield's or other hematoxylin stains). Many laboratories use automated instruments for hematology blood work. Parasite detection with these instruments has not proven to be acceptable; manual examination of stained blood films is recommended for the detection of all blood parasites. One negative set of blood films does not rule out a possible infection. Although for many years, Giemsa stain has been the stain of choice, the parasites can also be seen on blood films stained with Wright's stain, a Wright/Giemsa combination stain, or one of the more rapid stains such as Diff-Quik (American Scientific Products, McGraw Park, IL), Wright's Dip Stat Stain (Medical Chemical Corp., Torrance, CA), or Field's stain.

**Table 5.1** Body site, specimen, and recommended stain(s)<sup>a</sup>

Body site	Specimen	Recommended stain for suspect organism	Comments
Blood	Whole or anticoagulated blood	Blood stains (Giemsa, Wright's, Wright-Giemsa combination, Field's, rapid blood stains) All blood parasites Hematoxylin-based stain Microfilariae (sheathed)  Note: The QBC tube is also available as a screening method for blood parasites (hematocrit tube contains acridine orange) and has been used for malaria, <i>Babesia</i> , trypanosomes, leishmania, and microfilariae; <b>however, the sensitivity and specificity of the QBC tube do not equal those seen with thick and thin blood films.</b>	<b>STAT request (collection, processing, staining, examination, and reporting).</b> Most drawings and organism descriptions of blood parasites were originally based on Giemsa-stained blood films. Although Wright's stain (or Wright-Giemsa combination stain) works, stippling in malaria parasites may not be visible and the organism colors do not match the descriptions. However, using other stains (those listed above, in addition to some of the "quick" blood stains), the organisms should be visible on the blood film.  The use of anticoagulated blood (EDTA recommended) is acceptable. For detection of stippling, the smears should be prepared within 1 hr after the specimen is drawn. After that time, stippling may not be visible on stained films, and after 4 to 6 h, organisms begin to disappear. <b>Overall QC:</b> If the PMNs look good, any parasites present will also exhibit good morphology (both thick and thin blood films).
Bone marrow	Aspirate	Blood stains (Giemsa, Wright's, Wright/Giemsa combination, Field's, rapid blood stains) All blood parasites	See comments listed above for blood.

*(continued on next page)*

**Table 5.1** Body site, specimen, and recommended stain(s)<sup>a</sup> (continued)

Body site	Specimen	Recommended stain for suspect organism	Comments
Central nervous system	Spinal fluid, brain biopsy specimen	Blood stains <i>Trypanosomes, Toxoplasma gondii</i> Blood stains, Trichrome, or calcofluor Amebae ( <i>Naegleria</i> ; <i>Acanthamoeba</i> , <i>Balamuthia</i> , <i>Sappinia</i> ) Blood stains, acid-fast, PAS, modified trichrome, silver methenamine Microsporidia Hematoxylin and eosin (routine histology) Larval cestodes	<b>STAT request (collection, processing, staining, examination, and reporting).</b> If cerebrospinal fluid is received (with no suspect organism suggested), any of the blood stains would be acceptable; however, calcofluor is also recommended as a second stain. If brain biopsy material is received (particularly from an immunocompromised patient), electron microscopy studies may be required in order to identify microsporidia to the genus or species level. Immunofluorescent methods (along with routine histologic morphology) may be required to identify free-living amebae to the correct genus.
Cutaneous ulcers	Aspirate, biopsy specimen	Blood stains <i>Leishmaniae</i> Hematoxylin and eosin (routine histology) <i>Acanthamoeba</i> spp. <i>Entamoeba histolytica</i>	Most likely causative agent would be leishmaniae, all of which would stain with any of the blood stains. Hematoxylin and eosin (routine histology) could also be used to identify these organisms.
Eyes	Biopsy specimen, scrapings, contact lens, lens solution	Calcofluor (cysts only) Amebae ( <i>Acanthamoeba</i> ), Blood stains (trophozoites, cysts) Amebae Hematoxylin and eosin (routine histology) Cysticerci <i>Loa loa</i> <i>Toxoplasma gondii</i> Silver methenamine stain, PAS, acid-fast, electron microscopy Microsporidia	Some free-living amebae (most commonly <i>Acanthamoeba</i> ) have been implicated as a cause of keratitis. Although calcofluor stains the cyst walls, it does not stain the trophozoites. Therefore, in suspect cases of amebic keratitis, both stains should be used. Hematoxylin and eosin (routine histology) can be used to detect and confirm cysticercosis. The adult worm of <i>Loa loa</i> , when removed from the eye, can be stained with a hematoxylin-based stain (Delafield's) or can be stained and examined via routine histology. <i>Toxoplasma</i> infection could be diagnosed using routine histology and/or serology results. Microsporidia confirmation to the genus or species levels may require electron microscopy studies.

*(continued on next page)*

**Table 5.1** (continued)

Body site	Specimen	Recommended stain for suspect organism	Comments
Intestinal tract	Stool, sigmoidoscopy material, duodenal contents	Trichrome or iron hematoxylin Intestinal protozoa Modified trichrome Microsporidia Modified acid fast <i>Cryptosporidium</i> spp. <i>Cyclospora cayetanensis</i> <i>Isospora belli</i> Fecal immunoassays (enzyme immunoassay, FA, rapid cartridges) <i>E. histolytica</i> , <i>G. lamblia</i> <i>Cryptosporidium</i> spp. Microsporidia (experimental)	Although trichrome or iron hematoxylin stains can be used on almost all specimens from the intestinal tract, actual worm segments (tapeworm proglottids) can be stained with special stains. However, after routine dehydration through alcohols and xylenes (or xylene substitutes), the branched uterine structure is visible, thus allowing identification of the proglottid to the species level. Fecal immunoassay detection kits are also available for the identification of <i>Giardia lamblia</i> , <i>Entamoeba histolytica</i> , <i>Entamoeba dispar</i> , and <i>Cryptosporidium</i> spp. Microsporidia confirmation to the genus or species level may require electron microscopy studies.
	Anal impression smear	No stain, cellulose tape	Four to six consecutive negative tapes are required to rule out infection.
	Adult worm or worm segments	Carmine stains (rarely used)	Proglottids can usually be identified to the species level without using tissue stains.
	Biopsy specimen	Hematoxylin and eosin (routine histology) <i>Entamoeba histolytica</i> <i>Cryptosporidium</i> spp. <i>Cyclospora cayetanensis</i> <i>Isospora belli</i> <i>Giardia lamblia</i> Microsporidia	Special stains may be helpful in the identification of microsporidia: tissue Gram stains, silver stains, PAS, and Giemsa.
Liver, spleen	Aspirates, biopsy specimen	Blood stains Leishmaniae Hematoxylin and eosin (routine histology) Leishmaniae, microsporidia, trematodes (adults, eggs)	Aspirates and/or touch preparations from biopsy material can be routinely stained with blood stains. This allows identification of the leishmaniae. There are definite risks associated with spleen aspirates and/or biopsy specimens. Other parasites, such as larval cestodes, trematodes, amebae, or microsporidia, could be seen and identified from routine histologic staining.

(continued on next page)

**Table 5.1** Body site, specimen, and recommended stain(s)<sup>a</sup> (continued)

Body site	Specimen	Recommended stain for suspect organism	Comments
Lungs	Sputum, induced sputum, bronchoalveolar lavage fluid, transbronchial aspirate, tracheobronchial aspirate, brush biopsy specimen, open-lung biopsy specimen	Silver methenamine stain, Calcofluor (cysts only) <i>Pneumocystis jiroveci</i>  Giemsa (trophozoites only) <i>Pneumocystis jiroveci</i> Modified acid-fast stains <i>Cryptosporidium</i> spp. Hematoxylin and eosin (routine histology) <i>Strongyloides stercoralis</i> <i>Paragonimus</i> spp. Amebae  Silver methenamine stain PAS, acid-fast stains, tissue Gram stains, modified trichrome, electron microscopy Microsporidia	<i>Pneumocystis jiroveci</i> is the most common parasite (now classified with the fungi) recovered and identified from the lungs by using silver or Giemsa stains or monoclonal reagents (FA). Monoclonal reagents (FA) are also available for the diagnosis of pulmonary cryptosporidiosis. Routine histology procedures would allow the identification of any of the helminths or helminth eggs present in the lungs.
Muscle	Biopsy specimen	Hematoxylin and eosin (routine histology) <i>Trichinella</i> spp. Cysticerci <i>Sarcocystis</i> spp.  Silver methenamine stain PAS, acid-fast stains, tissue Gram stains, electron microscopy Microsporidia	If <i>Trypanosoma cruzi</i> were present in the striated muscle, it could be identified from routine histology preparations. Microsporidia confirmation to the genus or species level may require electron microscopy studies.
Nasopharynx, sinus cavities	Microsporidia  <i>Acanthamoeba</i> sp. <i>Naegleria</i> sp.	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor), methenamine silver, electron microscopy  Giemsa, trichrome Giemsa, trichrome	Microsporidia confirmation to the genus or species level may require electron microscopy studies. Some free-living amebae (most commonly <i>Acanthamoeba</i> or <i>Naegleria</i> ) have been found to colonize the nasopharynx and/or sinus cavities. Although calcofluor stains the cyst walls, it does not stain the trophozoites. Therefore, in suspect cases, both calcofluor and one of the routine stains should be used.

(continued on next page)

**Table 5.1** (continued)

Body site	Specimen	Recommended stain for suspect organism	Comments
Skin	Aspirates, skin snip, scrapings, biopsy specimens	See Cutaneous Ulcer above Hematoxylin and eosin (routine histology) <i>Onchocerca volvulus</i> <i>Dipetalonema streptocerca</i> <i>Acanthamoeba</i> spp. <i>Entamoeba histolytica</i>	Any of the potential parasites present could be identified using routine histology procedures and stains.
Urogenital system	Vaginal discharge, urethral discharge, prostatic secretions, urine  Biopsy specimen	Giems stain, immunoassay reagents (FA) <i>Trichomonas vaginalis</i> Delafeld's hematoxylin Microfilariae Modified trichrome Microsporidia Hematoxylin and eosin (routine histology) <i>Schistosoma haematobium</i> Microfilariae PAS, acid-fast stain, tissue Gram stains, electron microscopy Microsporidia	Although <i>T. vaginalis</i> is probably the most common parasite identified, there are others to consider, the most recently implicated organisms being in the microsporidian group. Microfilariae could also be recovered and stained.

<sup>a</sup> Modified from H. D. Isenberg, ed., *Clinical Microbiology Procedures Handbook*, 2nd ed., ASM Press, Washington, DC, 2004.

**Table 5.2** Approaches to stool parasitology: test ordering

Patient and/or situation	Test ordered <sup>a</sup>	Follow-up test ordered
Patient with diarrhea and AIDS or other cause of immune deficiency or Patient with diarrhea involved in a potential waterborne outbreak (municipal water supply)	<i>Cryptosporidium</i> or <i>Giardia</i> / <i>Cryptosporidium</i> immunoassay	If immunoassays are negative and symptoms continue, special stains for microsporidia (modified trichrome stain) and other coccidia (modified acid-fast stain) and O&P exam should be performed.
Patient with diarrhea (nursery school, day care center, camper, backpacker) or Patient with diarrhea involved in a potential waterborne outbreak (resort setting) or Patient from areas within the United States where <i>Giardia</i> is the most common parasite found	<i>Giardia</i> or <i>Giardia/Cryptosporidium</i> immunoassay	If immunoassays are negative and symptoms continue, special stains for microsporidia and other coccidia (see above) and O&P exam should be performed.
Patient with diarrhea and relevant travel history outside of the United States or Patient with diarrhea who is a past or present resident of a developing country or Patient in an area of the United States where parasites other than <i>Giardia</i> are found (large metropolitan centers such as New York, Los Angeles, Washington, DC, Miami)	O&P exam, <i>Entamoeba histolytica/E. dispar</i> immunoassay; immunoassay for confirmation of <i>E. histolytica</i> ; various tests for <i>Strongyloides</i> may be relevant (even in the absence of eosinophilia), particularly if there is any history of pneumonia (migrating larvae in lungs), sepsis, or meningitis (fecal bacteria carried by migrating larvae); the agar culture plate is the most sensitive diagnostic approach for <i>Strongyloides</i> .	The O&P exam is designed to detect and identify a broad range of parasites (amebae, flagellates, ciliates, <i>Isospora belli</i> , and helminths). If exams are negative and symptoms continue, special tests for coccidia (fecal immunoassays, modified acid-fast stains, autofluorescence) and microsporidia (modified trichrome stains, calcofluor white stains) should be performed; fluorescent stains are also options.
Patient with unexplained eosinophilia	Although the O&P exam is recommended, the agar plate culture for <i>Strongyloides stercoralis</i> (it is more sensitive than the O&P exam) is also recommended, particularly if there is any history of pneumonia (migrating larvae in lungs), sepsis or meningitis (fecal bacteria carried by migrating larvae).	If tests are negative and symptoms continue, additional O&P exams and special tests for microsporidia (modified trichrome stains, calcofluor white stains, fluorescent stains) and other coccidia (modified acid-fast stains, autofluorescence, fluorescent stains) should be performed.

*(continued on next page)*

**Table 5.2** (continued)

Patient and/or situation	Test ordered <sup>a</sup>	Follow-up test ordered
Patient with diarrhea (suspected food-borne outbreak)	Test for <i>Cyclospora cayetanensis</i> (modified acid-fast stain, autofluorescence, fluorescent stains).	If tests are negative and symptoms continue, special procedures for microsporidia and other coccidia and O&P exam should be performed.

<sup>a</sup> Depending on the particular immunoassay kit used, various single or multiple organisms may be included. Selection of a particular kit depends on many variables: clinical relevance, cost, ease of performance, training, personnel availability, number of test orders, training of physician clients, sensitivity, specificity, equipment, time to result, etc. Very few laboratories handle this type of testing exactly the same. Many options are clinically relevant and acceptable for good patient care. It is critical that the laboratory report indicate specifically which organisms could be identified by the kit; a negative report should list the organisms relevant to that particular kit. It is important to remember that sensitivity and specificity data for all of these fecal immunoassay kits (FA, enzyme immunoassay, cartridge formats) are comparable.

**Table 5.3** Laboratory test reports: optional comments<sup>a,b</sup>

***Entamoeba histolytica/E. dispar group***

Unless you see trophozoites containing ingested RBCs (these are from the true pathogen, *E. histolytica*), you cannot tell from the organism morphology whether you have actual pathogenic *E. histolytica* organisms or nonpathogenic *E. dispar* present.

**Report as *Entamoeba histolytica/E. dispar group*.**

Additional computer comments:

- A. Unable to determine pathogenicity from organism morphology
- B. Depending on patient's clinical condition, treatment may be appropriate.

If you have the kit reagents to differentiate the two organisms, comments could also be added:

- A. If you wish to determine which of the two organisms is present, please submit a fresh stool specimen.
- B. To determine the presence or absence of pathogenic *E. histolytica*, please submit a fresh stool specimen.

**Identification of nonpathogens**

Comments that can be used for reporting nonpathogens include the following. However, these statements assume that a complete stool exam was performed on multiple stools; you may detect nonpathogens in the first examination or an incomplete examination, but miss a pathogen (example: *Dientamoeba fragilis* requires the permanent stained smear for identification).

Additional computer comments:

- A. Considered nonpathogenic; treatment not recommended
- B. Nonpathogen; indication that patient has ingested something contaminated with fecal material (same method for acquiring pathogens).

**Reporting *Blastocystis hominis***

Several comments are optional for reporting *Blastocystis hominis*.

- A. Clinical significance is controversial; it appears that some strains are pathogenic.
- B. Status as a pathogen is controversial; it appears that some strains are pathogenic.

You may want to add a second comment:

- A. Other organisms capable of causing diarrhea should also be ruled out.

**Negative stool examination (O&P exam)<sup>c</sup>**

An additional comment may be helpful:

- A. Certain antibiotics such as metronidazole or tetracycline may interfere with the recovery of intestinal parasites, particularly the protozoa.
- B. Antibiotics such as metronidazole or tetracycline may prevent recovery and identification of the intestinal protozoa.

**Positive *Plasmodium* spp. report (ALWAYS a STAT request and report)<sup>d</sup>**

If *Plasmodium* spp. are detected but you are unable to identify to the species level, report as follows.

- A. *Plasmodium* spp. seen; unable to "rule out" *Plasmodium falciparum*
- B. If mixed infection: *Plasmodium* mixed infection possible; unable to "rule out" *Plasmodium falciparum*
- C. Quantitate all positive malarial smears (percentage of infected RBCs within 100 total RBCs); quantitate from thin blood film. **Always add quantitation to the report (this applies to *Babesia* spp. as well).**
- D. Send additional blood samples approximately every 6 h for 3 days (unless malaria is no longer a consideration).

<sup>a</sup> It is important to remember that educational information for your clients is critical to the success of your test reporting formats. The information in the table should be shared with your clients prior to changing your actual report formats. Your physician group may have a preference regarding additional comments. Information updates or newsletters are appropriate for this purpose.

<sup>b</sup> All of these comments are optional and wording can be changed to fit your circumstances. However, it is recommended that you select specific comments and try not to use "free text" so everyone reports test results the same way each time.

<sup>c</sup> This information could also be conveyed to your clients via the newsletter or update formats.

<sup>d</sup> It is very important to add the comments about *Plasmodium falciparum*; this is critical information for patient care.

**Table 5.4** Parasitemia determined from conventional light microscopy: clinical correlation

Parasitemia (%)	No. of parasites/ $\mu$ l	Clinical correlation
0.0001–0.0004	5–20	Number of organisms required for positive thick film (sensitivity) Examination of 100 thick-blood-film fields ( $0.25 \mu$ l) may miss up to 20% of infections (sensitivity). At least 300 fields should be examined before reporting a negative result (80–90%). Examination of 100 thin-blood-film fields ( $0.005 \mu$ l); at least 300 fields should be examined before reporting a negative result; both thick and thin blood films should be examined for every specimen submitted for a suspect malaria case (report final results with the $100\times$ oil immersion objective). <b>One set (thick plus thin blood films) of negative blood films does not rule out a malaria infection.</b>
0.002	100	<b>Patients may be symptomatic below this level, particularly if they are immunologically naive (with no prior exposure to malaria).</b>
0.02	1,000	Level seen in travelers (immunologically naive); results may also be lower than this.
0.2	10,000	Level above which immune patients exhibit symptoms; 0.1% (5,000) = BinaxNOW level of sensitivity for rapid malaria test (dipstick).
2	100,000	Maximum parasitemia of <i>P. vivax</i> and <i>P. ovale</i> (which infect young RBCs only)
2–5	100,000–250,000	Hyperparasitemia, severe malaria <sup>a</sup> ; increased mortality
10	500,000	Exchange transfusion may be considered; high mortality

<sup>a</sup> World Health Organization criteria for severe malaria are parasitemia of  $>10,000/\mu$ l and severe anemia (hemoglobin,  $<5$  g/liter). The prognosis is poor if  $>20\%$  of parasites are pigment-containing trophozoites and schizonts and/or if  $>5\%$  of neutrophils contain visible pigment.

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## S E C T I O N     6

# Commonly Asked Questions about Diagnostic Parasitology

As laboratory personnel become more widely cross-trained, the availability of people who have expertise in diagnostic parasitology will become more limited. Over the years, many questions have been asked about various aspects of diagnostic medical parasitology. Answers to these questions may be helpful for those working in this area of microbiology and may provide some “tips of the trade” that are learned through many years of bench experience.

## Stool Parasitology

### Specimen Collection

#### Intestinal Tract

1. What has been the “gold standard” for stool collection systems?

*The standard two-vial collection set has been used for many years and consists of one vial of 10% formalin from which the concentration is performed and one vial of PVA fixative (base fixative can be mercury or nonmercury to which has been added the plastic resin powder polyvinyl alcohol [PVA]) from which the permanent stained smear is prepared.*

2. What is the basis for the recommendation that three stools should be collected on alternate days, rather than three days in a row or three in one day?

*Intestinal protozoa are shed in the stool on a cyclic basis. It is generally accepted that this time frame is approximately 10 days, so if specimens are collected too close together, sampling may occur at a low point in the cycle and the organisms may be missed. By collecting specimens over a wider time frame, days with heavier shedding may be sampled as well.*

3. Is it acceptable to accept three stools collected on three consecutive days?

*Yes, but sampling during the total time frame for shedding (10 days) might be more likely to include days of heavy shedding.*

4. Is it ever appropriate to accept three stools in one day from the same patient?

*With rare exceptions, this is unacceptable. If a patient has severe diarrhea or dysentery, there is a large dilution factor that may make finding any organisms more difficult. Therefore, after consultation with the physician, more than one stool sample could be examined within any one day.*

5. Is it good laboratory practice to accept two (rather than three) stools for the O&P examination?

*Although the examination of three stool specimens provides a more statistically accurate result, many laboratories feel that the percentage of organism recovery with the examination of two stool samples is acceptable. The percent recovery varies from ~80% to over 95% with the submission and examination of two stool specimens from a single patient.*

6. Do you have to collect the stool specimen any differently if you are looking for the coccidia (*Cryptosporidium* spp., *Cyclospora cayetanensis*, or *Isospora belli*)?

*Fresh stool samples or specimens preserved in routine stool fixatives (formalin based) can be used for diagnostic procedures for the identification of the coccidian oocysts (special modified acid-fast stains for the coccidia or fecal immunoassays for *Cryptosporidium* spp.). Some of the single-vial collection options are also acceptable.*

7. Do you have to collect the stool specimen any differently if you are looking for microsporidia?

*Fresh stool samples or specimens preserved in routine stool fixatives (formalin based) can be used for diagnostic procedures (modified trichrome stains) for the identification of the microsporidial spores. Some of the single-vial collection options are also acceptable.*

## **Fixatives**

1. What are the pros and cons of using stool fixatives?

*Although it would be helpful to see motile organisms in the direct saline wet preparation, the trophozoite forms have often already begun to disintegrate by the time the stool specimen reaches the laboratory. To reduce the lag time between passage of the specimen and arrival in the laboratory, stool fixatives are recommended. The benefits of fixation and preservation of organism morphology far outweigh the benefits of receiving a fresh, unpreserved stool.*

2. When selecting a single-vial stool fixative, what questions should be asked?

*Can one perform a concentration, permanent stained smear and/or fecal immunoassay procedures from the specimen received in that vial? What stains work best with that particular fixative? Another issue for consideration involves the types and numbers of parasites that might be missed using this approach.*

3. What are the advantages and disadvantages of using 5% versus 10% formalin?

*Although 5% formalin is thought to provide a more "gentle" fixation of protozoa, this concentration does not always kill all helminth eggs. The 10% formalin kills most helminth eggs (except for *Ascaris lumbricoides*) but may be more damaging to protozoa. In reality, most people probably cannot tell the difference by examining clinical specimens preserved in either 5 or 10% formalin.*

4. What is the difference between buffered and nonbuffered formalin?

*Buffered formalin tends to produce fewer osmotic changes in the organisms during fixation; however, on a day-to-day basis, it may be difficult to detect morphologic differences from buffered or unbuffered formalin.*

5. Should the laboratory be concerned about the amount of formalin used within the parasitology lab?

*The amount of formalin used in diagnostic parasitology testing is very minimal. The regulations governing formalin use were developed for industry, where large amounts of formalin are sometimes used. However, the regulations do indicate that any place using formalin must be monitored. Once you have been monitored and your results have been found not to exceed the stated limits and your records are on file, there is no need to be rechecked (unless something dramatically changes in terms of your formalin volume use). We have never heard of any microbiology laboratory coming even close to the limits, so formalin use within diagnostic parasitology is perfectly acceptable. Also see a full discussion in the Concentration section.*

6. What is PVA?

*PVA stands for polyvinyl alcohol, a plastic powder/resin that is incorporated into the fixative (Schaudinn's or other fixatives) and serves as an adhesive to "glue" the stool material onto the slide. PVA itself has no preservation capability.*

7. What are some good stool fixative-stain combinations?

*The "gold standard" has been one vial of 10% formalin, from which the concentration is performed. The second vial contains mercuric chloride-based PVA, from which the permanent stained smear is prepared and then stained (trichrome or iron hematoxylin). Other options in terms of overall quality are*

- Sodium acetate-acetic acid-formalin (SAF) and iron hematoxylin stain*
- UNIFIX or Z-PVA (Medical Chemical Corp., Torrance, CA) and trichrome stain*
- ECOFIX and ECOSTAIN (Meridian Biosciences)*
- SAF and trichrome stain*

*Other options may be available; check the published literature.*

## **Specimen Processing**

### **O&P Exam**

1. What procedures constitute the ova and parasite examination (O&P exam)?

*The direct wet smear, concentration, and permanent stained smear constitute the routine O&P exam on fresh stool specimens. If the specimens are submitted to the laboratory in stool preservatives, the concentration and permanent stained smear should be performed. If a laboratory indicates they provide an O&P exam, the College of American Pathologists (CAP) checklist indicates that the O&P exam must include the concentration and permanent stained smear. Also, the CAP checklist requires the direct wet smear be performed on fresh liquid or soft stool only (looking for motile trophozoites). There is no need to perform a direct wet mount on fresh*

*formed stool; the likelihood of seeing motile trophozoites is low, since formed stools tend to contain only the cyst forms.*

2. Why is it necessary to perform a permanent stained smear examination on every stool specimen submitted for an O&P exam?

*Intestinal protozoa are not always seen and identified from the concentration examination. Since the permanent stained smear is designed to facilitate identification and/or confirmation of the intestinal protozoa, it is important that this procedure be performed on all stools for which the O&P exam has been ordered. Also, since trophozoite stages are not visible on the concentration examination (rare exceptions, e.g., trophozoites can be seen in concentration sediments prepared from SAF-preserved specimens), it is even more important to examine the permanent stained smear. Even if organisms (trophozoites and/or cysts) are seen in the concentration wet mount, they might not be identified accurately and will require confirmation from examination of the permanent stained smear. Patients who are symptomatic with diarrhea are more likely to have protozoan trophozoites in the stool, not the more resistant cyst form. This approach is consistent with the O&P exam (CAP Checklist for laboratory inspections).*

3. Why do you need to pour out some PVA onto paper towels prior to preparation of slides for permanent staining?

*Many people were transferring the PVA-stool from the vial directly to the slide, and too much PVA was being carried over onto the slide. It takes quite a bit of time for PVA to dry, and so the material often falls off during staining because the thick PVA is not yet dry. Also, the amount of PVA (plastic powder) needed to glue the stool onto the slide is extremely small. So, if the excess PVA is removed prior to making the smears, the slides require less drying time, the stool adheres to the glass, and a better stained smear will be obtained. However, if the material (PVA/stool mixture) is taken right out of the vial onto the glass slide and this approach is working, there is no need to change protocols.*

4. Can you use concentrated sediment to prepare slides for permanent staining?

*It is important to remember that a routine trichrome stain cannot be performed from the concentration sediment if the specimen was originally preserved in formalin or has been rinsed using formalin, saline, or water. A trichrome stain can be performed on SAF-preserved material, but not if the specimen has been rinsed with formalin, saline, or water. If you centrifuge the fecal-fixative material (without adding any rinse reagents), then provided your fixative is compatible with the permanent stain you are using, you can use some of the sediment for smear preparation prior to staining. However, once you continue with the rinse steps, the final concentration sediment is often (again depending on the rinse fluids) not compatible with trichrome staining. If you want to use a single-vial fixative system, you can centrifuge the stool-fixative mixture (using no additional rinse reagents),*

*prepare your slide for permanent staining from the sediment, and then proceed with the regular rinses called for in the concentration procedure.*

## Diagnostic Methods

### Direct Wet Examinations

1. What is the purpose of the direct wet examination?

*The direct wet examination is designed to allow the viewer to detect motile protozoa; this procedure should not be performed on preserved specimens and should be reserved for fresh stool specimens that are very soft or liquid. Organism identification is often presumptive; permanent stained smears must also be examined.*

2. How should the direct wet preparation be examined?

*The entire coverslip preparation (22 by 22 mm) should be examined under low magnification ( $\times 100$ ); approximately one-third to one-half of the coverslip preparation should be examined under high dry magnification ( $\times 400$ ). It is not practical to examine this preparation using oil immersion magnification ( $\times 1,000$ ). Saline and/or iodine mounts can be examined; however, iodine kills any organisms present, so trophozoite motility will no longer be visible.*

3. What do you expect to see during a wet preparation examination?

*Helminth eggs and/or larvae can be seen, as well as some protozoan cysts, white blood cells (WBCs), some yeast, and fecal debris. Many of the intestinal protozoa will need to be confirmed using the oil immersion magnification ( $\times 1,000$ ) for the permanent stained smear.*

### Concentrations

1. What is the recommended time and speed for centrifugation for the concentration method? Why is this important?

*The current recommendation is for every centrifugation step in the concentration method (sedimentation) to be performed for 10 min at 500  $\times$  g. If this recommendation is not followed, small coccidian oocysts and microsporidial spores may not be recovered in the sediment. The number of organisms obtained after centrifugation is greatly increased over taking the sample from the uncentrifuged specimen.*

2. What is the purpose of the concentration procedure?

*The purpose of the concentration is to concentrate the parasites present, through either sedimentation or flotation. The concentration is specifically designed to allow recovery of protozoan cysts, coccidian oocysts, microsporidian spores, and helminth eggs and larvae.*

3. Why is the flotation concentration used less frequently than the sedimentation concentration?

*There are several reasons. First, not all parasites float; therefore, you need to examine both the surface film and the sediment before indicating that the concentration examination is negative. Second, the organisms*

*must not be left in contact with the high-specific-gravity zinc sulfate for too long or the protozoa will tend to become distorted, so the timing of the examination is more critical. Also, the specific gravity of the fluid must be checked periodically.*

4. What specific gravity zinc sulfate should be used for the flotation concentration procedure?

*If the concentration is being performed on fresh stool, the specific gravity of the solution should be 1.18. However, if the concentration is being performed on stool preserved in a formalin-based fixative, the specific gravity of the zinc sulfate should be 1.20.*

5. How should the concentration wet preparation be examined?

*Formalin-ethyl acetate (substitute for ether) sedimentation concentration is the most commonly used method. Zinc sulfate flotation does not detect operculated or heavy eggs; both the surface film and sediment must be examined before a negative result is reported. Smears prepared from concentrated stool are normally examined as for the direct wet mount under the low power objective (10 $\times$ ) and the high dry power objective (40 $\times$ ); use of the oil immersion objective (100 $\times$ ) is not recommended (organism morphology is not clear). The addition of too much iodine may obscure helminth eggs (they will mimic debris).*

6. What semi-automated methods are available to read the concentration sediments?

*The traditional sampling approach using pipettes and the preparation of wet smears on glass slides and coverslips can be replaced with a semi-automated sampling and viewing system from DiaSys Corp. The specimen is drawn through tubing from the mixed concentrated stool sediment into two viewing chambers that fit onto the microscope stage. The quality of the glass is excellent, and organism morphology can be easily seen within the viewing chambers. Selection of such a system often depends on the laboratory workload.*

7. Are there any tips for specimen processing for detection of the microsporidia?

*The earlier studies of microsporidia were performed mainly at the Centers for Disease Control and Prevention (CDC). When early comparisons were performed on methods, the authors compared slow centrifugation with using uncentrifuged material. Of these methods, they felt that using uncentrifuged material was better for recovery of microsporidial spores. However, at the University of California Los Angeles when we looked carefully at this approach compared with centrifugation at 500  $\times$  g for 10 min (the standard centrifugation speed and time), we found considerably more spores in the sediment after centrifugation. If the stool contains a lot of mucus or is runny, formalin should be added before centrifugation. It is not necessary to use ethyl acetate since it may pull much of the material you want to examine up into the mucus layer. If the stool is not particularly watery or does not contain a lot of mucus, it can be treated just like a regular concentration (but every centrifugation should be at 500  $\times$  g for*

10 min). Also, the more a stool specimen is manipulated, the more likely it is that some organisms will be lost (this applies to all parasites in stool). So, you may want to eliminate the wash steps and work with the sediment you obtain from the first centrifugation step. The smears should be pretty thin to help visualize the spores, but also remember not to decolorize too much. Filtration is not a problem for any of the commercial concentration systems. If you are using gauze, make sure you use woven gauze and use only two layers (do not use pressed gauze, which is too thick).

8. How safe is the use of formalin within the microbiology laboratory?

The formalin regulations were originally developed for industry (plywood, fabrication, etc.), where great amounts of formalin are used in the manufacturing process. The amount of formalin we are exposed to in the laboratory is very minimal; I have never heard of any microbiology laboratory (including a full-service parasitology service) even coming close to the limits.

The Occupational Safety and Health Administration (OSHA) amended the original regulations for occupational exposure to formaldehyde in May of 1992 (1: Fed Regist 1992 May 27;57(102):22290-328). The final amendments lower the permissible exposure level for formaldehyde from 1 ppm (part per million) as an 8-hour time-weighted average (TWA) to an 8-hour time-weighted average of 0.75 ppm. The amendments also add medical removal protection provisions to supplement the existing medical surveillance requirements for those employees suffering significant eye, nose or throat irritation and for those suffering from dermal irritation or sensitization from occupational exposure to formaldehyde. In addition, changes have been made to the standard's hazard communication and employee training requirements. These amendments establish specific hazard labeling for all forms of formaldehyde, including mixtures and solutions composed of 0.1% or greater of formaldehyde in excess of 0.1 ppm. Additional hazard labeling, including a warning that formaldehyde presents a potential cancer hazard, is required where formaldehyde levels, under reasonably foreseeable conditions of use, may potentially exceed 0.5 ppm. The final amendments also provide for annual training of all employees exposed to formaldehyde at levels of 0.1 ppm or higher.

Laboratories that have been monitored have not come close to either measurement. Once a laboratory has been measured and the results (below thresholds for regulatory requirements) are on file, this information does not have to be generated again. No badges are required. Even without a fume hood (many labs do not use a fume hood), performing the routine formalin-ethyl acetate concentration does not seem to be a problem. A number of people who have indicated that they want to remove formalin from the laboratory probably do not really understand the history of the regulation or the actual issues. The only possible problem seen in the clinical and pathology laboratory setting might be a routine anatomical pathology laboratory where very large amounts of formalin are used, and with sloppy use. However, within the microbiology laboratory (even large laboratories), it does not seem to be a problem.

## **Permanent Stains**

1. What is the purpose of the permanent stained smear?

*The purpose of the permanent stained smear is to provide contrasting colors for the background debris and parasites present; it is designed to allow examination and recognition of detailed organism morphology under oil immersion examination (100 $\times$  objective, for a total magnification of  $\times 1,000$ ). This examination is designed primarily to allow recovery and identification of the intestinal protozoa.*

2. How long should the permanent stained smear be examined?

*Rather than responding with a specific number of minutes, the recommendation is to examine at least 300 oil immersion ( $\times 1,000$  total magnification) fields; additional fields may be required if suspect organisms have been seen in the wet preparations from the concentrated specimen.*

3. What recent changes have influenced the overall quality of the permanent stained smear?

*The use of mercury substitutes in PVA generally leads to diminished quality of the overall morphology of the intestinal protozoa. However, some of the mercury substitutes provide a morphologic quality close to that of mercury, allowing identification of most of the intestinal protozoa. Differences in detection and identification are usually comparable unless very few organisms are present or the organisms are quite small. In these circumstances, some organisms may be missed using mercury substitutes.*

4. What is the purpose of the iodine dish in Wheatley's trichrome stain protocol?

*Mercury is removed from the smear when it is placed in the iodine dish; there is a chemical substitution of iodine for mercury. The iodine is removed during the next two alcohol rinses. Therefore, at the point the slide is placed in trichrome stain, neither mercury nor iodine is left on the smear.*

5. Why don't you need to use the iodine dish when staining fecal smears prepared from specimens preserved in the newer single-vial systems (zinc sulfate-based PVA)?

*The zinc sulfate-based PVA is water soluble, and so the dry smears can be placed directly into the trichrome dish without having to go through the iodine and subsequent rinse steps. The zinc sulfate is removed by the water in the trichrome stain.*

6. Why might you have to use the iodine dish and subsequent rinses in your staining setup when staining slides from the proficiency testing agencies (American Association of Bioanalysts, various states, etc.)?

*Some smears used for proficiency testing are prepared from fecal specimens that have been preserved in mercury-based fixatives, so the iodine dish and subsequent rinse steps are required to remove mercury and iodine prior to*

*staining with either trichrome or iron hematoxylin stains. For several years, CAP proficiency testing specimens have been preserved in nonmercury fixatives; therefore, the iodine dish is not required.*

7. What role does the acetic acid play in the trichrome stain?

*Both the trichrome and iron hematoxylin stains are considered regressive stains; the fecal smears are overstained and then destained. The acetic acid in the 90% alcohol rinse step in the trichrome stain removes some of the stain and provides better contrast. However, in some cases differences in the quality of staining between stained protozoa that have been subjected to the 90% alcohol rinse with and without the acetic acid may be difficult to detect. It is important not to destain the smears too much; overall organism morphology will be diminished.*

8. What causes the xylene (or xylene substitute) dehydration solutions to turn cloudy when a slide from the previous alcohol dish is moved forward into the xylene dish?

*If there is too much water carryover from the last alcohol dish, the xylene solution may turn cloudy. When this occurs, replace the 100% alcohol dishes, back up the slide into 70% alcohol (you can also use a series of steps, 95% and then 70%), allow it to stand for 15 min, and then move the slide forward through the 100% alcohol and xylene steps.*

9. Why is absolute ethanol (100%) recommended as the best approach?

*Although many laboratories use the commercially available 95/5% denatured alcohol mix as their “absolute alcohol,” the dehydration of stained fecal smears is not as good as that obtained with 100% ethanol. You may want to add an additional dish of the denatured alcohol (absolute alcohol) to your staining setup to obtain better dehydration.*

10. What is the difference between xylene and xylene substitutes?

*There are several differences. Most laboratories have made the decision to eliminate xylene from their laboratories as a safety measure. However, xylene substitutes generally do not dehydrate as well. Also, after the slides are removed from the last dish of xylene substitute, they take longer to dry. You may want to add an additional dish of the xylene substitute to your staining setup to obtain better overall dehydration.*

## **Stool Immunoassay Options**

1. What are some of the immunoassay options available for stool protozoa?

*Currently, immunoassays are available for Giardia lamblia, Cryptosporidium spp., the Entamoeba histolytica/E. dispar group, and E. histolytica. Reagents for the detection of Dientamoeba fragilis and the microsporidia are under development.*

2. What methods are available commercially?

*Direct fluorescence (DFA), enzyme immunoassay (EIA), and cartridge formats (membrane flow or a solid-phase qualitative immunochromatographic procedure) are currently available.*

3. Why might someone want to use a fecal immunoassay option?

*If the most common organisms found in the area are Giardia, Cryptosporidium, and/or the E. histolytica/E. dispar group, then fecal immunoassays are certainly options. Specific patient histories and symptoms would suggest the use of fecal immunoassays. Both the O&P exams and fecal immunoassays are recommended for a laboratory test menu; both would be orderable, billable procedures. Refer to Table 5.2, which contains order recommendations based on the patient's history; sharing this table with your physician clients is highly recommended.*

4. How would stool immunoassay requests fit into a laboratory that also performs O&P exams?

*Any diagnostic laboratory performing routine parasitology testing should offer both routine O&P exams and fecal immunoassays. Both tests are recommended for a laboratory test menu; both would be orderable, billable procedures (refer to Table 5.2).*

5. What do you mean by the "routine O&P exam"?

*The routine O&P exam includes (for liquid or semiliquid fresh stools) a direct wet smear, the concentration, and the permanent stained smear; preserved specimens would require a concentration and permanent stained smear (no direct wet mount is required).*

6. How would you fit the fecal immunoassays into your laboratory?

*You could offer the routine O&P exam, and you could also offer on request the stool immunoassay as a separate option. Both options should be in the laboratory ordering test menu.*

7. What type of educational initiatives would have to be undertaken prior to offering these options?

*Physicians would need to know the pros and cons of ordering either the immunoassay or the routine O&P examination (refer to Table 5.2).*

8. What are some of the pros and cons for the fecal immunoassays?

*Refer to Section 5, "Comments on the Performance of Fecal Immunoassays."*

*Pros:*

- a. *Depending on the format selected, the immunoassays are fast and relatively simple to perform.*
- b. *The result can rule in or out some very specific organisms.*
- c. *If the patient becomes asymptomatic at the time the immunoassay is negative, additional testing may not be necessary.*
- d. *They may help reduce personnel costs (time to perform procedures).*

- e. The fecal immunoassays are more sensitive than the routine O&P exams and/or the special stains for the coccidia or microsporidia.

Cons:

- a. The fecal immunoassay kits test **only** for selected organisms.
  - b. Depending on the format, they might be somewhat complex to perform.
  - c. Test requests may not justify certain formats (cost, equipment, or training).
  - d. It is critical that the physician realize that a negative immunoassay does not rule out all possible parasitic etiologic agents causing diarrhea.
  - e. In tests for Giardia lamblia, fecal immunoassays on two different stool specimens may be required to get a positive result. This is not the case for other protozoa.
9. Can the fecal immunoassays be used for duodenal fluid (giardiasis)?  
*The tests have not been approved or validated for this type of specimen. Also, the duodenal fluid or aspirate would contain primarily the trophozoite form, not the cyst stage for which the reagents have been designed. As an example, if one is using the FA (rather than EIA or cartridge) the trophozoites may appear to be fluorescent at a very pale 1+ while the cysts are a strong 3± to 4+. So, while a few antigenic sites may be shared by the cysts and trophozoites, the commercial tests for Giardia are detecting the cyst antigens. You can try using the reagents on these specimens, but if the result is negative, the result in no way has "ruled giardiasis out." I would recommend testing stool only; if the result was positive, this approach might avoid the need for duodenal aspirate testing altogether.*
10. In the Giardia fecal immunoassay, how many specimens should be tested before assuming the patient is not infected?  
*Since the evidence indicates that Giardia is shed sporadically and that more than one immunoassay might be required to diagnose the infection, the recommendation is similar to that seen for stool collection for the routine O&P exam: perform the immunoassay on two different stools (assuming the first specimen is negative) collected within no more than a 10-day period—a good collection schedule would be day 1 and then day 3 or 4. That way, one of those collections should yield a positive result if the organisms (in sufficient numbers) are present. However, it is important to remember that if the patient is a carrier with a low organism load, even the second immunoassay might be negative.*
11. Although an FA for *Cryptosporidium/Giardia* was negative, the O&P concentrate showed *Giardia* trophozoites on the wet mount. Might we miss a *Giardia*-positive sample if only the FA is ordered? Also, do any of the antigen EIA detect both trophozoites and cysts, and should we switch to one of those?  
*Although the antibody in the immunoassay kits is to the cyst antigen (primarily, but it is a polyclonal reagent), in some of the kits the trophozoites*

do fluoresce, but much less intensely (around a 1+ or even a 2+, thus indicating some shared epitopes). In almost all patients, there is a combination of cysts and trophozoites, unless the patient has active diarrhea and is passing only trophozoites (no time for cysts to form with rapid passage through the gastrointestinal [GI] tract). However, most patients harbor cysts as well, and the results (if above the test limits for sensitivity) will be positive. The situation described in the question can happen, but it is probably not that common. However, with only trophozoites present, you may get a negative result. Some of the kits tend to provide a bit higher fluorescence with the trophozoite, but it varies.

12. After the patient has been treated, how long does the Giardia antigen test remain positive?

*It has been recommended that patients be tested about 7 days after therapy, hopefully to avoid picking up residual antigen. However, if you wait too long (several weeks), you always run the risk of picking up antigen from a possible reinfection. Some also feel that low antigen levels can be found for up to about 2 weeks. A good time frame for retesting would probably be about 7 to 10 days after therapy. If the first specimen at 7 days is still positive, then retesting at 10 to 12 days would be appropriate. Also, we know that the immunoassays (for diagnosis) may not pick up low antigen loads (organism shedding issues); therefore, the recommendation for diagnostic immunoassay testing for giardiasis has been changed and now involves performing immunoassay testing on one additional stool specimen (if the first one is negative). The testing on two different stool specimens should be performed within about 3 to 5 days.*

13. Why do fecal immunoassay kits that test for either *Entamoeba histolytica* or the *E. histolytica/E. dispar* group require fresh or frozen stools?

*Unfortunately, at present these reagents do not function properly on preserved fecal specimens. Although the manufacturers are trying to develop such kits, they are not yet available.*

14. How long does antigen survive in fresh stools?

*It is recommended that fresh stools be tested within 24 h of collection; they can be stored overnight in the refrigerator. They can also be frozen or preserved in 10% formalin before being tested (both freezing and formalin preservation methods preserve antigen for long periods, even years).*

## Organism Identification

### Protozoa

1. What is the most effective technique for the identification of the intestinal protozoa?

*Although some protozoan cysts can be seen and identified on the wet preparation smear (direct mount, concentration sediment wet mount), the permanent stained smear is recommended as the most relevant and accurate procedure for identification of the intestinal protozoa. These preparations*

*are examined using the oil immersion objective (100 $\times$ ) for a total magnification of  $\times 1,000$ . At least 300 oil immersion fields should be examined prior to reporting the result of the permanent stained smear.*

2. Are trophozoites ever seen in the wet mounts of stool?

*Usually the trophozoites are not seen in the concentration sediment wet mount preparations unless they are prepared from SAF-fixed material. Motile trophozoites are occasionally seen in the direct wet smear, but this is rare.*

3. What are some of the tips to consider when reporting *Entamoeba hartmanni*?

*The measurements of 10  $\mu\text{m}$  or less for the *Entamoeba hartmanni* cyst refer to wet preparation measurements, so the measurement should be decreased  $\sim 1 \mu\text{m}$  on the permanent stained smear. When you see a cyst on the permanent stained smear, there is often a halo representing shrinkage. The cyst must be measured to include that halo. On the bench, the measurements for this cyst generally run from around 9.5 down to about 8  $\mu\text{m}$  and they are morphologically definitely *E. hartmanni*. The *E. hartmanni* cyst generally contains more chromatoidal bars than are seen in *E. histolytica*/*E. dispar*. Also, the *E. histolytica*/*E. dispar* cysts tend to measure routinely on the bench (trichrome slides) from about 10.5 up to about 13  $\mu\text{m}$ . Also, on the bench, the *E. hartmanni* nuclei, particularly in the trophozoite, tends to look like a "bulls eye," consisting of a very sharp line of nuclear chromatin with the karyosome right in the middle.*

4. Do nonpathogenic protozoa ever cause symptoms?

*Endolimax nana, Iodamoeba bütschlii, Chilomastix mesnili, and Pentatrichomonas hominis (as examples) have been categorized as nonpathogens. Patients have been documented to be symptomatic when infected with a nonpathogen, although this is rare. However, it is sometimes difficult to determine from the case history the extent of the workup, including coccidia and the microsporidia. Before assigning symptoms to nonpathogenic protozoa, a comprehensive search for other proven pathogens should be performed.*

5. What color is the autofluorescence seen with *Cyclospora cayetanensis*?

*The color depends on the particular FA filters used. If the filters are used for calciofluor white, the oocysts appear as pale blue rings; if the filters are used for fluorescein isothiocyanate, the oocysts appear to be more yellow-green. Fluorescence intensity varies from about 1+ to 2+; it is rare to see stronger autofluorescence with these oocysts.*

## **Helminths**

1. Why can't all helminth eggs be recovered using the flotation concentration rather than the sedimentation concentration?

*Some helminth eggs are quite heavy and will not float, even when zinc sulfate with a specific gravity of 1.20 is used. Other helminth eggs are operculated; when the egg is placed in a high-specific-gravity solution, the*

*operculum "pops" open and the egg fills with fluid and sinks to the bottom of the tube. Thus, both the surface film and the sediment should be examined before the specimen is reported as negative.*

2. Why must helminth larvae be identified to the species level? Shouldn't all larvae recovered in stool be those of *Strongyloides stercoralis*?

*Although helminth larvae in stool are normally *Strongyloides stercoralis*, there is always the possibility that hookworm eggs have continued to mature in fresh stool and may hatch before the stool is processed and/or placed in fixatives. It is important to make sure that the larvae seen are, in fact, the rhabditiform (noninfectious) larvae of *S. stercoralis* rather than larvae of hookworm. The agar plate culture is the most sensitive method for the recovery of *S. stercoralis* larvae. Also, migrating larvae could also be recovered from respiratory specimens (sputum, bronchoalveolar lavage fluid, etc.).*

3. Are there any specific recommendations for the detection of schistosome eggs?

*When trying to diagnose schistosomiasis, regardless of the species suspected you should be examining both stool (several different stool specimens) and urine (spot urine samples plus a 24-h specimen), collected with no preservatives. Occasionally adult worms get into blood vessels where they are not normally found (for example, *S. mansoni* eggs found in urine only rather than stool, the more common specimen). When you perform a sedimentation concentration, you should use saline to prevent premature egg hatching. Once you are ready to try a hatching procedure, you can put the sediment into spring water (dechlorinated water) to stimulate hatching. When examining wet mounts under the microscope, you need to look for the movement of cilia on the larvae within the eggshell (hence the need to collect the specimens with no preservatives). You should be able to tell the physician whether the eggs, if present, are viable or if you see only dead eggshells. If you suspect schistosomiasis, it is recommended that you examine a number of wet mounts, particularly if you are not going to perform a hatching test.*

4. What is the most sensitive test for the diagnosis of strongyloidiasis?

*Agar plate cultures are recommended for the recovery of *S. stercoralis* larvae and tend to be more sensitive than some of the other diagnostic methods such as the O&P exam. Stool is placed onto agar plates, and the plates are sealed to prevent accidental infections and held for 2 days at room temperature. As the larvae crawl over the agar, they carry bacteria with them, thus creating visible tracks over the agar. The plates are examined under the microscope for confirmation of larvae, the surface of the agar is then washed with 10% formalin, and final confirmation of larval identification is made via wet examination of the sediment from the formalin washings (see Section 5).*

*In a study of the prevalence of *S. stercoralis* in three areas of Brazil, the diagnostic efficacy of the agar plate culture method was as high as 93.9% compared to only 28.5% and 26.5% by the Harada-Mori filter paper culture and fecal concentration methods, respectively, when fecal spec-*

imens were processed using all three methods. Among the 49 positive samples, about 60% were confirmed as positive by using only the agar plate method. These results indicate that the agar plate approach is probably a much more sensitive diagnostic method and is recommended for the diagnosis of strongyloidiasis.

It is important to remember that more than half of *S. stercoralis*-infected individuals tend to have low-level infections. The agar plate method continues to be documented as a more sensitive method than the usual direct smear or fecal concentration methods. A daily search for furrows on agar plates for up to six consecutive days results in increased sensitivity for diagnosis of both *S. stercoralis* and hookworm infections. Also, a careful search for *S. stercoralis* should be made for all patients with comparable clinical findings before a diagnosis of idiopathic eosinophilic colitis is made, because consequent steroid treatment may have a fatal outcome by inducing widespread dissemination of the parasite.

5. Where can one get serologies for a *Baylisascaris procyonis* infection?

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## Reporting

### Organism Identification

1. Should common or scientific names be used when reporting the presence of parasites?

*The scientific (genus and species) names should be used on the final report that goes to the physician and on the patient's chart. It is also recommended that the stage of the organism be included (trophozoite, cyst, oocyst, spore, egg, larvae, adult worm); various stages of the malaria parasites affect the outcome of therapy.*

2. What happens if several different names are used in the literature (e.g., *Giardia lamblia*, *G. intestinalis*, *G. duodenalis*)?

*It is appropriate to use the most commonly accepted name (*Giardia lamblia*); you can also let the proficiency testing organism lists be your guide. When a replacement name begins to appear on the proficiency test list, then it's appropriate to notify your clients and institute the name change. Remember, it is very important to notify all clients prior to making any name changes.*

3. How has the reporting of *Cryptosporidium parvum* changed and why?

*It is now known that more than one species of *Cryptosporidium* can cause disease in humans (*C. parvum* infects humans and other mammals, while *C. hominis* infects only humans). However, the different species cannot be*

*differentiated on the basis of morphology. It is now recommended that the more correct reporting format would be Cryptosporidium spp. (rather than Cryptosporidium parvum).*

4. Should WBCs and/or other cells or yeast be reported and why?

*The reporting and quantitation (rare, few, moderate, many) of WBCs (polymorphonuclear leukocytes [PMNs], macrophages, eosinophils) provide some additional information for the physician. If the patient continues to have diarrhea, this may give the physician something more to consider, particularly if a stool culture has not been ordered. Conditions related to noninfectious diarrheas may also result in WBCs and macrophages in the stool. We always include this type of information/explanation in our educational process regarding reporting formats. Physicians can then decide on the relevance of the information. When reporting WBCs, we can also identify eosinophils from the permanent stained smear (this may or may not be related to parasitic infection and may or may not correlate with peripheral eosinophilia); this information may also be helpful.*

*Reporting yeast infections is a bit different. To report anything about yeast, it is important to know that the stool was fresh or immediately put in preservative. If there are large numbers of yeast, budding yeast, and/or pseudohyphae, this provides some additional information for the physician, often depending on the patient's general condition and whether the patient is immunosuppressed. However, if you do not know whether the collection criteria were met, reporting anything about yeast is NOT recommended since this type of report may be misleading.*

5. How should *Blastocystis hominis* be reported?

*In the past there was some agreement that the number of organisms present (moderate, many, packed) was more likely to be associated with symptoms. However, in the past few years, there have been anecdotal reports of patients being symptomatic after infection with rare or few organisms. The current recommendation is to report the organism and quantitate as rare, few, moderate, many, packed (using your proficiency testing quantitation scheme). It is important to confirm that the physicians know what the report means and understand the controversial issues surrounding this organism; they can then correlate the numbers with symptoms. If the organisms are rare or few, there are no solid data on the relevance of numbers other than the anecdotal case reports (many of which are word of mouth). There is still a lot of controversy about pathogenicity. However, many physicians are treating patients who are symptomatic and in whom no other organisms are found (including coccidia and/or microsporidia).*

*Blastocystis hominis may actually be a mix of several strains, some of which are pathogenic and some of which are nonpathogenic. Based on continuing molecular biology studies, the classification of this group could change soon. If both types of strains currently make up what is called "Blastocystis hominis," this could explain the controversy regarding pathogenicity and the fact that some patients are symptomatic while some are asymptomatic.*

**6. How should intestinal protozoa be reported?**

*Reporting trophozoites and cysts has been the accepted way of reporting intestinal protozoa for a couple of reasons. (i) Different drugs are used to treat E. histolytica cysts and trophozoites. (ii) Since the cyst form is the infective stage for the protozoa (except for D. fragilis and the trichomonads), the report does convey some epidemiological information. For these reasons, the current recommendation is to continue to report all protozoa (genus, species, and stage).*

**7. What additional reporting comments should be added to the reporting of the O&P examination and the fecal immunoassays?**

*It is important to add the following comment to the O&P examination: "The O&P examination is not designed to detect the intestinal coccidia (Cryptosporidium spp., Cyclospora cayetanensis) or the microsporidia. Isospora belli oocysts (coccidia) can be detected from the concentration sediment examination."*

*The results of the fecal immunoassays should be reported based on the specific organism(s) relevant to the kit:*

*"No Giardia lamblia antigen detected." OR "Giardia lamblia antigen present."*

*"No Giardia lamblia or Cryptosporidium spp. antigen detected."*

*OR*

*"Negative for Giardia lamblia."*

*"Negative for Giardia lamblia and Cryptosporidium spp."*

## **Quantitation**

**1. What organisms should be quantitated in the final report to the physician and patient's chart?**

*Organisms and nonorganism cells and other structures (stool blood cells, Charcot-Leyden crystals) that are recommended for quantitation include the following:*

- a. *Intestinal protozoa: Blastocystis hominis*
  - b. *Helminths: Trichuris trichiura eggs, Clonorchis sinensis eggs, schistosome eggs (also report the viability of Schistosoma eggs).*
  - c. *Blood parasites: all malaria organisms and Babesia spp.*
  - d. *Blood cells (PMNs, macrophages, RBCs)*
  - e. *Charcot-Leyden crystals*
- 2. Why are all proficiency testing specimen answers reported and quantitated, while clinical specimen reports are rarely quantitated in terms of organism numbers?**

*There are two issues to consider.*

*(i) When reporting proficiency testing specimen results, you are asked to quantitate organisms in both the formalin wet mounts and permanent stained smears. This information serves as a quality control check for the proficiency testing agency to ensure that organism numbers are consistent throughout the challenge vials/slides.*

(ii) Most laboratories (with few organism exceptions) do not quantitate organisms on the concentration or permanent stained smear reports. One exception is *Trichuris trichiura* eggs in a concentration wet mount (light infections might not be treated). Since many organisms are shed on a random basis, quantitation may change dramatically from day to day and generally has little clinical relevance.

## Proficiency Testing

### Wet Preparations

1. How should you examine a wet preparation for proficiency testing?

*Most directions recommend that you shake the vial and take a sample from the mixed vial contents. However, if only a few eggs are present, you may be better off taking a very small drop from the settled material. If you take too large a drop, it will be too thick to examine properly. Make sure you do not add too much iodine; very darkly stained helminth eggs can resemble debris.*

2. Are there any "tips" regarding the microscope set up for the examination of an unstained wet preparation?

*The microscope should be aligned properly using Köhler illumination. Make sure the light is not too bright; otherwise, you may shine right through the organisms and miss the parasites. Also, you may want to close the diaphragm a bit to provide a bit more contrast. Extra contrast is particularly important when reading with saline only (no iodine added).*

### Permanent Stained Smears

1. How should you examine a permanent stained fecal smear for proficiency testing?

*Make sure the light is very bright (with the condenser all the way up) and the diaphragm is open. Review both thin and thick areas of the smear; examine at least 300 oil immersion fields (using the 100 $\times$  oil immersion objective) before you report the specimen as a negative. If you use the 50 $\times$  or 60 $\times$  scanning oil immersion lens, you still need to review 300 oil fields as indicated above (using the 100 $\times$  oil immersion objective).*

### Tissues or Fluids

1. How should tissues be submitted to the laboratory?

*Tissue specimens should be immediately sent to the laboratory and kept moist during transit. If the tissue is to be processed for culture (*Acanthamoeba*, *Naegleria*, *Leishmania* spp., *Trypanosoma* spp., or *Toxoplasma gondii*), the specimen should be kept sterile and submitted in a sterile container. If the tissue is to be processed for wet examinations and permanent stained smears, any type of container is acceptable. Remember that the specimen must be kept moist; if it dries out in transit, neither culture nor stained smears will be acceptable.*

2. How should eye specimens be submitted for *Acanthamoeba* culture?  
*If you are sending the specimen in a screw cap tube or vial (or other capped container), make sure that the container is filled to the top with transport fluid (saline is acceptable). This will prevent the small tissue specimen from drying out if it is shaken onto the side of the container during transit. If the container is not full of fluid, the tissue may dry out and will be unacceptable for culture.*
3. How should duodenal drainage specimens be handled?  
*Duodenal drainage specimens must be submitted to the laboratory as quickly as possible for processing. Delays may prevent organism recovery and identification. These specimens may be very liquid and may contain a lot of mucus. It is best to centrifuge the tube, discard any supernatant fluid left, and examine the mucus only as wet preparations (low light) and/or permanent stained smears (trichrome, iron hematoxylin).*

## Blood

Many questions have been asked regarding various aspects of diagnostic medical parasitology and the examination of blood specimens. Answers to these questions may be helpful for those working in this area of microbiology and/or hematology and may provide some "tips of the trade" that are learned through many years of bench experience.

### Specimen Collection

1. If *Plasmodium falciparum* parasites are sequestered in the capillaries, why not do a finger stick rather than a venipuncture?  
*The capillaries are generally in the deep tissues (spleen, liver, and bone marrow), so a finger stick blood sample is no more likely to be positive than a venipuncture blood. There may be some differences with *P. falciparum*, but not sufficient to warrant eliminating venipunctures. The anticoagulant tube also provides additional blood for multiple thick and thin blood films and/or buffy coat films.*
2. What is the best anticoagulant to use for blood specimens?  
*Although heparin (green top) or EDTA (lavender top) can be used, EDTA is recommended as providing better organism morphology, particularly for *Plasmodium* spp. Blood collected using EDTA anticoagulant is acceptable; however, if the blood remains in the tube for >1 to 2 h, true stippling may not be visible within the infected RBCs (*Plasmodium vivax*, *P. ovale*). Also, when anticoagulants are used, the proper ratio between blood and anticoagulant is necessary for good organism morphology. The lavender-top tube should be filled to the top with blood to provide the proper blood/anticoagulant ratio. Although heparin can be used, EDTA is preferred. Finger stick blood is recommended when the volume of blood required is minimal (i.e., when no other hematologic procedures have been ordered). The blood should be free-flowing when taken for smear preparation and should not be contaminated with alcohol used to clean the finger prior to the stick. However, finger stick blood is no longer commonly used in many parts of*

the world. When blood is collected in EDTA, specimens should be processed immediately after blood collection. Parasite numbers may decrease if processing is delayed, even 4 to 6 h. Adhesion of the blood to the slide can be a problem if the ratio of anticoagulant to blood is high, the patient is anemic, or the blood was held in EDTA too long.

Organism	Morphology (normal)	Morphology (EDTA) contact with EDTA for >1–2 h
<i>Plasmodium falciparum</i>	Rings Typical, small to medium rings; double rings/cell	Numbers tend to remain constant.
	Gametocytes Crescent-shaped	Gametocytes may round up and be confused with other species.
<i>Plasmodium vivax</i>	Trophozoites Ameboid trophozoites, for both early and late rings	May round up and lose their characteristic shape
	Schüffner's dots Typical dots appear in late rings; present throughout the rest of the life cycle stages	Stippling (Schüffner's dots) may not be visible at all, in any of the life cycle stages (regardless of buffer pH used). Note: If slides are prepared soon after collection in EDTA, the dots will be seen after staining; however, if slides are prepared after the blood has been in contact with EDTA for several hours, the dots may not be visible after staining.
<i>Plasmodium spp.</i> Male gametocytes	No exflagellation	As blood cools and becomes oxygenated, the parasites "think" they are now in the mosquito (this cycle may continue). The male gametocyte may exflagellate; microgametes may resemble <i>Borrelia</i> (this is also related to pH and pCO <sub>2</sub> , e.g., when the lid is left off the tube).

### 3. Why do new slides have to be cleaned with alcohol prior to use?

Even new slides are coated with a very fine layer of oil (to allow the slides to be "pulled apart" from each other). If the coating is removed, the blood will flow more smoothly over the glass during blood film preparation.

"Holes" in the blood film are evidence of oil or grease on the slide. **Use Standard Precautions:** remember that both slides and spreaders must be held on the edges and not on any part of the slide that will come in contact with blood.

4. When should blood specimens be drawn for a suspect malaria diagnosis?

The majority of patients with malaria in the United States have never been exposed to the organism before; therefore, they have no antibody and when they present they do NOT have a synchronized fever cycle. These immunologically naive patients may present with nonspecific symptoms that can mimic many other diseases. The rule of thumb is to draw immediately; do not wait for some "magic" periodic cycle that may never appear. Patients with a very low parasitemia with *P. falciparum* can become quite ill before they have any type of fever cycle or gametocytes. For patients who are suspected of having malaria or who have a fever of unknown origin, blood should be drawn and both thick and thin blood films should be prepared and examined immediately. This request is always considered a STAT request. In areas of the world where malaria is endemic and people have been exposed to the parasite before (and therefore have some antibody), they may not become symptomatic until they actually have some sort of periodic fever cycle. However, you should always use the general guideline to draw immediately. Also, remember that one set of negative blood films does not rule out malaria. If the first set (both thick and thin films) is negative, you can recommend that additional blood films be drawn in about 4 to 6 h. Also, any decision to delay treatment should be left to the physician, not the laboratory.

## Specimen Processing

1. Why is it important that the EDTA-blood be processed as quickly as possible?

If a tube of blood containing EDTA cools to room temperature and the cap has been removed, several parasite changes can occur. The parasites within the RBCs will respond as if they were now in the mosquito after being taken in with a blood meal. The morphology of these changes in the life cycle and within the RBCs can cause confusion when blood films prepared from this blood are examined.

- a. Stippling (Schüffner's dots) may not be visible.
- b. The male gametocyte (if present) may exflagellate.
- c. The ookinetes of *Plasmodium* spp. other than *P. falciparum* may develop as if they were in the mosquito and may mimic the crescent-shaped gametocytes of *P. falciparum*.
- d. Smears left longer than 24 h can autofix; if this occurs, lysis of the RBCs will be difficult, if not impossible, to achieve. This autofixation tends to occur more quickly in warm, humid climates.

- e. Thick smears can be dried in a 37°C incubator for 10 to 15 min without fixation of the RBCs; do not go beyond 15 min when using this method.
2. Why is it important to keep thick films from getting hot (heat fixation)?

*Heat fixes the RBCs, and they subsequently do not lyse in the staining process (see 1e above).*

3. What should you do if you have blood films that can't be stained for several days?

*If staining with Giemsa is delayed for more than 3 days or if the film is to be stained with Wright's stain, lyse the RBCs in the thick film by placing the slide in buffered water (pH 7.0 to 7.2) for 10 min, remove it from the water, and place it in a vertical position to air dry. The laked thick films and thin films should be dipped in absolute methanol and placed in a vertical position to air dry. This is particularly important if the blood films are to be stored for days or weeks before being stained.*

## Diagnostic Methods

1. Why is it important to always examine both thick and thin blood films prior to reporting the specimen as negative for blood parasites?

*Thick blood films allow a larger amount of blood to be examined, which increases the possibility of detecting light infections. However, only experienced workers can usually make a species identification from a thick film, particularly for malaria parasites. The morphologic characteristics of blood parasites are best seen in thin films.*

2. What are the advantages of the thick blood film?

*The purpose of using a thick film is to have a drop of blood with 20 or 30 layers of RBCs on the slide, then to lyse the RBCs, wash off the hemoglobin, and stain the parasites, which remain intact in the process. As a consequence, RBCs are normally not visible, but WBCs and parasites may be seen. It is essential to become familiar with the characteristics of malaria parasites and know what to look for on such a preparation. An experienced microscopist should be able to detect 20 parasites per  $\mu\text{l}$  of blood (i.e., a parasitemia of 0.0001%) after examining 100 fields in 10 min. A greater volume of blood can be examined in the same time as that taken for examination of the thin blood film. The presence of phagocytized malaria pigment within WBCs (particularly in cases with low levels of parasitemia) can be very helpful. Occasionally, one can see Schüffner's dots in a thick film.*

3. What are the disadvantages of the thick blood film?

*Due to lysis of the RBCs during processing of thick blood films, the thick film cannot be used to compare the size of the parasite within the RBCs or to compare the size of the infected RBCs to the uninfected RBCs. Rec-*

ognition of organism distortion and identification to the species level are generally more difficult from the thick film.

4. What are some of the problems associated with thick blood films?

*The thick film flakes off during the staining process.*

- a. *The film was not dry.*
- b. *The film did not dry evenly.*
- c. *The film was too thick (refer to the combination thick-thin blood film protocol).*
- d. *The blood was too diluted with anticoagulant or the patient was anemic (in this case, centrifuge blood at  $500 \times g$  for 1 min and repeat the thick film).*
- e. *The slides were greasy or dirty.*
- f. *The blood was in EDTA too long (prepare thick films thinner than normal; dry for a longer period).*

*The thick film does not stain adequately.*

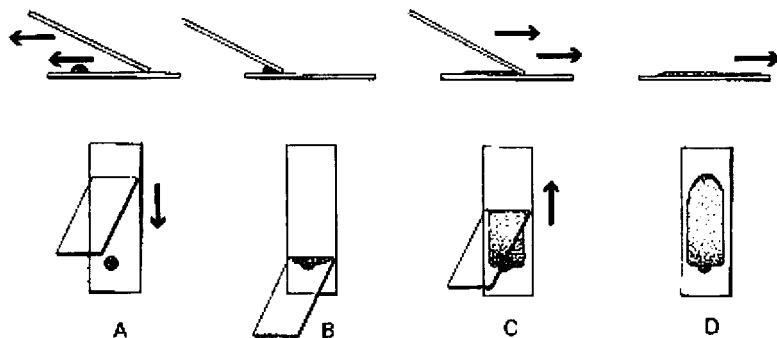
- g. *The film was too thick.*
- h. *The staining solution was too dilute or the staining time was too short.*

5. What are some of the tips for improving thick blood films?

*After the thick films are dry, fix the smear with acetone for a few seconds (only dip twice), then dry the film. This approach improves the durability of the thick film, and does not interfere with the lysis of the RBCs. These films tend to have a clean background, making the parasites easier to see. Overall adherence to the slide is enhanced by using this approach.*

6. How do you prepare thin blood films?

*Keep the spreader at about a 40 to 45° angle to allow blood to flow to almost the edge of the slide. Lower the angle to about 30 to 35° to pull the film in one smooth motion (Figure 6.1). The finished blood film should appear as a feathered edge in the center of the slide, with a free margin*



**Figure 6.1** The traditional method for preparing a thin blood film; the blood can be either “pushed” or “pulled” by the spreader slide. (Illustration by Sharon Belkin.)

**on either side.** Before fixing the thin blood films in absolute methanol, the film must be completely dry. If slides must be stored unfixed, they should be frozen. If fixation lasts too long (>30 to 45 s), stippling may be reduced. If fixation is too short, RBCs may be distorted (crescent-shaped) or partially lysed. During fixation it is better to use a dispensing bottle for methanol, rather than a Coplin jar, in which the methanol will pick up water from the air. Methanol used on one day (Coplin jar) should not be reused the next day; it is important to begin with fresh stock.

7. What are the advantages of the thin blood film?

The RBC morphology can be seen, as well as the size of the parasite with respect to the infected RBC in which the parasite resides. The sizes of the infected RBCs can be compared to that of the uninfected RBCs. It is much easier to identify malaria organisms to the species level by using the thin blood film. The parasitemia can be calculated much more easily from the thin blood film than the thick film.

8. What are the disadvantages of the thin blood film?

The thin blood film has a much lower sensitivity than does the thick blood film; thus, infections with a low parasitemia may be missed.

9. What are some of the problems associated with thin blood films?

Films are too thick.

- a. Too large a drop of blood was used.
- b. The RBCs may be crowded together with no thin feathered edge.
- c. The film was prepared too quickly or with too large an angle >30–35°.

Films are too thin.

- d. Blood from anemic patients does not spread well; it should be spread more quickly. Allow the blood to settle or centrifuge, remove some plasma, and repeat the thin-film preparation procedure.
- e. The angle between the spreader and slide was too small (<30°).

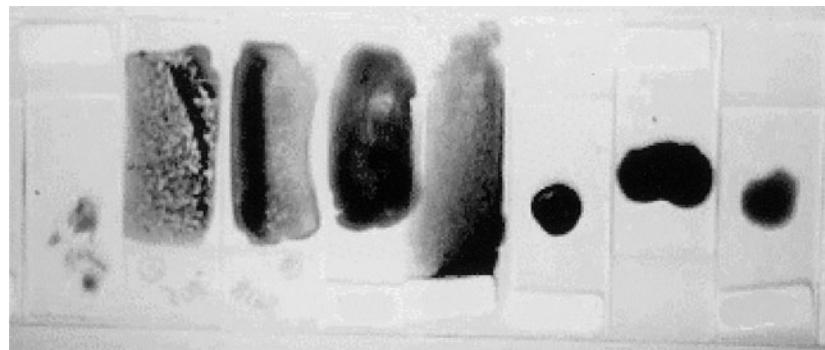
Smears have ragged edges.

- f. The drop of blood may have been in front of the spreader.
- g. The slides were greasy or dirty.
- h. The spreader slide was chipped.
- i. The spreader slide was reused (a new slide should be used each time).
- j. If the blood contained no anticoagulant, a delay in spreading may have led to fibrin formation, causing streaking.
- k. The problem may be due to a high plasma fibrinogen level and not to the technique.

Poorly prepared thick and thin blood films can be seen in Figure 6.2.

10. How does one prepare a combination thick-thin blood film?

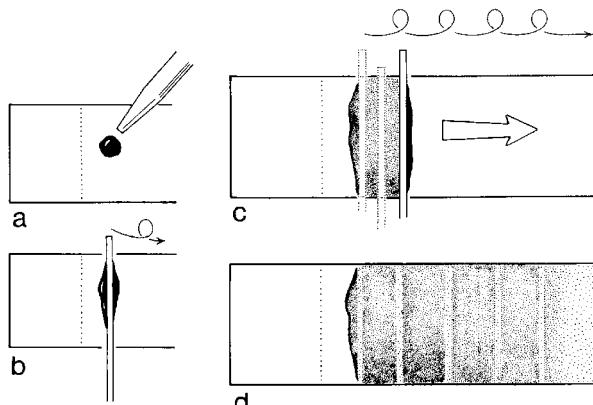
The combination thick-thin blood film provides both options on one glass slide and the slide can be stained as either a thick or a thin blood film



**Figure 6.2** Poorly prepared thin and thick blood films (dirty slides, oil on slides, excessively thick preparations, poor spreading of the blood); organism morphology is very poor on the stained films.

(Figure 6.3). If the film is fixed prior to being stained, the smear will be read as a thin blood film; if RBCs are lysed during staining, the preparation will be read as a thick blood film (parasites, platelets, and WBCs). This combination blood film dries more rapidly than the traditional thick blood film, thus allowing staining and examination to proceed with very little waiting time for the slide(s) to dry.

- Place a drop (30 to 40  $\mu\text{l}$ ) of blood onto one end of a clean slide about 0.5 in. from the end.
- Using an applicator stick lying across the glass slide and keeping the applicator in contact with the blood and glass, rotate (do not "roll")



**Figure 6.3** Method of thick-thin combination blood film preparation. (a) Position of the drop of EDTA-containing blood. (b) Position of the applicator stick in contact with blood and the glass slide. (c) Rotation of the applicator stick. (d) Completed thick-thin combination blood film prior to staining. (Illustration by Sharon Belkin.)

*the stick in a circular motion while moving the stick down the glass slide to the opposite end.*

- c. *The appearance of the blood smear should be alternate thick and thin areas of blood.*
- d. *Immediately place the film over some small print and be sure that the print is just barely readable.*
- e. *Allow the film to air dry horizontally and protected from dust for at least 30 min to 1 h. Do not attempt to speed the drying process by applying any type of heat, because the heat will fix the RBCs and they subsequently will not lyse in the staining process.*
- f. *This slide can be stained as either a thick or thin blood film.*

11. How should malaria smears be stained with Giemsa stain?

*Giemsa is a mixture of eosin and methylene blue. Stock solutions of Giemsa can be purchased commercially. Some brands are better than others. The stock solution of Giemsa stain is easily prepared from commercially available Giemsa powder.*

*Stock solutions of Giemsa stain must always be diluted by mixing an appropriate amount of stain with distilled neutral or slightly alkaline water; buffered saline is preferred because it provides a cleaner background and better preservation of parasite morphology. Although most people do not filter the working stain prior to use (if using a Coplin jar), results are better overall if the working stain is filtered through Whatman no.1 filter paper immediately before use. The working stain is stable for 1 day. Make sure to use absolute methanol (acetone free) for fixing thin blood films. Stock buffered water is stable for 1 year at room temperature. Working-stock buffered water is stable for 1 month at room temperature. Stock Giemsa stain is stable at room temperature indefinitely; stock stain appears to improve with age (similar to iron hematoxylin stains). A 45- to 60-min staining time appears to work better than 15 min; staining times depend on stain dilution. Some workers feel that the use of a 10% Triton solution is helpful, while some feel that it is detrimental to the overall quality.*

12. How should you handle a delay between thick-film preparation and staining?

*Thick films can be preserved, particularly if there will be a delay prior to staining. Dip the slides in a buffered 0.65% methylene blue solution for 1 to 2 s, and allow them to dry.*

- a. *Methylene blue ..... 0.65 g  
Disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) ..... 2.0 g  
Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) ..... 0.65 g  
Distilled water ..... 1.0 liter*

*Mix and store in a stoppered brown bottle.*

*The benefits of such an approach are as follows:*

- b. The RBCs are hemolyzed.
  - c. Color is introduced into the cytoplasm of the parasites.
  - d. Prestaining with methylene blue helps preserve the organisms on the smear.
13. Can blood stains other than Giemsa stain be used to stain the blood films?

*Detection of parasites in the blood has been made possible by the use of Romanovsky-type differential stains which selectively color the nuclear material red and the cytoplasm blue. This reaction takes place under optimal pH conditions: rather than using a neutral pH, optically perfect results are obtained using the slightly basic pH of 7.2 (due to the optical dominance of red pigmentation). Although Giemsa stain has been the stain of choice for many years, the parasites can also be seen on blood films stained with Wright's stain, a Wright-Giemsa combination stain, or one of the more rapid stains such as Diff-Quik (American Scientific Products, McGraw Park, IL), Wright's Dip Stat Stain (Medical Chemical Corp., Torrance, CA), or Field's stain. It is more appropriate to use a stain with which you are familiar than to use Giemsa, which is somewhat more complicated to use, if you are not familiar with it. PMNs serve as the quality control (QC) organism for any of the blood stains. Any parasites present stain like the PMNs, regardless of the stain used. Also, the CAP checklist does not mandate the use of Giemsa stain.*

14. How should malaria blood films (both thick and thin films) be examined?

*A minimum of 300 oil immersion fields should be examined using the 100× objective. The blood film can be scanned using a 50× or 60× oil immersion lens, but final reporting of the results should be based on the use of the 100× oil immersion lens for a total magnification of ×1,000.*

15. What type of QC slides should be used for blood parasite work?

*Regardless of the stain you are using (Giemsa, Wright's, Wright-Giemsa, rapid stains), your QC slide is the actual slide you are staining. This approach to QC is acceptable to CAP as well. Any parasites present stain like WBCs, so your QC is built into the system. Another good source of teaching slides is Meridian Bioscience, Inc. This company currently holds the CAP contract for proficiency testing and has extra slides and specimens available for sale. Since these have been reviewed for CAP proficiency testing, the quality is good. Contact them for a brochure: (800) 543-1980 ext. 335 or [jross@meridianbioscience.com](mailto:jross@meridianbioscience.com).*

## **Organism Identification**

1. Why is it so important to rule out infections with *P. falciparum*?

*P. falciparum causes more serious disease than the other species (*P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*). *P. falciparum* tends to invade all ages of RBCs, and the proportion of infected cells may exceed 50%.*

*Schizogony occurs in the internal organs (spleen, liver, bone marrow, etc.) rather than in the circulating blood. Ischemia caused by the plugging of vessels within these organs by masses of parasitized RBCs causes various symptoms, depending on the organ involved.*

*The onset of a P. falciparum malaria attack occurs 8 to 12 days after infection and is preceded by 3 to 4 days of vague symptoms such as aches, pains, headache, fatigue, anorexia, or nausea. The onset is characterized by fever, a more severe headache, and nausea and vomiting, with occasional severe epigastric pain. There may be only a feeling of chilliness at the onset of fever. Periodicity of the cycle will not be established during the early stages, and the presumptive diagnosis may be totally unrelated to a possible malaria infection. If the fever does develop a synchronous cycle, it is usually a cycle of somewhat less than 48 h. An untreated primary attack of P. falciparum malaria usually ends within 2 to 3 weeks. True relapses from the liver do not occur, and after a year, recrudescences are rare. Severe or fatal complications of P. falciparum malaria can occur at any time during the infection and are related to the plugging of vessels in the internal organs; the symptoms depend on the organ(s) involved. Note: The primary objective when performing a blood film examination for parasites is to "rule out P. falciparum."*

2. What are some of the problems associated with the differentiation between *Plasmodium* spp. and *Babesia* spp.? Can *Babesia* spp. cause serious infections?

*The ring forms of all five species of Plasmodium can mimic the ring forms of Babesia spp. Multiple rings per cell are more typical of P. falciparum than of the other species causing human malaria. Babesia rings are often numerous and smaller and tend to be very pleomorphic, while those of P. falciparum tend to be fewer and more consistent in size and shape. Remember that P. falciparum rings may appear somewhat larger if the blood has been drawn in EDTA and there is any lag time prior to thick- and thin-film preparation. Differentiation between Plasmodium spp. and Babesia spp. may be impossible without examining a thin blood film (rather than a thick blood film). Often, the parasitemia in a Babesia infection is heavier than that in a P. falciparum infection, particularly when a patient presents early in the infection. It is also important to remember that some of the Babesia spp. can cause severe illness (B. divergens from Europe, etc. [42% mortality], Babesia from California, Oregon, Washington, Missouri), while some infections such as those due to B. microti cause less serious or sub-clinical infections. However, any of the Babesia spp. can cause severe disease in immunocompromised patients, particularly patients who have undergone splenectomy.*

3. What is the significance of finding only ring forms on two sets of blood films drawn 6 h apart?

*Remember that all of the life cycle stages (rings, developing trophozoites, early schizonts, late schizonts, mature schizonts, and gametocytes) can be seen on the blood films in infections with P. vivax, P. ovale, P. malariae, and P. knowlesi. Due to the unique characteristics of the life cycle, only*

*rings and gametocytes (and occasional mature schizonts) are seen in the peripheral blood of a patient with a P. falciparum infection. Therefore, if you see two sets of blood films (collected 6 h apart) that contain ring forms only, there is an excellent chance that the patient is infected with P. falciparum, which causes the most serious infections of the five Plasmodium spp.*

4. Why aren't gametocytes of *P. falciparum* seen in many patients presenting to the Emergency Room?

*Many patients present to the emergency room (ER) early in the infection, prior to the formation of the gametocytes; thus, the diagnosis must be made on the basis of seeing the ring forms only. As you can imagine, identification of Plasmodium organisms to the species level can be very difficult when only rings are present. These patients tend to be travelers who have had no prior exposure to *P. falciparum* (immunologically naive persons) and who become symptomatic very early after being infected. It normally takes approximately 10 days for the crescent-shaped *P. falciparum* gametocytes to form.*

## Reporting

1. Why is it important to identify malaria organisms to the species level?

*Since *P. falciparum* can cause severe disease and death, it is very important for the physician to know whether this infection can be ruled out. It is also important to know if any of the other four species are present, particularly *P. vivax* or *P. ovale*, which would require therapy for both the liver and RBC stages due to potential relapse from the liver stages. It is also important because of potential drug resistance (chloroquine resistance of *P. falciparum* and *P. vivax*; primaquine tolerance or resistance of *P. vivax* [rare but documented]).*

2. How should a positive malaria blood film be reported?

a. *Using the thin-blood-film method, report the percentage of parasite-infected RBCs per 100 RBCs counted.*

*Example:* Plasmodium falciparum, parasitemia = 0.01%

b. *Using the thick/thin-blood-film method, report the number of parasites per microliter of blood.*

c. *Example:* Plasmodium falciparum, parasitemia = 10,000 per  $\mu\text{l}$  of blood

3. How should results be reported if *Plasmodium* spp. parasites are seen but *P. falciparum* infection cannot be ruled out?

*It is important to convey to the physician that *P. falciparum* cannot be ruled out; therapy may be initiated on the assumption that this species might be present. The report should read, "Plasmodium spp. present; unable to "rule out" Plasmodium falciparum."*

4. How often do mixed infections occur, and how should they be reported?

*It is important to remember that mixed infections are much more common than suspected and/or reported. When rings are present, along with other*

*developing stages (P. vivax, P. ovale, P. malariae, P. knowlesi), always look for the presence of two populations of ring forms, one of which might be P. falciparum! The report should read, Plasmodium spp. present, possible mixed infection; unable to "rule out" P. falciparum. Another report example might be, "Plasmodium vivax rings, developing schizonts, and gametocytes; possible mixed infection: unable to "rule out" P. falciparum.*

5. Why is it important to report the *Plasmodium* stages seen in the blood films?

*Patients who have been diagnosed with Plasmodium infection and who are not suspected of having drug-resistant malaria (P. falciparum, P. vivax) are often treated with chloroquine. Chloroquine does not eliminate any gametocytes present, and there are mosquitoes within the United States that can transmit malaria if they take a blood meal from an individual with gametocytes in the blood. Thus, it is important for the physician to know which stages are present in the blood (rings, developing trophozoites, schizonts, and/or gametocytes).*

6. How do parasitemia and malaria severity correlate?

*The following percentages are helpful in interpretation of malaria severity:*

<i>Parasitemia of &gt;10,000/<math>\mu</math>l, heavy infection 0.002%, 100/<math>\mu</math>l, immunologically naive, symptomatic below this level 0.2%, 10,000/<math>\mu</math>l, immune patients symptomatic 0.1%, 5,000/<math>\mu</math>l, minimum sensitivity of rapid malaria test (BinaxNOW) 2%, 100,000/<math>\mu</math>l, maximum parasitemia for P. vivax, P. ovale (rarely go above 2%) 2 to 5%, up to 250,000/<math>\mu</math>l, severe malaria, mortality 10%, 500,000/<math>\mu</math>l, exchange transfusion</i>
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## Proficiency Testing

1. How should blood films be examined for proficiency testing?

*Since you have no idea what organisms might be present, always review the blood films with the 10 $\times$  objective (review the entire slide). This examination is likely to reveal any microfilariae that are present; however, small parasites like Plasmodium and Babesia may be missed. Before reporting the smear as negative, examine at least 300 oil immersion fields with the 100 $\times$  oil immersion lens.*

2. Do proficiency testing blood films match those seen from actual patients?

*Yes and no! Blood films for PT are actual patient specimens; however, they may have a higher parasitemia than is seen in many patients reporting to the ER, clinic, etc. Often, smears contain a higher parasitemia than is commonly seen. Therefore, the PT smears represent a mix, some of which are fairly typical and some of which contain a large number of organisms. As in the case of specimens from travelers, the thin blood film may appear to be negative, while the thick film will be positive!*

3. What blood parasites might be seen in proficiency testing specimens?

*The following parasites may be seen in PT specimens: P. falciparum, P. vivax, P. ovale, P. malariae, mixed malaria infections, Babesia spp., Trypanosoma cruzi, Trypanosoma rhodesiense/gambiense, and microfilariae.*

## General Questions

1. What stains are recommended for staining microfilariae?

*Although Giemsa stain is generally recommended, it does not stain the sheath of Wuchereria bancrofti; hematoxylin-based stains (Delafield's stain) are recommended. Also, the sheath of Brugia malayi stains pink with Giemsa stain.*

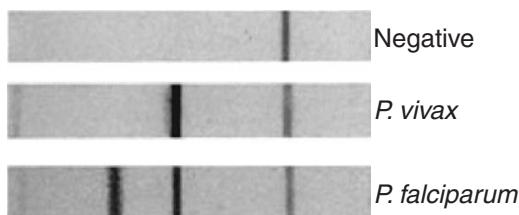
2. What other methods can be used for the identification of blood parasites?

*Concentrations (trypanosomes, microfilariae, leishmaniae), buffy coat thick and thin blood films (all blood parasites, including Plasmodium spp.), skin biopsies and bone marrow aspirates (Giemsa or other blood stains) (leishmaniae), and rapid methods/dipstick formats (malaria and microfilariae) can all be used. (Figure 6.4). NOTE: The Inverness/Binax rapid test for Plasmodium falciparum and Plasmodium spp. has been approved by the Food and Drug Administration (June, 2007); however, the company does not yet have a positive external control available for customers. The Binax rapid test is as sensitive as a good microscopist; however, often these skilled individuals may not be available on off-hour shifts. It is important to recognize the pros and cons of the rapid test compared to microscopic examination of blood films.*

3. How good are serologic tests for malaria, and when should they be performed?

*Serologic tests for malaria are available through CDC. These tests contain antigens of all four species, but the reactions are not species specific; there*

**Figure 6.4** Rapid malaria test. (Top) The negative test shows the control line only; (middle) the control line plus the *Plasmodium vivax* line indicates the presence of a panspecific antigen (common to all *Plasmodium* spp., but more sensitive for *P. vivax* than for *P. ovale* and *P. malariae*); (bottom) the control line, panspecific antigen line, and line specific for *P. falciparum* antigen.



*is a lot of cross-reactivity. Plasmodium spp. can be determined in about 80% of nonimmune patients with their primary infection (such as members of the U.S. Army during the Vietnam War); however, the tests cannot determine species in patients with long-term exposure and multiple infections (i.e., Africans). CDC does not recommend serologic testing for identification to the species level; PCR is the best tool for this purpose if pretreatment EDTA-blood is available.*

4. How do you categorize malaria resistance (seen in both *P. falciparum* and *P. vivax*)?

Resistance definition	Comments
Sensitive	From initiation of therapy, asexual parasites are cleared by day 6; no evidence of recrudescence up to day 28 • Peripheral blood films appear to go from positive to negative very quickly (can be a change from one draw to the second draw 6 h later).
Resistance type I (RI)	From initiation of therapy, asexual parasites have cleared for at least two consecutive days (the latest day being day 6); recrudescence follows. • Parasite count initially drops and blood films appear to be negative; patient should be monitored for a period of days, particularly if drug-resistant <i>P. falciparum</i> is suspected.
Resistance type II (RII)	Within 48 h of initiation of therapy, marked reduction of asexual parasitemia to <25% of pretreatment count; however, no subsequent disappearance of parasitemia (smear positive on day 6) • Patient appears to be improving; parasite count drops, but blood films always appear positive.
Resistance type III (RIII)	Modest reduction in parasitemia may be seen; no change or increase in parasitemia seen during first 48 h after treatment; no clearing of asexual parasites • In some cases, the parasite count continues to increase with no visible decrease at any time; blood films show overall parasite increase.

5. How soon after initiation of therapy for *P. vivax* malaria would one expect to see clearance in blood smears? How soon should follow-up blood smears be submitted for evaluation after therapy?

*Although the number of cases seen in the United States is certainly smaller than in areas of endemic infection, we begin to see a drop in parasite numbers very quickly (within a few hours) after the initiation of therapy. If an individual presents in the ER, the parasitemia is often below 1 to*

*2% (0.1 to 0.01%); the patient is given therapy and is not admitted to the hospital (in the case of P. vivax, P. malariae, or P. ovale infection). If the organism is identified as P. falciparum or a mix (the most common would be P. falciparum plus P. vivax or P. falciparum plus P. malariae), the patient is admitted. In some cases, we do not receive blood for follow-up examination (from ER patients for whom P. falciparum has been ruled out). When treating P. falciparum, we receive blood samples approximately every 4 to 6 h for routine checks (patients are admitted to the hospital for treatment). Although the number of cases of resistant P. vivax infections is small, this scenario has been confirmed, so this is always a consideration, as are mixed infections.*

6. Where can one get PCR performed for the malaria diagnosis and identification to the species level?

*CDC performs PCR for malaria, which identifies the organisms to the species level. CDC requires about 0.5 ml of pretreatment EDTA-blood. Also, if possible, they like to review both thick and thin smears if extra blood films are available. Specimens can be sent overnight to:*

*Stephanie Johnston, MS  
Microbiologist  
Division of Parasitic Diseases  
Centers for Disease Control and Prevention  
Building 109, Room 1302  
4770 Buford Highway NE  
Atlanta, GA 30341  
Phone: (770) 488-7044*

7. What is the current status of rapid testing for malaria?

*This issue has been a difficult one to resolve in the United States. The Binax rapid test was approved in June 2007 (Figure 6.4) (see table below). However, the company does not yet have an external positive control available for customers. Workers are therefore reluctant to use the kits to actually diagnose patients. Certainly the use of these rapid tests would be a tremendous advantage for personnel on the night shift. However, if the test was negative, it would not rule out a Plasmodium infection. If it was positive, follow-up testing would need to be performed on slides to detect mixed infections. All kits are helpful (according to published data), but they are no better and somewhat less accurate than a good microscopist. However, as we all know, these individuals are often not available on off-hour shifts. Many places have solved the problem by just bringing someone in to prepare and read slides on off hours. Often, the afternoon shifts prepare and stain smears (Wright-Giemsa or one of the rapid stains) and another individual would examine the thick and thin stained blood films. We tried to expose our residents, postdocs, etc., to these kits and found them to be helpful. However, when dealing with immunologically naive patients with very low parasitemias, it would be easy to miss an infection unless careful examination of the thick films was performed. Once the external QC specimen is commercially available, it will be very*

*helpful to have this rapid test available. However, the rapid test will not take the place of careful examination of both thick and thin blood films.*

% Parasitemia	Comments
>10,000/ $\mu$ l	Heavy infection
0.0001–0.0004, 5–20/ $\mu$ l	Required for a positive thick blood film
0.002%, 100/ $\mu$ l	Immunologically naive patients may be symptomatic below this level
0.02%, 1,000/ $\mu$ l	Can be seen in travelers in ER (these patients are symptomatic very early with low parasitemia, meaning that they are immunologically naive)
0.1%, 5,000/ $\mu$ l	Minimum sensitivity of the BinaxNOW rapid lateral-flow method
0.2%, 10,000/ $\mu$ l	Immune patients are symptomatic
2%, 100,000/ $\mu$ l	Maximum parasitemia for <i>P. vivax</i> and <i>P. ovale</i> ; rarely goes above 2%
2–5%, up to 250,000/ $\mu$ l	Severe malaria, high mortality
10%, 500,000/ $\mu$ l	Exchange transfusion usually required

8. Can mosquitoes be infected with more than one species of *Plasmodium*?

*There appears to be no barrier to infection of Anopheles with mixed Plasmodium species. Mosquitoes doubly infected with *P. falciparum* and *P. vivax* were able to transmit both species to humans following deliberate feeding on volunteers. In natural situations, however, suppressive effects in the human host may lead to overlapping waves of gametocytes of different species, so that there is a tendency for mosquitoes to be infected with only one species. In some cases there may be specific suppression of sporogony of one species compared to the other. For example, in areas where *P. falciparum* and *P. malariae* are sympatric, the cold temperatures associated with altitude may be disadvantageous to *P. falciparum*, which requires a higher temperature for sporogony than *P. malariae*. This may lead to seasonal changes in the prevalence of the two species.*

9. What is the general status of PCR for leishmaniasis?

*PCR for leishmaniasis has been through some very rigorous validation at the Leishmaniasis Laboratory at Walter Reed Army Institute of Research and is pending Food and Drug Administration approval. Contact Glen Wortmann at glenn.wortmann@us.army.mil.*

10. Are serologic tests available for the African trypanosomes?

*CDC does not perform PCR for African trypanosomiasis. It is unnecessary for diagnosing *T. b. rhodesiense* (these parasites are easily detectable by*

*microscopy). Testing for T. b. gambiense would need to be arranged at a laboratory in Europe. It is viewed as a research tool only. The CDC consultant is Anne Moore [(770) 488-7776].*

### **Suggested Reading**

**Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

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**Hanson, K. L., and C. P. Cartwright.** 2001. Use of an enzyme immunoassay does not eliminate the need to analyze multiple stool specimens for sensitive detection of *Giardia lamblia*. *J. Clin. Microbiol.* 39:474–477.

**Isenberg, H. D. (ed.).** 2004. *Clinical Microbiology Procedures Handbook*, 2nd ed. ASM Press, Washington, DC. Parasitology section in vol. 2 of 3 volumes.

**NCCLS/CLSI.** 2000. *Use of Blood Film Examination for Parasites*. Approved guideline M15-A. NCCLS/CLSI, Wayne, PA.

**NCCLS/CLSI.** 1997. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract*, 2nd ed. Approved guideline M28-A. NCCLS/CLSI, Wayne, PA.

S E C T I O N      7

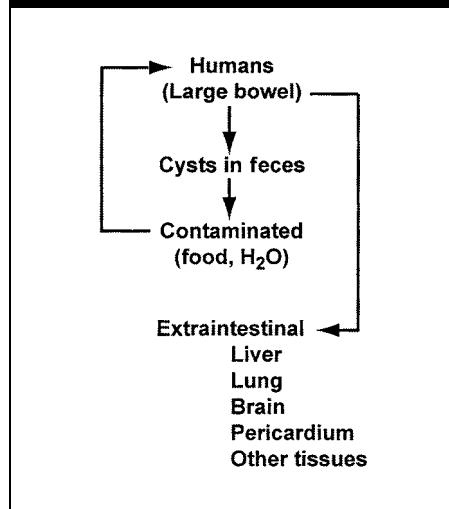
## Parasite Identification

## PROTOZOA • Amebae (Intestinal)

### Entamoeba histolytica

<b>Pathogenic</b>	Yes
<b>Disease</b>	Amebiasis
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine, liver, skin, miscellaneous body sites
<b>Symptoms</b>	<i>Intestinal:</i> Diarrhea, dysentery <i>Extraintestinal</i> (liver): Right upper quadrant pain, fever
<b>Clinical specimen</b>	<i>Intestinal:</i> Stool, sigmoidoscopy specimens <i>Extraintestinal:</i> Liver aspirate, biopsy, serum for antibody
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *E. histolytica* in stool specimens. Microscopic examination of a direct saline wet mount may reveal motile trophozoites, which may contain RBCs. However, in many patients who do not present with acute dysentery, trophozoites may not contain RBCs. An asymptomatic individual may have few trophozoites or possibly only cysts in the stool. Although the concentration technique is helpful in demonstrating cysts, the most important technique for recovery and identification of protozoan organisms is the permanent stained smear (normally stained with trichrome or iron hematoxylin). At least three specimens collected over no more than 10 days is often recommended.

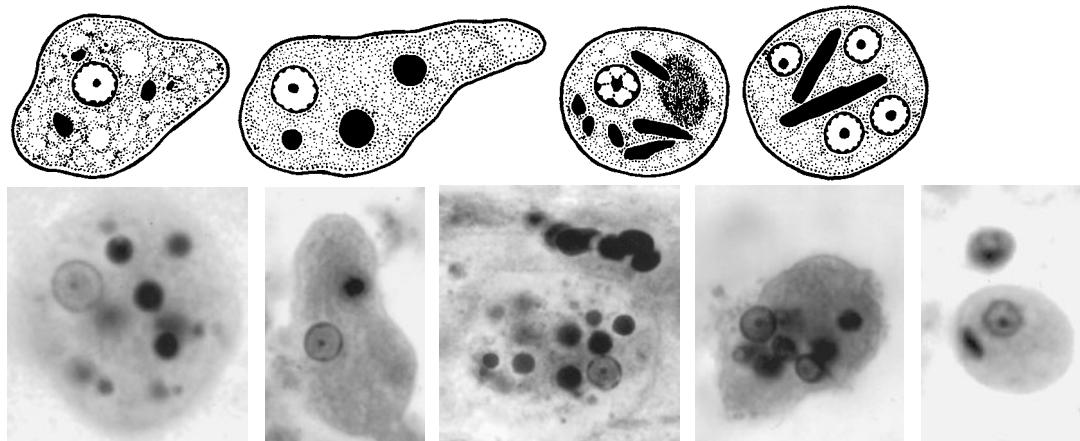
**Note:** If the permanent stained smear is quite dark, a delicate *E. histolytica* cell may appear more like *Entamoeba coli*; on a very thin, pale smear, *E. coli* can appear more like *E. histolytica*. Also, when organisms are dying or have been poorly fixed, they appear more highly vacuolated, looking like *E. coli* trophozoites rather than *E. histolytica*. Diagnosis of liver abscess can be made by identification of organisms from liver aspirate material, a procedure which is rarely performed. Serologic tests are also available.

### General Comments

Although many people worldwide are infected, only a few develop clinical symptoms. Morbidity and mortality due to *E. histolytica* vary depending on geographic area, organism strain, and the patient's immune status. For many years, the issue of pathogenicity has been very controversial: some felt that what was called *E. histolytica* was really two separate species, one being pathogenic and causing invasive disease and the other being nonpathogenic and causing mild or asymptomatic infections, and others felt that all organisms designated *E. histolytica* were potentially pathogenic, with symptoms depending on the result of host or environmental factors, including intestinal flora.

Based on current knowledge, pathogenic *E. histolytica* is considered the etiologic agent of amebic colitis and extraintestinal abscesses, while nonpathogenic *E. dispar* produces no intestinal symptoms and is not invasive in humans. The division of *Entamoeba* into invasive *E. histolytica* and noninvasive *E. dispar* by isoenzyme analysis is supported by genetic differences; pathogenesis differences also help explain the epidemiology, clinical syndromes, and pathology of amebiasis. The total body of evidence supports the differentiation of pathogenic *E. histolytica* from nonpathogenic *E. dispar*.

Fecal immunoassays can identify the *E. histolytica/E. dispar* group in stool, or can confirm the presence of the true pathogen *E. histolytica*; fresh or frozen stool is required.



**Images from left to right:** The first four images are *E. histolytica* trophozoites (note the ingested RBCs). Image 5 is a precyst with one chromatoidal bar. This organism would need to be identified as "Entamoeba histolytica/E. dispar" (might or might not be the true pathogen, *E. histolytica*)."

### Description (Trophozoite)

Living trophozoites vary in diameter from about 10 to 60  $\mu\text{m}$ . Organisms recovered from diarrheic or dysenteric stools are generally larger than those in a formed stool from an asymptomatic individual. Motility is rapid and unidirectional, with pseudopods forming quickly in response to the conditions around the organism. The motility may appear sporadic. Although this characteristic motility is often described, it is rare to diagnose amebiasis on the basis of motility seen in a direct mount. The cytoplasm is differentiated into a clear outer ectoplasm and a more granular inner endoplasm.

When the organism is examined on a permanent stained smear (trichrome or iron hematoxylin), the morphologic characteristics are readily seen. The nucleus is characterized by having evenly arranged chromatin on the nuclear membrane and a small, compact, centrally located karyosome. The cytoplasm is usually described as finely granular with few ingested bacteria or debris in vacuoles. In a patient with dysentery, RBCs may be visible in the cytoplasm, and this feature is considered diagnostic for *E. histolytica*. Most often, infection with *E. histolytica*/ *E. dispar* is diagnosed on the basis of trophozoite morphology without the presence of RBCs or cyst morphology (the organisms look identical).

### Description (Cyst)

For unknown reasons, the trophozoites may condense into a round mass (precyst), and a thin wall is secreted around the immature cyst. There may be two types of inclusions within this immature cyst, a glycogen mass and highly refractile chromatoidal bars with smooth, rounded edges. As the cysts mature (metacyst), there is nuclear division with the production of four nuclei; occasionally, eight nuclei are produced, and the cysts range in size from 10 to 20  $\mu\text{m}$ . Often, as the cyst matures, the glycogen completely disappears; the chromatoidals may also be absent in the mature cyst. Cyst formation occurs only within the intestinal tract, not when the stool has left the body. The one-, two-, and four-nucleated cysts are infective and represent the mode of transmission from one host to another.

After cyst ingestion, no changes occur in an acid environment; however, once the pH becomes neutral or slightly alkaline, the encysted organism becomes active, with the outcome being four separate trophozoites (small, metacystic trophozoites). These develop into the normal trophozoites when they become established in the large intestine.

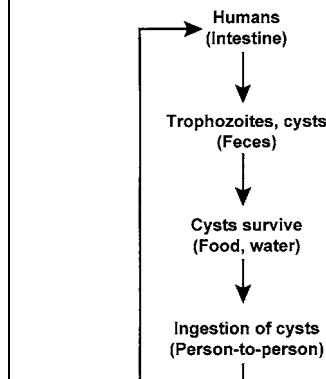
*E. histolytica* trophozoites and cysts can be confused with human macrophages and PMNs, respectively.

## PROTOZOA • Amebae (Intestinal)

### Entamoeba dispar

<b>Pathogenic</b>	No
<b>Disease</b>	None
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	None
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *E. dispar* in stool specimens. Microscopic examination of a direct saline wet mount may reveal motile trophozoites, which do not contain RBCs. Any trophozoite that is the appropriate size but contains ingested RBCs in the cytoplasm should be identified as the true pathogen, *E. histolytica*.

Although the concentration technique is helpful in demonstrating cysts, *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

**Note:** If the permanent stained smear is quite dark, a delicate *E. dispar* cell may appear more like *Entamoeba coli*; on a very thin, pale smear, *E. coli* can appear more like *E. histolytica* or *E. dispar*. Also, when the organisms are dying or have been poorly fixed, they appear more highly vacuolated, looking like *E. coli* trophozoites rather than *E. histolytica* or *E. dispar*.

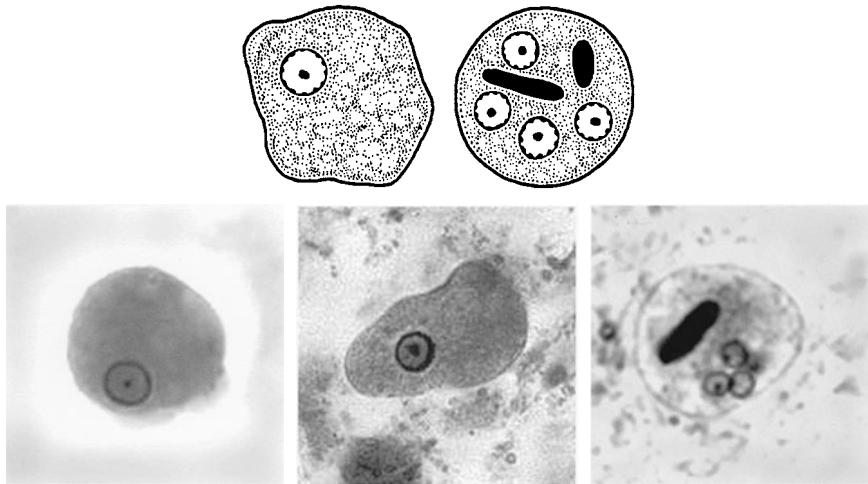
Fecal immunoassays can identify the *E. histolytica/E. dispar* group in the stool, or can confirm the presence of the true pathogen *E. histolytica*; fresh or frozen stool is required.

### General Comments

Although many people worldwide are infected, only a small percentage develop clinical symptoms and actually have *E. histolytica* rather than the nonpathogen, *E. dispar*. For many years, the issue of pathogenicity has been very controversial. Some felt that what was called *E. histolytica* was really two separate species of *Entamoeba*, one being pathogenic and causing invasive disease and the other being nonpathogenic and causing mild or asymptomatic infections. Others felt that all organisms designated *E. histolytica* were potentially pathogenic, with symptoms depending on host or environmental factors, including intestinal flora.

Based on current knowledge, pathogenic *E. histolytica* is considered the etiologic agent of amebic colitis and extraintestinal abscesses, while nonpathogenic *E. dispar* produces no intestinal symptoms and is not invasive in humans. The division of *Entamoeba* into invasive *E. histolytica* and noninvasive *E. dispar* by isoenzyme analysis is supported by genetic differences; pathogenesis differences also help explain the epidemiology, clinical syndromes, and pathology of amoebiasis.

The total body of evidence supports the differentiation of the pathogenic *E. histolytica* from the nonpathogenic *E. dispar* as two distinct species.



Images from left to right: The first two images in the *Entamoeba histolytica*/*E. dispar* group are trophozoites (no ingested RBCs within the trophozoite cytoplasm). The third image is a cyst containing a chromatoidal bar. This organism would need to be identified as *Entamoeba histolytica*/*E. dispar* (it might be the true pathogen, *E. histolytica*, or the nonpathogenic species, *E. dispar*).

### Description (Trophozoite)

Living trophozoites vary in diameter from about 10 to 60  $\mu\text{m}$ . Organisms recovered from diarrheic or dysenteric stools are generally larger than those in formed stool from asymptomatic individuals. Motility is rapid and unidirectional, with pseudopods forming quickly in response to the conditions around the organism; it may appear sporadic. Although this characteristic motility is often described, it is rare to diagnose amebiasis on the basis of motility seen in a direct mount. The cytoplasm is differentiated into a clear outer ectoplasm and a more granular inner endoplasm.

When the organism is examined on a permanent stained smear (trichrome or iron hematoxylin), the morphologic characteristics are readily seen. The nucleus is characterized by having evenly arranged chromatin on the nuclear membrane and the presence of a small, compact, centrally located karyosome. The cytoplasm is usually described as finely granular with few ingested bacteria or debris in vacuoles. In a patient with dysentery, RBCs may be visible in the cytoplasm, and this feature is considered diagnostic for *E. histolytica*. Infection with *E. histolytica*/*E. dispar* is usually diagnosed on the basis of organism morphology without the presence of RBCs. *E. dispar* will never contain RBCs in the cytoplasm; it is nonpathogenic.

### Description (Cyst)

For unknown reasons, the trophozoites may condense into a round mass (precyst), and a thin wall is secreted around the immature cyst. There may be two types of inclusions within this immature cyst, a glycogen mass and highly refractile chromatoidal bars with smooth, rounded edges. As the cysts mature (metacyst), there is nuclear division with the production of four nuclei; occasionally, eight nuclei are produced, and the cysts range in size from 10 to 20  $\mu\text{m}$ . Often, as the cyst matures, the glycogen completely disappears; the chromatoids may also be absent in the mature cyst. Cyst formation occurs only within the intestinal tract, not when the stool has left the body. The one-, two-, and four-nucleated cysts are infective and represent the mode of transmission from one host to another.

After cyst ingestion, no changes occur in an acid environment; however, once the pH becomes neutral or slightly alkaline, the encysted organism becomes active, with the outcome being four separate trophozoites (small, metacystic trophozoites). These organisms develop into the normal trophozoites when they become established in the large intestine.

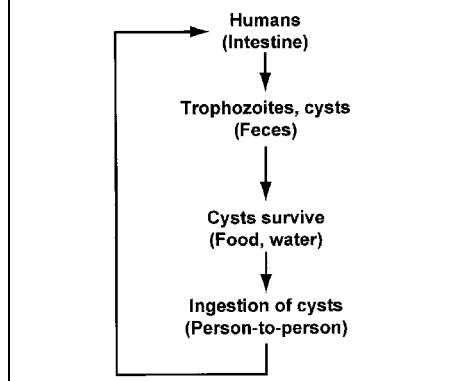
*E. dispar* trophozoites and cysts can be confused with human macrophages and PMNs, respectively.

## PROTOZOA • Amebae (Intestinal)

### Entamoeba hartmanni

<b>Pathogenic</b>	No
<b>Disease</b>	None
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	None
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *E. hartmanni* in stool specimens. Microscopic examination of a direct saline wet mount may reveal small motile trophozoites. An asymptomatic individual may have few trophozoites and possibly only cysts in the stool. Although the concentration technique is helpful in demonstrating cysts, *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

Because *E. hartmanni* can be easily confused with other small amebae, particularly in a wet preparation, it is almost mandatory that the final identification be obtained from the permanent stained smear. Accurate measurement of organisms also confirms the tentative visual diagnosis.

The mode of transmission is similar to that of other protozoa and is related to ingestion of cysts in contaminated food or water. In areas where accurate identifications have been made, the prevalence of *E. hartmanni* is similar to that of *E. histolytica*.

**Note:** Depending on the intensity of the stain, the trophozoite stage can mimic *Endolimax nana* or *Dientamoeba fragilis* trophozoites.

### General Comments

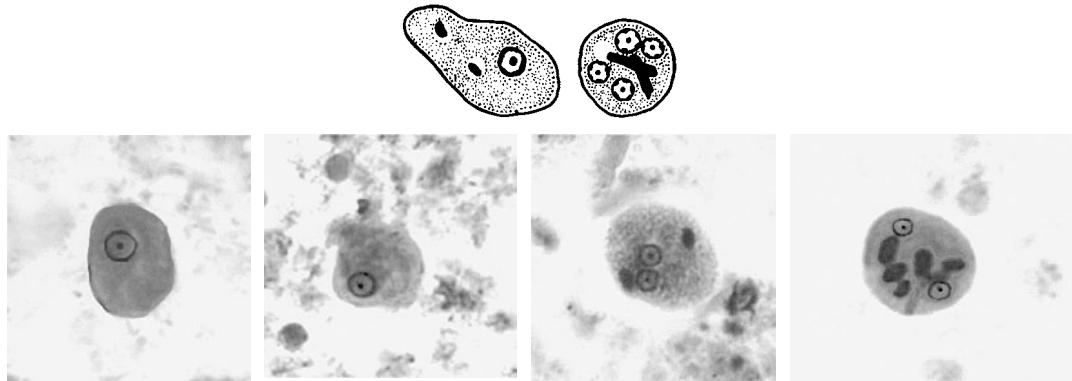
Although many people worldwide may be infected, they will remain asymptomatic. The main problem is the correct identification and differentiation between this nonpathogen and *E. histolytica*/*E. dispar*.

*E. histolytica* is considered the etiologic agent of amebic colitis and extraintestinal abscesses, while nonpathogenic *E. dispar* produces no intestinal symptoms and is not invasive in humans. *E. dispar* can be confused morphologically with the nonpathogenic *E. hartmanni*, although the latter is smaller; since both are nonpathogens, there is essentially no clinical relevance if *E. hartmanni* is identified as *E. dispar* or vice versa.

*E. hartmanni* can also be confused with human cells, including PMNs and macrophages.

Generally, *E. hartmanni* is not as common as *Entamoeba coli* or *E. dispar*. The morphology is usually very clear. The only problem with identification might occur if one was examining the permanent stained smear with a 50 or 60× oil immersion objective rather than the 100× oil immersion objective.

Although early studies suggested that *E. hartmanni* could transform from small "nonpathogenic" strains to large "pathogenic" ones, none of this information has been confirmed by subsequent studies. Because *E. hartmanni* is a nonpathogen, treatment is not recommended.



Images from left to right: The first two images are *E. hartmanni* trophozoites. The next two are *E. hartmanni* cysts containing chromatoidal bars (often the cyst contains only two nuclei). Note these amebae can often be confused with *E. histolytica* or *E. dispar*; they would be separated on the basis of size.

### Description (Trophozoite)

The life cycle is essentially identical to that of *E. histolytica* and *E. dispar*, and morphologic differences involve size, although there is even an overlap in size between the two species. In wet preparations, trophozoites range in size from 4 to 12  $\mu\text{m}$  and cysts range in size from 5 to 10  $\mu\text{m}$ . However, on the permanent stained smear there is a certain amount of artificial shrinkage due to dehydration; thus, the sizes of all of the organisms, including pathogenic *E. histolytica* and nonpathogenic *E. dispar*, may be somewhat smaller on a permanent stained smear (from 1 to 1.5  $\mu\text{m}$  smaller) than the sizes quoted for the wet preparation measurements.

Trophozoites do not ingest RBCs, and the motility is usually less rapid. Otherwise, nuclear and cytoplasmic characteristics are very similar to those seen in *E. histolytica*/*E. dispar*.

In some situations there may be confusion among *E. hartmanni*, *Endolimax nana*, and *Dientamoeba fragilis*, particularly in the trophozoite stage. However, the nucleus in the trophozoite of *E. hartmanni* tends to be very precise and resembles a "bull's eye" with very even chromatin and central karyosome.

### Description (Cyst)

Frequently cysts contain only one or two nuclei, even though the mature cyst contains four nuclei. Mature *E. hartmanni* cysts also tend to retain their chromatoidal bars, a characteristic often not seen in *E. histolytica* or *E. dispar*.

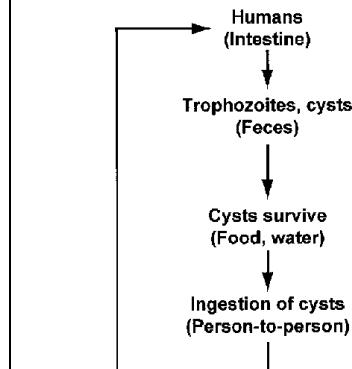
Chromatoidal bars are similar to those in *E. histolytica* and *E. dispar* but are smaller and more numerous. Because differentiation between *E. hartmanni*, *E. histolytica*, and *E. dispar* at the species level depends primarily on size, it is mandatory that laboratories use calibrated microscopes which are periodically rechecked. Although the CAP checklist does not require yearly microscope calibration, it is recommended if the microscope receives heavy use and/or is moved frequently during the year. *E. hartmanni* trophozoites and cysts can be confused with PMNs and macrophages, particularly since these human cells appear somewhat shrunk on the permanent stained smear; they always appear smaller in a permanent stained smear of fecal material than on a stained thin blood film.

## PROTOZOA • Amebae (Intestinal)

### Entamoeba coli

<b>Pathogenic</b>	No
<b>Disease</b>	None
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	None
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *E. coli* in stool specimens. Microscopic examination of a direct saline wet mount may reveal motile trophozoites. An asymptomatic individual may have few trophozoites and possibly only cysts in the stool. Although the concentration technique is helpful in demonstrating cysts, the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

**Note:** If the permanent stained smear is quite dark, a delicate *E. histolytica* or *E. dispar* cell may appear more like *E. coli*; on a very thin, pale smear, *E. coli* can appear more like *E. histolytica* or *E. dispar*. Also, when the organisms are dying or have been poorly fixed, they appear more highly vacuolated, thus looking like *E. coli* trophozoites rather than *E. histolytica*.

Except for the mature cyst, the morphologies of *E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. coli* are similar. It is very important to examine permanent stained smears, even if a tentative identification has been made from a wet preparation examination. Correct differentiation is critical to good patient care. Also, all four species can be found in the same patient. If few *E. histolytica/E. dispar* organisms are present among many *E. coli* organisms, additional searching and/or species-specific immunoassay testing may be necessary to correctly identify the different species.

### General Comments

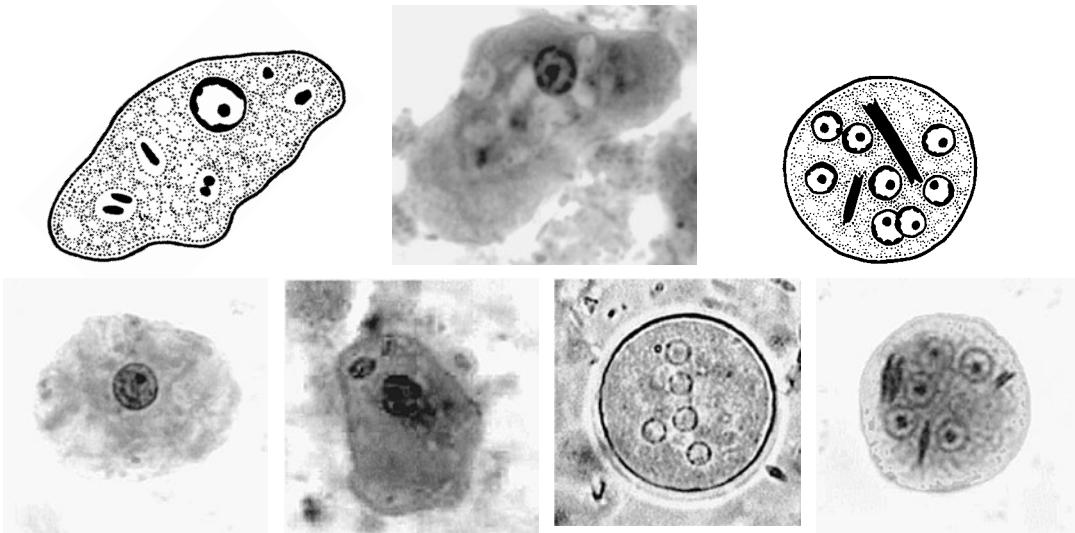
Although a large number of people throughout the world are infected with this organism, they will remain asymptomatic. The main problem is the correct identification and differentiation between this nonpathogen and *E. histolytica/E. dispar*.

*E. histolytica* is considered the etiologic agent of amebic colitis and extraintestinal abscesses, while nonpathogenic *E. dispar* produces no intestinal symptoms and is not invasive in humans. *E. dispar* can be confused morphologically with the nonpathogenic *E. coli*; since both are nonpathogens, there is essentially no clinical relevance if *E. coli* is identified as *E. dispar* or vice versa. Specific treatment is not recommended for this nonpathogen.

*E. coli* can also be confused with human cells, including PMNs and macrophages.

*E. coli* is generally described as a "dirty-looking" organism and is more commonly found in human stool specimens than the nonpathogen *E. dispar* or the pathogenic *E. histolytica*.

The mode of transmission is ingestion of cysts from contaminated food or water. Apparently, the infection is readily acquired, and in some warmer climates or areas with primitive hygienic conditions, the infection rate can be quite high.



Images from left to right: The far left two images in the bottom row are *E. coli* trophozoites (as is the middle image in the top row). The next two images are *E. coli* cysts. Note that there are five or more nuclei in the cysts.

### Description (Trophozoite)

Living trophozoites tend to be somewhat larger than those of *E. histolytica* and range from 15 to 50  $\mu\text{m}$ . Motility has been described as sluggish with broad, short pseudopods. In wet preparations, it may be extremely difficult to differentiate nonpathogenic *E. coli* from pathogenic *E. histolytica* or nonpathogenic *E. dispar*. On the permanent stained smear, the cytoplasm appears granular with few to numerous vacuoles containing bacteria, yeasts, and other food materials. The nucleus has a moderately large karyosome that is frequently eccentric. The chromatin on the nuclear membrane is usually clumped and irregular in placement. If there are RBCs in the intestinal tract, *E. coli* may ingest them rather than bacteria; occasionally the cytoplasm also contains ingested *Sphaerista* spores and possibly a *G. lamblia* cyst.

*E. coli* trophozoites (which resemble macrophages) and immature cysts with four nuclei (which resemble PMNs) can be confused with human cells.

### Description (Cyst)

Trophozoites discharge their undigested food and begin to round up prior to precyst and cyst formation. Early cysts usually contain a dense glycogen mass and may also contain chromatoidal bars which tend to be splinter shaped and irregular. Eventually the nuclei divide until the mature cyst, containing 8 (occasionally 16) nuclei, is formed. The cysts measure 10 to 35  $\mu\text{m}$  and almost always lose their chromatoidal bars as they mature. As the cyst of *E. coli* matures, it becomes more refractive to fixation with various preservatives. It may therefore be seen on the wet preparation and not on the permanent stained smear. Occasionally on trichrome smears, the cysts appear distorted and somewhat pink. This is not an indication of poor reagents or techniques but reflects the poor fixation of the cysts. Better fixation and more detailed morphology can be obtained by heating some of the fixatives prior to specimen preservation.

After cyst ingestion, the metacyst undergoes division of the cytoplasm, thus becoming metacystic trophozoites that will grow and divide within the lumen of the intestine. Usually fewer than eight trophozoites are formed from the mature cyst.

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**PROTOZOA • Amebae (Intestinal)**

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***Entamoeba gingivalis***

<b>Pathogenic</b>	No
<b>Disease</b>	None
<b>Acquired</b>	Droplet spray from the mouth; close contact, contaminated cups
<b>Body site</b>	Mouth, vagina, cervix
<b>Symptoms</b>	None
<b>Clinical specimen</b>	Material from gums or teeth
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Proper care of teeth and gums; removal of IUDs

***Entamoeba polecki***

<b>Pathogenic</b>	No
<b>Disease</b>	None
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	None
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, usually found in pigs and monkeys, less often in humans
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

**Comments**

*Entamoeba gingivalis* was the first parasitic ameba of humans to be described. It was recovered from the soft tartar between the teeth. It has also been recovered from the tonsillar crypts and can multiply in bronchial mucus, thus appearing in sputum. Since morphologically it is very similar to *E. histolytica*, it is important to make the correct identification from a sputum specimen (nonpathogenic *E. gingivalis* rather than *E. histolytica* from a possible pulmonary abscess).

Organisms identified as *E. gingivalis* have been recovered in vaginal and cervical smears from women using intrauterine devices (IUDs); the organisms spontaneously disappeared after removal of the devices. In an unusual case, *E. gingivalis* was identified in a left upper neck nodule by fine-needle aspiration. Apparently, the patient had an increased number of amebae within the oral cavity secondary to radiation therapy, which may have contributed to a fistula tract between the oral cavity and the surgical incision site, resulting in the formation of a small inflammatory nodule in the upper neck. Generally, no treatment is indicated, regardless of the body site from which the parasites are recovered. The infection suggests a need for better oral hygiene and can be prevented by proper care of teeth and gums.

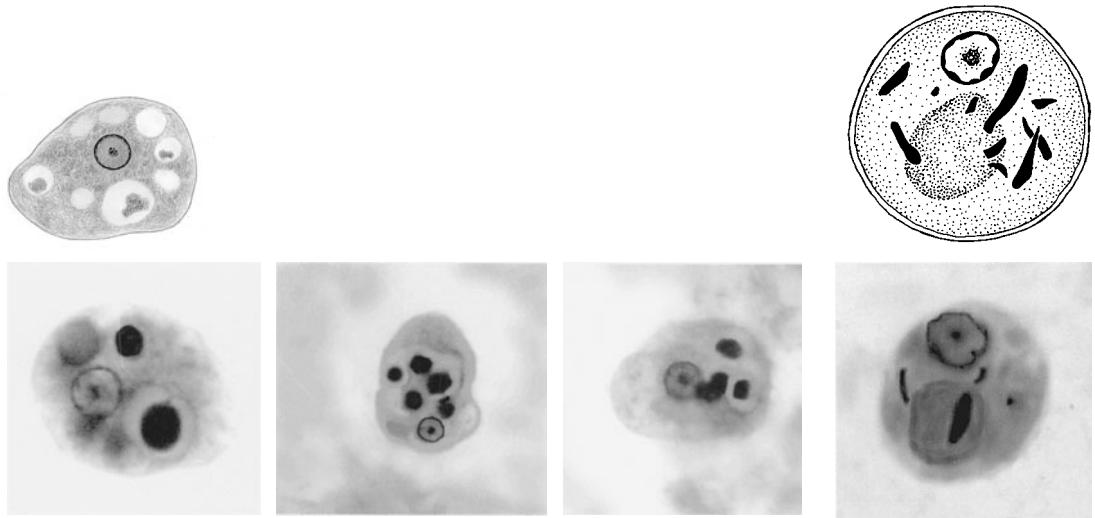
**Comments**

*Entamoeba polecki* was originally found in the intestines of pigs and monkeys and has also been found as a human parasite. Where humans and pigs live in close association and where sanitation is poor, pig-to-human transmission is considered the most likely source of human infection (via ingestion of infective cysts). However, human-to-human transmission is very likely if the prevalence rate and intensity of infection are high.

This ameba has been reported infrequently in the Western world; most reported cases have been from the New Guinea region. In most described patients, no definite gastrointestinal symptoms could be directly attributed to *E. polecki* infection.

Due to increased travel and numbers of immigrants to the United States, *E. polecki* may be identified more frequently than in the past. Physicians and laboratory personnel should be familiar with this organism because it may be confused with *E. histolytica*, a true pathogen.

In the earlier literature, one patient was reported to experience intermittent episodes of abdominal cramps, diarrhea, nausea, and malaise associated with large numbers of *E. polecki* cysts in the stool. However, this is probably a rare occurrence of symptoms.



Images from left to right: The first three images are *E. gingivalis* trophozoites; note the ingested PMNs. Image 4 is an *E. polecki* cyst; note the large inclusion in the cyst (not seen in other *Entamoeba* species).

## Description

The *E. gingivalis* trophozoite measures approximately 5 to 15  $\mu\text{m}$ , and the cytoplasm most often contains ingested leukocytes. On the permanent stained smear, nuclear fragments of the WBCs can be seen within food vacuoles, which are usually larger than the vacuoles seen in *E. histolytica*. This helps differentiate the two, since *E. gingivalis* is the only species that ingests WBCs. No cysts are formed by this species. Although *E. gingivalis* is most often recovered from patients with pyorrhea alveolaris, it is still considered to be nonpathogenic.

## Description

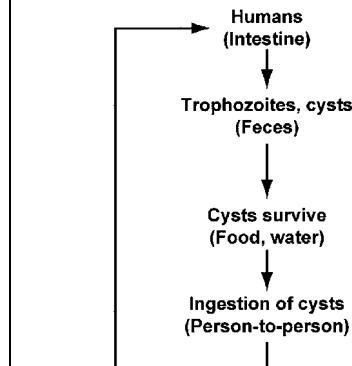
In certain areas of the world, such as Papua New Guinea, *E. polecki* is the most common intestinal ameba of humans. Few cases are reported, possibly because it resembles *E. histolytica*/*E. dispar*, *E. moshkovskii*, and *E. coli*. The trophozoites resemble *E. coli* in that the cytoplasm is granular, containing ingested bacteria, and the motility tends to be sluggish. *The nuclear morphology is almost a composite of those of E. histolytica/E. dispar, E. moshkovskii, and E. coli*. Without some of the cyst stages for comparison, it would be very difficult to identify this organism to the species level on the basis of the trophozoite alone. The cyst normally has only a single nucleus, chromatoidal material like that in *E. histolytica*, and often an inclusion body. This mass tends to be round or oval and is not sharply defined on the edges. The material, which is not glycogen, remains on the permanent stained smear and stains less intensely than nuclear material or chromatoidal bars. This organism is rarely differentiated from *E. histolytica* and *E. coli* by a wet preparation examination. The size on the permanent stained smear ranges from 10 to 12  $\mu\text{m}$  for the trophozoite and 5 to 11  $\mu\text{m}$  for the cyst.

## PROTOZOA • Amebae (Intestinal)

### *Endolimax nana*

<b>Pathogenic</b>	No
<b>Disease</b>	None
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	None
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *E. nana* in stool specimens. Microscopic examination of a direct saline wet mount may reveal small, motile trophozoites. An asymptomatic individual may have few trophozoites and possibly only cysts in the stool. Although the concentration technique is helpful in demonstrating cysts, *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

Although *E. nana* is a nonpathogen and no therapy is recommended, it is still important to differentiate it from other amebae, some of which are pathogenic. As indicated above, because these organisms are small, definitive diagnosis of *E. nana* may have to be based on the permanent stained smear. The four nuclear karyosomes appear very refractile in the wet preparation.

*E. nana* (particularly the cyst stage) does not fix well with mercury-substitute fixatives and may be very difficult to find and identify on the permanent stained smear.

### General Comments

*E. nana* is one of the smaller nonpathogenic amebae and was distinguished as a separate ameba around 1908. Its distribution is worldwide; it is seen in most populations at least as frequently as *E. coli*.

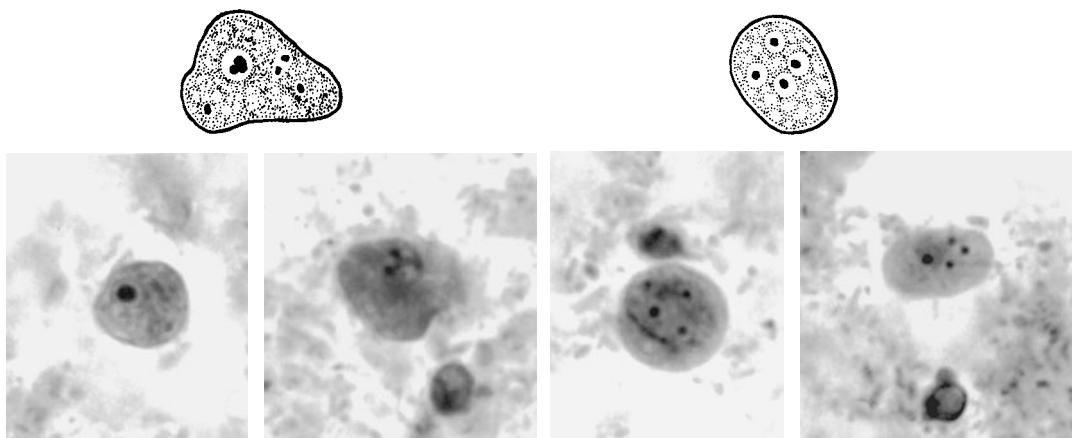
Although many people worldwide are infected with this organism, they remain asymptomatic. The main problem is the correct identification and differentiation between this nonpathogen and the pathogenic *Dientamoeba fragilis* or the nonpathogenic *Entamoeba hartmanni*.

This organism is one of the smaller amebae, and its trophozoite stage and that of *Iodamoeba bütschlii* look very similar. However, misidentification of these two organisms has little clinical significance since both are considered nonpathogens. In general, *E. nana* is much more common than *I. bütschlii* and the two organisms can also be found in the same specimen.

Of all the nonpathogenic amebae, *E. nana* is the most common and often seen in mixed protozoan infections.

Although *E. nana* is a nonpathogen, it is acquired via the fecal-oral route just like the pathogenic protozoa. Its presence in a stool specimen should always be reported to the physician.

*E. nana* cysts tend to be less resistant to desiccation than do those of *E. coli*. This organism is also found in the same areas of the world as are the other amebae, that is, in warm, moist climates and in other areas where there is a low standard of personal hygiene and poor sanitary conditions.



Images from left to right: The left-hand image is a trophozoite of *E. nana*. The next image is another *E. nana* trophozoite (note the unusual nuclear chromatin arrangement; there is tremendous nuclear variation in this species). The two images to the right are *E. nana* cysts (note that some are round while some are the more typical oval shape).

### Description (Trophozoite)

Motility has been described as sluggish and nonprogressive with blunt, hyaline pseudopods. In the permanent stained smear, the nucleus is more easily seen. There is normally no peripheral chromatin on the nuclear membrane, and the karyosome tends to be large and has a central or eccentric location within the nucleus. The trophozoites measure 6 to 12  $\mu\text{m}$ , with a usual range of 8 to 10  $\mu\text{m}$ .

There is tremendous nuclear variation; occasionally the overall morphology mimics *Dientamoeba fragilis* and *E. hartmanni*. The more organisms there are on the smear, the more likely it is that some of them will mimic other species of the amebae. The cytoplasm may have small vacuoles containing ingested debris or bacteria.

Nuclear morphology can be described as a "Y" shape, a band of chromatin across the nucleus, peripheral chromatin, or numerous other variations.

### Description (Cyst)

Trophozoites discharge their undigested food and begin to round up prior to precyst and cyst formation. Early cysts may contain very thin, curved chromatoidal bars (of-often difficult to see). Eventually the nuclei divide until the mature cyst, containing four nuclei, is formed.

Cysts usually measure 5 to 10  $\mu\text{m}$ , with a normal range of 6 to 8  $\mu\text{m}$ . In some instances, cysts as large as 14  $\mu\text{m}$  have been seen. They are usually oval to round. Occasionally very small, slightly curved chromatoidal bars are present. It is unusual to see the two-nucleated stage. Clinical specimens often contain both trophozoites and cysts.

Since there tends to be no peripheral nuclear chromatin, the cyst nuclei may be difficult to see, particularly if the fecal specimen was originally fixed in non-mercury fixatives.

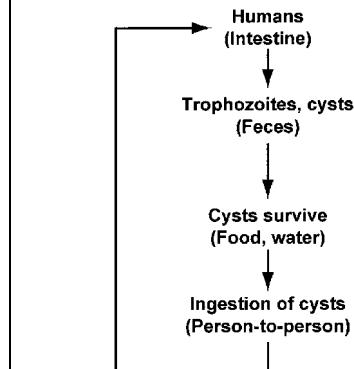
Cysts can be difficult to fix and may be very difficult to identify, particularly when using the non-mercury fixatives. If a two-nucleated cyst is seen, it may mimic the cyst of *Enteromonas hominis*—a small oval cyst with two small nuclei. However, the appearance of two-nucleated cysts of *E. nana* is quite unusual; almost all cysts contain four nuclei.

## PROTOZOA • Amebae (Intestinal)

### *Iodamoeba bütschlii*

<b>Pathogenic</b>	No
<b>Disease</b>	None
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	None
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *I. bütschlii* in stool specimens. Microscopic examination of a direct saline wet mount may reveal small, motile trophozoites. An asymptomatic individual may have few trophozoites and possibly only cysts in the stool. Although the concentration technique is helpful in demonstrating cysts, *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

This organism can be easily confused with other small amebae, particularly in the trophozoite stage. The cyst form is visible when the organisms are stained with iodine in the wet preparation.

The mode of transmission is ingestion of infective cysts in contaminated food or water. Prevention depends on improved personal hygiene and sanitary conditions.

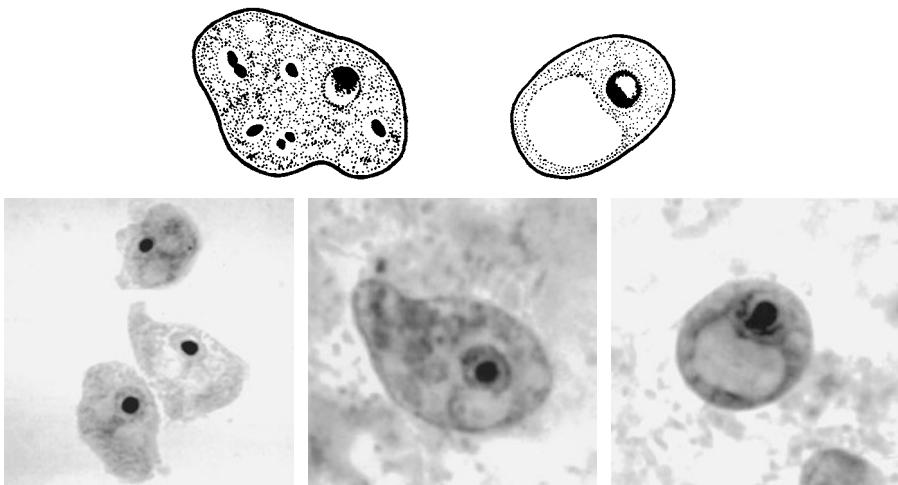
### General Comments

Although *I. bütschlii* is found in areas where the other amebae have been recovered, its incidence is not as high as that of *E. coli* or *E. nana*. One of the most striking morphologic features of this organism is the large glycogen vacuole which appears in the cyst and readily stains with iodine on a wet preparation smear.

Many people are infected with this organism worldwide, but they remain asymptomatic. The main problem is correct identification and differentiation between this nonpathogen and the pathogenic *Dientamoeba fragilis* or the nonpathogens *Entamoeba hartmanni* and *Endolimax nana*.

This organism is one of the smaller amebae; its trophozoite stage looks very similar to that of *E. nana*. However, misidentification of these two organisms has little clinical significance since both are considered nonpathogens. In general, *E. nana* is much more common than *I. bütschlii* and they can also be found in the same specimen.

When you identify both *E. nana* and *I. bütschlii* trophozoites in a fecal specimen, look for two different organism populations which differ mainly in size. There is no solid evidence why *E. nana* tends to be more common than *I. bütschlii* in human fecal specimens.



Images from left to right: (Top) *I. bütschlii* trophozoite and cyst; (bottom), two trophozoites and one cyst containing the large glycogen vacuole.

### Description (Trophozoite)

Motility is active in the wet preparation. In the permanent stained smear, the nucleus has a large karyosome which can be either central or eccentric and may appear to have a halo. Chromatin granules fan out around the karyosome. If the granules are on one side, the nucleus may appear to have the "basket nucleus" arrangement of chromatin, which is often seen in the cyst stage. The cytoplasm is rather granular, containing numerous vacuoles with ingested debris and bacteria. In general, the vacuolated cytoplasm is more obvious than that in *E. nana* trophozoites. The trophozoites measure 8 to 20  $\mu\text{m}$ .

The trophozoites of *E. nana* and *I. bütschlii* may be very similar and difficult to differentiate, even on the permanent stained smear. Both are considered nonpathogens, and *E. nana* is recovered more frequently than *I. bütschlii*.

### Description (Cyst)

Trophozoites discharge their undigested food and begin to round up prior to precyst and cyst formation. The cyst of *I. bütschlii* contains a single nucleus, like that in the trophozoite.

Cysts usually measure 5 to 20  $\mu\text{m}$  and are rarely confused with other protozoa. Occasionally, cysts may appear collapsed and the typical, large glycogen vacuole may not be clearly visible. The cyst is usually oval to round.

The cyst form is often seen in the concentration wet sediment preparation, particularly when iodine is added to the smear; the iodine stains the glycogen in the vacuole, making it easier to see the large vacuole within the cyst. However, the typical morphology is usually clear on the permanent stained smear.

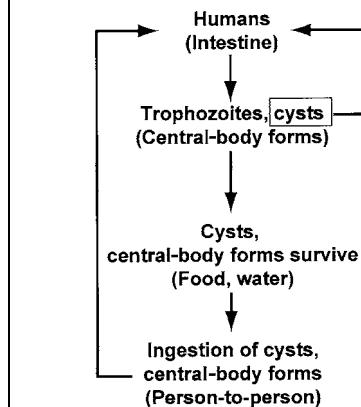
In a specimen containing both *E. nana* and *I. bütschlii* trophozoites, there are usually two populations of organisms, with the main difference usually being size. Although their sizes overlap, *I. bütschlii* is almost always the larger of the two.

## PROTOZOA • Amebae (Intestinal)

### Blastocystis hominis

<b>Pathogenic</b>	Occasionally causes illness
<b>Disease</b>	May be responsible for symptoms
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine, rare reports of dissemination to lymph nodes in debilitated patients
<b>Symptoms</b>	Generally none
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables.

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *B. hominis* in stool specimens. Microscopic examination of a direct saline wet mount may reveal small to large "central-body" forms. An asymptomatic individual may have few organisms present, all of which are the same central-body forms. Although the concentration technique is helpful in demonstrating these organisms, *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

This organism can be easily confused with other small protozoa and/or yeast cells.

Some directions for the stool concentration recommend rinsing the stool specimen with water prior to fixation with formalin. *This is not recommended* for any of the protozoa, but especially because *B. hominis* organisms may be ruptured by contact with tap or distilled water. Fecal specimens should be placed into formalin (or other comparable) fixative before the concentration procedure. However, identification and quantitation (rare, few, moderate, many, packed) are normally based on the permanent stained smear.

The classic form usually seen in human stool specimens varies tremendously in size, from 6 to 40  $\mu\text{m}$ , and is characterized by a large central body, which may be involved with carbohydrate and lipid storage (visually like a large vacuole). The more amebic form is occasionally seen in diarrheal fluid but may be extremely difficult to recognize. Generally, *B. hominis* is identified on the basis of the more typical round form with the central body.

### General Comments

Although many people worldwide are infected, most probably remain asymptomatic. The main problem is correct identification and differentiation between this organism and pathogenic intestinal protozoa.

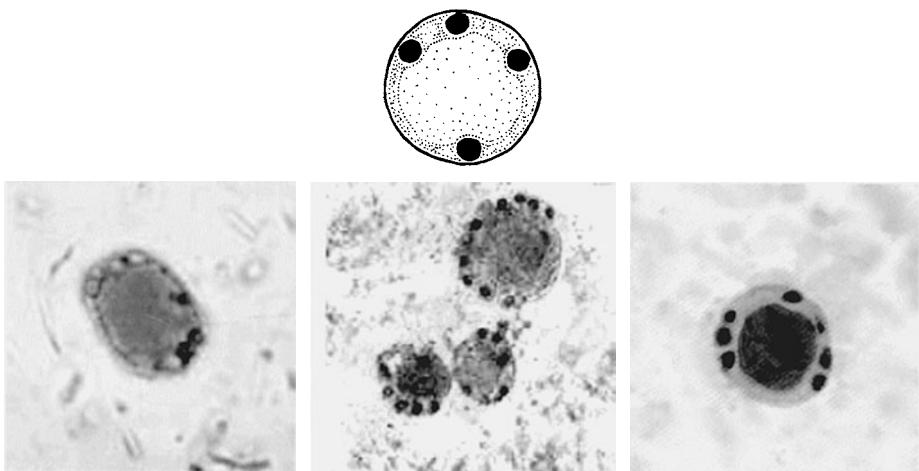
This organism is currently classified with the protozoa (amebae), but molecular studies may lead to reclassification. The more amebic form is occasionally seen in diarrheal specimens but may be very difficult to recognize, even on the permanent stained smear.

This organism is much more prevalent worldwide than other pathogenic and nonpathogenic intestinal protozoa.

The organisms should be quantitated when reported (rare, few, moderate, many, packed); both large and small numbers of organisms may cause symptoms. However, clinical specimens should be examined thoroughly before pathogenic status is assigned. Therapy is also effective in eradicating other intestinal protozoa (*Giardia*, etc.).

PCR-based genotype classification indicates ca. 12 different species. This variety may explain why some patients are asymptomatic and some have clinical symptoms. Currently, the species designation remains *B. hominis*.

Both thin- and thick-walled cysts have been confirmed. The thin-walled cysts may be autoinfectious, leading to multiplication of the organism in the intestinal tract. The thick-walled cysts are probably responsible for external transmission via the fecal-oral route. This life cycle might explain the unusually high percentage of positive carriers in many studies.



**Images:** (Top) Drawing of *B. hominis* central-body form. (Bottom) Central-body forms on a trichrome-stained smear.

## Description

*B. hominis* is capable of pseudopod extension and retraction; it reproduces by binary fission or "sporulation" and has a membrane-bound body that takes up about 90% of the organism. This form is called the "central-body form"; it is usually seen in the stool specimen and measures from 6 to 40  $\mu\text{m}$ . It is characterized by having a large central body that can be seen in the wet preparation from the concentration and in the permanent stained smear.

The more amebic form can be very difficult to identify and is seen in patients with more severe diarrhea. However, patients with *B. hominis* infections tend to have the central-body forms in the stool, regardless of whether the amebic forms are seen.

The presence of thin- and thick-walled cysts has been suggested. The thin-walled cysts may be autoinfectious, leading to multiplication of the organism in the intestinal tract. The thick-walled cysts are responsible for external transmission via the fecal-oral route. This life cycle might explain the high percentage of positive *B. hominis* carriers relative to those infected with other protozoa in many studies.

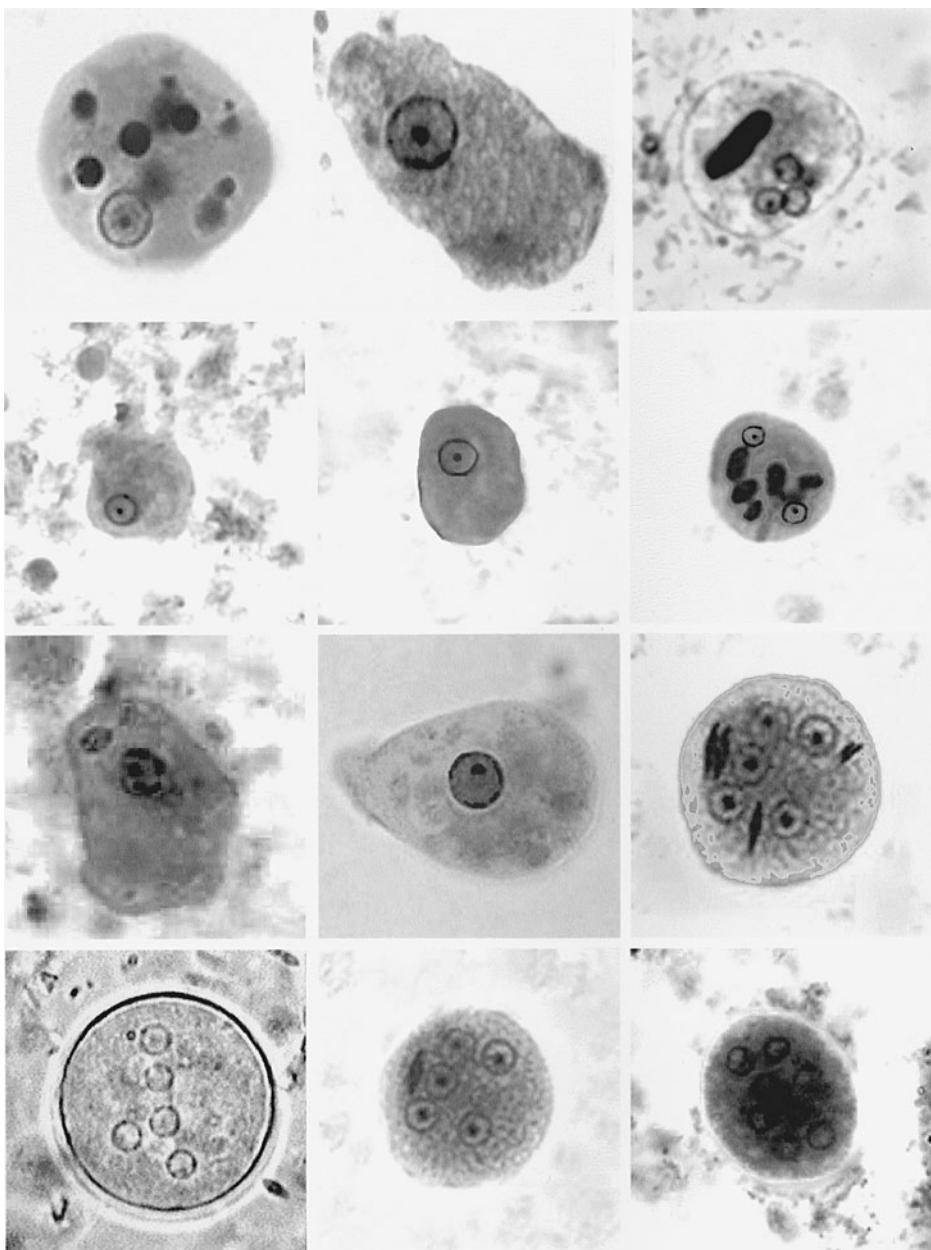
EAs and FA tests have been used to detect serum antibody to *B. hominis*. A strong antibody response is consistent with the ability to cause symptoms. Also, serum antibody production during and after *B. hominis* symptomatic disease is immunologic evidence for the pathogenic role of this protozoan.

## Additional Information

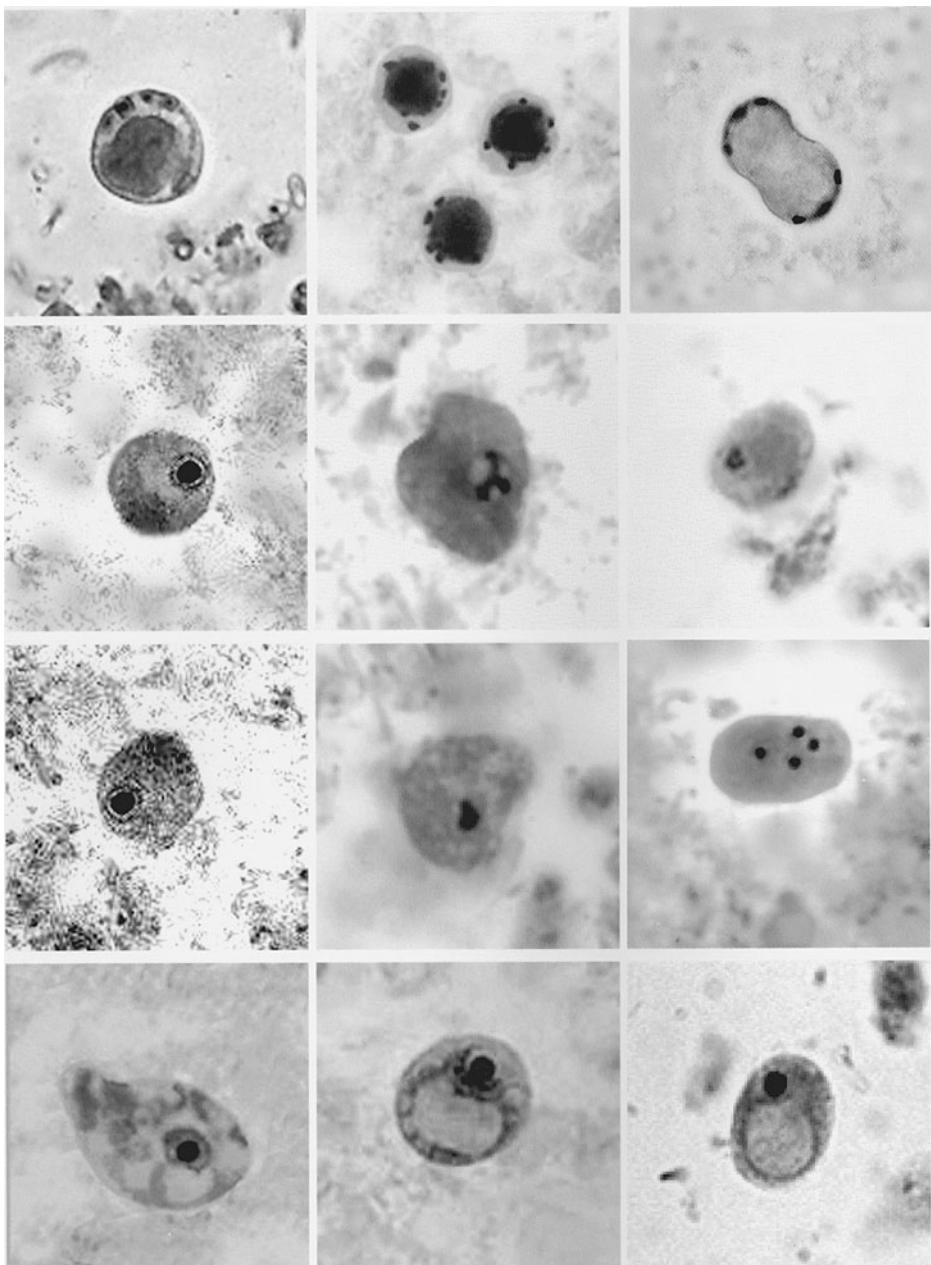
Terminology for *B. hominis* may change, depending on the outcome of molecular studies attempting to classify this organism more accurately. Some use the term "cyst" to denote the central-body form, while others continue to use "central-body form." Either term is acceptable at present.

The organisms may resemble yeast cells, debris, and/or amebic cysts. In some preparations, the organisms appear to be dividing, a finding not consistent with a cyst form of the intestinal protozoa.

In the absence of other organisms, *B. hominis* may cause diarrhea, cramps, nausea, fever, vomiting, and abdominal pain and may require therapy. It has also been linked to infective arthritis. In patients with other underlying conditions, the symptoms may be more pronounced. The incidence of this organism in stools submitted for parasite examination appears to be higher than suspected. In symptomatic patients in whom no other etiologic agent has been identified, *B. hominis* should be considered the possible pathogen. When a symptomatic *B. hominis* infection responds to therapy, the improvement may represent elimination of some other undetected pathogenic organism (*E. histolytica*, *G. lamblia*, *D. fragilis*). However, it is also clear that some organisms within the *B. hominis* group are almost certainly pathogenic. In symptomatic patients in whom no other etiologic agent has been identified, *B. hominis* should certainly be considered the possible pathogen.



**Plate 7.1** Row 1 (left to right): *Entamoeba histolytica* trophozoite, *Entamoeba histolytica/E. dispar* trophozoite, and *Entamoeba histolytica/E. dispar* cyst; row 2: *Entamoeba hartmanni* trophozoites (left and middle) and *Entamoeba hartmanni* cyst (right); row 3: *Entamoeba coli* trophozoite, *Entamoeba coli* trophozoite, and *Entamoeba coli* cyst; row 4: *Entamoeba coli* cyst (wet preparation), *Entamoeba coli* cyst, and *Entamoeba coli* cyst.



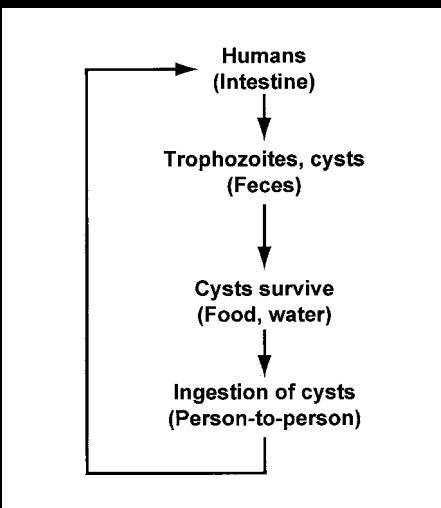
**Plate 7.2** Row 1 (left to right): *Blastocystis hominis* central-body forms (note that the cell in the right image is dividing; this is occasionally seen in fecal specimens); row 2: *Endolimax nana* trophozoites (note that the middle image has nuclear variation, which is common with this organism; the right organism mimics *Dientamoeba fragilis*); row 3: *Endolimax nana* trophozoites and (on the right) *Endolimax nana* cyst; row 4: *Iodamoeba bütschlii* trophozoite and *Iodamoeba bütschlii* cysts.

## PROTOZOA • Flagellates (Intestinal)

### Giardia lamblia

<b>Pathogenic</b>	Yes
<b>Disease</b>	Giardiasis
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine, occasionally gallbladder, and rarely bronchoalveolar lavage fluid
<b>Symptoms</b>	Diarrhea, epigastric pain, flatulence, increased fat and mucus in stool; gallbladder colic and jaundice
<b>Clinical specimen</b>	<i>Intestinal:</i> Stool <i>Extraintestinal:</i> Fluids
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *G. lamblia* in stool specimens; however, even after a series of four to six stool examinations, the organisms may not be seen. Duodenal aspirate fluid and/or the Entero-Test capsule may be used. Microscopic examination of a direct saline wet mount or mucus from the Entero-Test capsule may reveal motile trophozoites and/or nonmotile cysts. Although the concentration technique is helpful in demonstrating these organisms, *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

It is important to remember that this organism can be easily missed, even with multiple stool examinations.

Fecal immunoassays such as EIA, FA, and the rapid cartridges (membrane flow) are more sensitive than the routine O&P exam. Test menus should contain both the O&P exam and one or more of the fecal immunoassays. Appropriate ordering by the physician depends on patient history (travel history, possible outbreaks, etc.). Unfortunately, serodiagnostic procedures for giardiasis do not yet fulfill the criteria necessary for wide clinical use, particularly since they may indicate either past or present infection.

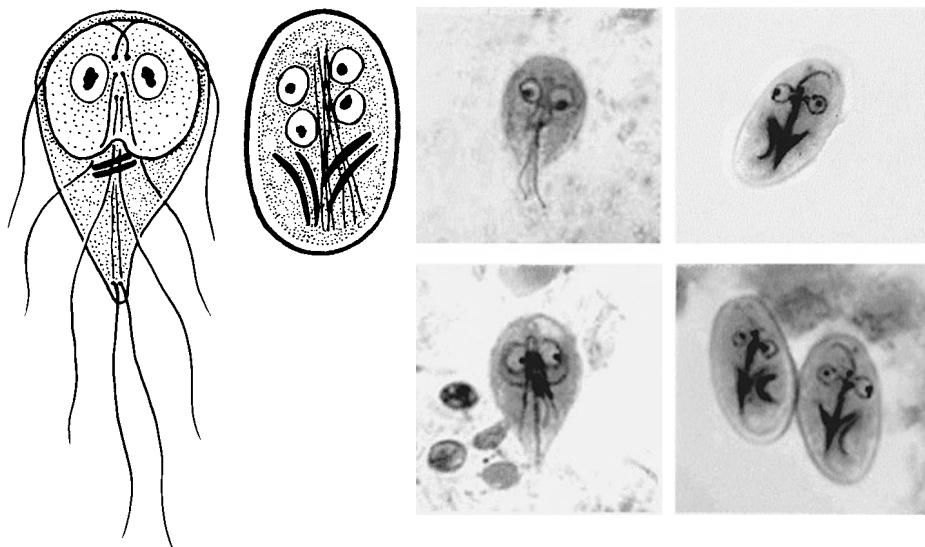
### General Comments

The incidence of giardiasis worldwide may be as high as a billion cases. Although many people worldwide are infected, not all patients present with continuous symptoms. Another challenge is making the correct identification and differentiation between this organism and other intestinal protozoa that may be nonpathogenic.

This organism is currently classified with the protozoa (flagellates), and studies at the molecular level have led to differentiation among *G. lamblia*, *G. duodenalis*, and *G. intestinalis*. Consistent with common terminology used in the United States, the term *G. lamblia* is used throughout this text. The actual term used for identification is not as important as consistent use by both laboratory and clinician. The flagellate trophozoites are often seen in diarrheal specimens but may be very difficult to recognize, even on the permanent stained smear.

Treatment failures have been reported with all the common antigiardial agents, and resistance to these drugs has been demonstrated in the laboratory. Clinical resistance has been reported, including resistance to both me tronidazole and albendazole in the same individual.

Potential wild-animal reservoirs have complicated control measures, particularly with waterborne outbreaks. The single most effective practice that prevents the spread of infection in the child care setting is thorough hand-washing by the children, staff, and visitors.



Images from left to right: Drawing of trophozoite and cyst (left); *Giardia* trophozoites (center); *Giardia* cysts (right).

### Description (Trophozoite)

The standard O&P exam is recommended for recovery and identification of *G. lamblia* in stool specimens. The trophozoite is usually described as being teardrop shaped from the front, with the posterior end being pointed. From the side, it resembles the curved portion of a spoon. The concave portion is the area of the sucking disk, used for attachment to the mucosal lining. There are four pairs of flagella, two nuclei, two linear axonemes, and two curved bodies called the median bodies. Trophozoites usually measure 10 to 20  $\mu\text{m}$  long and 5 to 15  $\mu\text{m}$  wide.

Although the concentration technique is helpful in demonstrating these organisms, *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). As with most intestinal protozoa, the trophozoite is usually seen in diarrheic or soft stools while the cyst is seen in the more normal stool or when the patient may be asymptomatic. Trophozoites may remain attached or detach from the mucosal surface. Since the epithelial surface sloughs off the tip of the villus every 72 h, apparently the trophozoites detach at that time.

If mucus from the Entero-Test or fluid from a duodenal aspirate is submitted, the organisms may be trapped in the mucus. Keep the microscope light low, particularly when examining wet preparations. Make sure your eye becomes adjusted to each field before moving the stage.

### Description (Cyst)

Cyst formation takes place as the organisms move through the jejunum after exposure to biliary secretions. Trophozoites discharge their undigested food and begin to round up prior to precyst and cyst formation. They retract the flagella into the axonemes, the cytoplasm becomes condensed, and the cyst wall is secreted. As the cyst matures, the internal structures are doubled, so that when excystation occurs, the cytoplasm divides, producing two trophozoites. Excystation would normally occur in the duodenum or appropriate culture medium.

*G. lamblia* cysts may be round or oval; they contain four nuclei, axonemes, and median bodies. Often some cysts appear to be distorted or shrunk; there may be two halos, one around the cyst wall and one inside the cyst wall around the shrunken organism. The halo around the outside of the cyst wall is often easily seen in the permanent stained fecal smear. Cysts normally measure 11 to 14  $\mu\text{m}$  long and 7 to 10  $\mu\text{m}$  wide.

Occasionally, shrunken cysts mimic large *Isospora belli* oocysts; however, if the organisms are measured, they can be very easily differentiated.

Antigenic variation occurs with surface antigen changes during *G. lamblia* infections; although the biological importance of this work is not clear, it suggests that this variation may allow the organism to escape the host immune response.

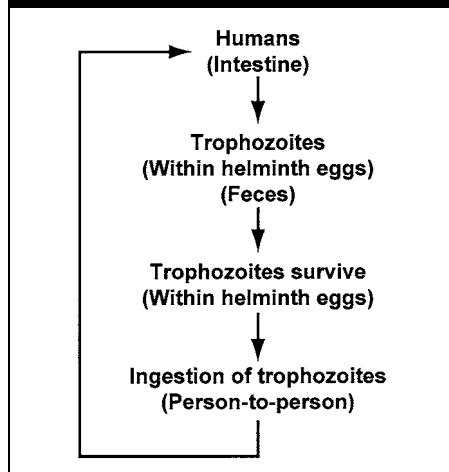
Although patients with symptomatic giardiasis usually have no underlying abnormality of serum immunoglobulins, giardiasis is common in patients with immunodeficiency syndromes, particularly in those with common variable hypogammaglobulinemia.

## PROTOZOA • Flagellates (Intestinal)

### Dientamoeba fragilis

<b>Pathogenic</b>	Yes
<b>Disease</b>	Dientamoebiasis
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water; may be transmitted within helminth eggs ( <i>Ascaris, Enterobius</i> )
<b>Body site</b>	Intestine
<b>Symptoms</b>	Intermittent diarrhea, abdominal pain, nausea, anorexia, malaise, fatigue, poor weight gain, unexplained eosinophilia
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *D. fragilis* in stool specimens; however, even after a series of several stool examinations, the organisms may not be seen, particularly if wet preparations are the only examination performed. Although the concentration technique is helpful in demonstrating some intestinal protozoa, *the most important technique for the recovery and identification of D. fragilis is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

*D. fragilis* has been recovered in formed stool; therefore, a permanent stained smear must be prepared for every stool sample submitted. Organisms seen in direct wet mounts may appear as refractile, round forms; the nuclear structure cannot be seen without examination of the permanent stained smear.

This organism can be easily missed, even with multiple stool examinations. There is no cyst form, and the trophozoites can be easily missed, even with the permanent stained smear.

Fecal immunoassays such as EIA, FA, or the rapid cartridges (membrane flow) would probably be more sensitive than the routine O&P exam. However, these methods are still under development and are not yet commercially available.

### General Comments

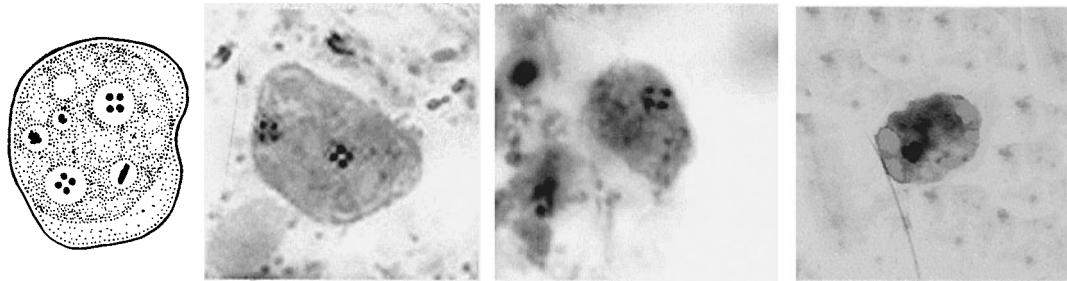
Although a large number of people throughout the world are infected with *D. fragilis*, not all patients have continuous symptoms. In some areas of the United States, this organism is recovered as often as *Giardia*. It has a cosmopolitan distribution, and past surveys show incidence rates of 1.4 to 19%.

*D. fragilis* is currently classified with the protozoa (flagellates); studies at the molecular level may lead to reclassification and differentiation. The flagellate trophozoites are often seen in diarrheal specimens but may be very difficult to recognize, even on the permanent stained smear. The flagella are internal, and the organism resembles a small ameba rather than a flagellate.

Incidence is hard to assess; laboratories that do not routinely examine a permanent stained smear will tend to miss this organism entirely.

These infections have been seen in nursery schools, along with giardiasis and cryptosporidiosis.

The significance of two genetically distinct forms of *D. fragilis* may ultimately clarify the issues of virulence and clinical perceptions regarding pathogenicity. Case reports of children and adults infected with *D. fragilis* reveal a number of symptoms, including intermittent diarrhea, abdominal pain, nausea, anorexia, malaise, fatigue, poor weight gain, and unexplained eosinophilia. The most common symptoms in patients infected with this parasite appear to be intermittent diarrhea and fatigue. In some patients, both the organism and the symptoms persist or reappear until appropriate treatment is initiated.



Images from left to right: The first drawing is a *Dientamoeba* trophozoite; the next three photographs are *D. fragilis* trophozoites (note that organisms can have either a single nucleus or multiple nuclei, most of which contain fragments of nuclear chromatin).

## Description

The standard O&P exam is recommended for recovery and identification of *D. fragilis* in stool specimens. Motility is usually nonprogressive. The trophozoite is characterized by having one (20 to 40%) or two (60 to 80%) nuclei. The nuclear chromatin tends to be fragmented into three to five granules, and there is normally no peripheral chromatin on the nuclear membrane. In some organisms, the nuclear chromatin mimics that of *E. nana*, *E. hartmanni*, or even *Chilomastix mesnili*. The cytoplasm is usually vacuolated and may contain ingested debris or bacteria. There may also be some large granules. The cytoplasm can also appear quite clean with few inclusions. The trophozoites usually measure 5 to 15  $\mu\text{m}$ , with the usual range being 9 to 12  $\mu\text{m}$ .

Again, the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear (normally stained with trichrome or iron hematoxylin).

## Additional Information

Although its pathogenic status is still not well defined, *D. fragilis* has been associated with a wide range of symptoms. In one study, 11 pediatric patients, 7 of whom had peripheral eosinophilia, a history of recent travel, and symptoms of anorexia, intermittent vomiting, abdominal pain, and diarrhea, were diagnosed with *D. fragilis*. Based on these and other findings, including bovine protein allergy and eosinophilic colitis, the authors recommend that *D. fragilis* should be included in the differential diagnosis of chronic diarrhea and eosinophilic colitis.

Clinical improvement has been observed in adults receiving tetracycline; symptomatic relief was reported in children receiving diiodohydroxyquin, metronidazole, or tetracycline. Current recommendations include iodoquinol, paromomycin, or tetracycline. Since symptomatic relief has been observed to follow appropriate therapy, *D. fragilis* is probably pathogenic in infected individuals who are symptomatic.

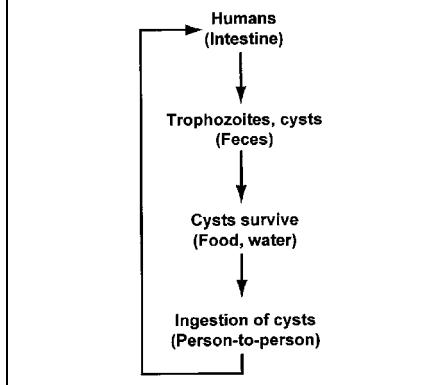
Since fecal-oral transmission has not been documented, it is difficult to determine preventive measures. However, if transmission occurs from the ingestion of certain helminth eggs (*Enterobius vermicularis*, *Ascaris lumbricoides*), hygienic and sanitary measures to prevent contamination with fecal material would be appropriate. There is speculation that *D. fragilis* may be infrequently recovered and identified; its low incidence or absence from survey studies may be due to poor laboratory techniques and a general lack of knowledge concerning the organism.

## PROTOZOA • Flagellates (Intestinal)

### *Chilomastix mesnili*

<b>Pathogenic</b>	No
<b>Disease</b>	None
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	None
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *C. mesnili* in stool specimens. Microscopic examination of a direct saline wet mount may reveal motile trophozoites and/or nonmotile cysts. Although the concentration technique is helpful in demonstrating these organisms, *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

This organism can be easily missed, even with multiple stool examinations.

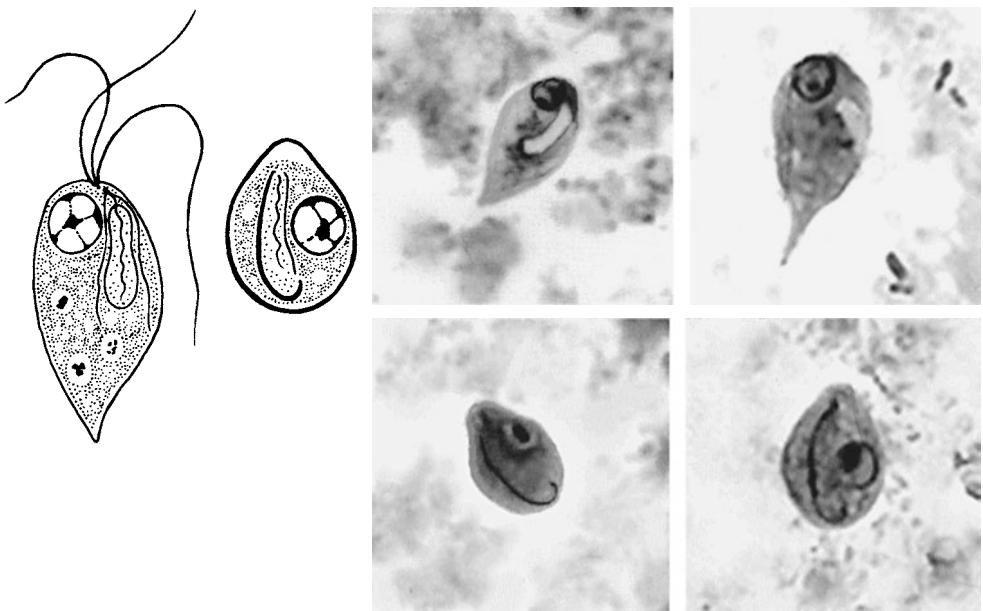
These organisms normally live in the cecal region of the large intestine, where they feed on bacteria and debris. They are considered nonpathogens, and no treatment is recommended. Since transmission is through ingestion of infective cysts, prevention depends on improved personal hygiene and upgraded sanitary conditions.

### General Comments

*C. mesnili* tends to have a cosmopolitan distribution, although it is found more frequently in warm climates. It has both trophozoite and cyst stages and is somewhat more easily identified than are some of the smaller flagellates such as *E. hominis* and *R. intestinalis*. Although many people worldwide are probably infected, the organisms may not always be seen, particularly if a permanent stained smear is not examined. Another challenge is correct identification and differentiation between this organism and other intestinal protozoa that may be pathogenic.

This organism is currently classified with the protozoa (flagellates).

The presence of this organism should always be reported, since nonpathogenic organisms are transmitted in the same way as pathogens. Confirmation of the presence of this organism indicates that the patient has ingested something (food, water, etc.) contaminated with fecal material. If nonpathogenic protozoa are found, pathogens may also be found on additional examination of the specimen.



Images from left to right: The top row shows a drawing of a *C. mesnili* trophozoite and cyst and then two *C. mesnili* trophozoites. The bottom row shows two cysts (note the curved fibril, the "shepherd's crook").

### Description (Trophozoite)

The trophozoite is pear shaped, measuring 6 to 24  $\mu\text{m}$  long and 4 to 8  $\mu\text{m}$  wide. There is a single nucleus and a distinct oral groove or cytostome close to the nucleus. Flagella are difficult to see unless there is obvious motility in a wet preparation. Morphology can be confirmed by a permanent stained smear, particularly when the cytostome is visible.

Although the concentration technique is helpful in demonstrating these organisms, *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). As with most intestinal protozoa, the trophozoite form is usually seen in diarrheic or soft stools while the cyst stage is seen in the more normal stool or when the patient may be asymptomatic.

If this trophozoite appears as somewhat round to oval and the cytostome is not visible, *C. mesnili* can easily be confused with other small amebae and/or flagellates.

### Description (Cyst)

Trophozoites discharge their undigested food and begin to round up prior to precyst and cyst formation. The cysts are pear or lemon shaped and range from 6 to 10  $\mu\text{m}$  long and from 4 to 6  $\mu\text{m}$  wide. There is also a single nucleus in the cyst and the typical curved cytostomal fibril, which is called the shepherd's crook.

These cysts can be confused with other small flagellate cysts, especially *Retortamonas intestinalis* cysts. The cytostomal fibril (shepherd's crook) may not always be visible, and if the cyst is somewhat shrunk, confusion with other small organisms is possible. Often one can see shrinkage (halo) surrounding the cyst on the permanent stained smear; thus, the organism may measure at the small end of the size range due to shrinkage.

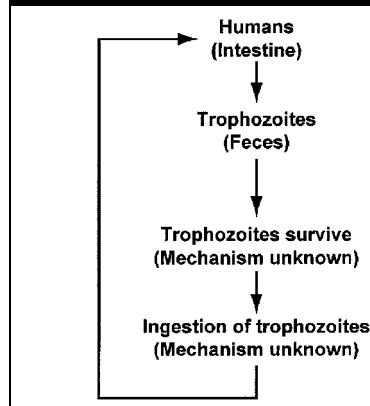
Due to the small size of the cyst, identification is not as easy as identification of *G. lamblia*. Also, when preserved with mercuric-chloride substitutes, the overall morphology, even on the permanent stained smear, is not as clear.

## PROTOZOA • Flagellates (Intestinal)

### *Pentatrichomonas hominis*

<b>Pathogenic</b>	No
<b>Disease</b>	None
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	None
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *P. hominis* in stool specimens. Microscopic examination of a direct saline wet mount may reveal motile trophozoites; there are no known cyst forms. The concentration technique is not very useful; *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

This organism can be easily missed, even with multiple stool examinations and the permanent stained smear.

This organism is found in the intestinal tract only; *Pentatrichomonas* and *Trichomonas* spp. tend to be site specific, and only *T. vaginalis* is pathogenic (it is found in the urinary-genital tract).

**Note:** *Trichomonas tenax* was first recovered in tartar from the teeth. Its distribution is worldwide. There is no known cyst stage. The trophozoite is pyriform and is smaller and more slender than that of *P. hominis*. The undulating membrane extends the length of the body, as in *P. hominis*. There is also the typical axostyle and a single nucleus. *T. tenax* does not survive passage through the stomach and cannot be established in the vagina. The mode of transmission is not known but is assumed to be via direct contact or use of contaminated dishes and glasses. Diagnosis is based on recovery of organisms from the teeth, gums, or tonsillar crypts, and no therapy is indicated. Better oral hygiene rapidly eliminates the infection.

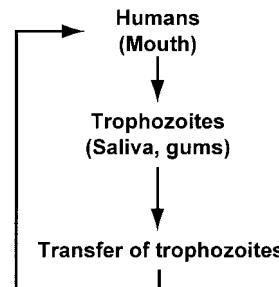
### General Comments

The genus name has been changed from *Trichomonas* to *Pentatrichomonas* based on the five anterior flagella and a granular parabasal body. This organism is currently classified with the protozoa (flagellates).

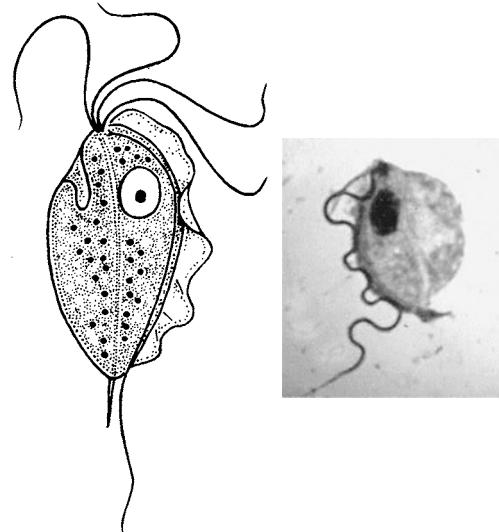
Many people worldwide may be infected; however, the organisms are not always seen, particularly if a permanent stained smear is not examined. Correct identification and differentiation between this organism and other intestinal protozoa that may be pathogenic (*G. lamblia*) are difficult.

The presence of this nonpathogen should always be reported; confirmation of its presence indicates that the patient has ingested something (food, water, etc.) contaminated with fecal material, which also contains pathogens.

*P. hominis* is probably the most commonly identified flagellate other than *G. lamblia* and *D. fragilis*. It has been recovered worldwide, in both warm and temperate climates.



Life cycle for *Trichomonas tenax*.



Images from left to right: Drawing of *P. hominis*; photograph of *P. hominis* trophozoite (note that the undulating membrane extends to the bottom of the organism and the axostyle/supporting rod extends through the bottom).

## Description

The standard O&P exam is recommended for recovery and identification of *P. hominis* in stool specimens. The trophozoite measures 5 to 15  $\mu\text{m}$  long and 7 to 10  $\mu\text{m}$  wide. It is pyriform and has both an axostyle and undulating membrane, which help in identification. The undulating membrane extends the entire length of the body, in contrast to that of *T. vaginalis*. Flagella are difficult to see unless there is obvious motility in a wet preparation.

Although the concentration technique is helpful in demonstrating these organisms, the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear (normally stained with trichrome or iron hematoxylin). As with most intestinal protozoa, the trophozoite form is usually seen in diarrheic or soft stools.

*Pentatrichomonas* trophozoites tend to stain very pale and can be easily missed, even on the permanent stained smear. Often one can see the axostyle protruding from the bottom of the organism. On the permanent stained smear, look for the small granules that may be clustered along the axostyle. These small granules are often typically found in trichomonads.

These trophozoites can easily be confused with those of other small flagellates such as *Enteromonas hominis* and *Retortamonas intestinalis*.

There are no known cyst forms.

## Additional Information

The trophozoites live in the cecal area of the large intestine and feed on bacteria. The organism is not considered invasive.

When urine is examined for *T. vaginalis* (pathogen), it is important not to accidentally misidentify *P. hominis* as *T. vaginalis* if the urine is contaminated with fecal material. *T. vaginalis* is a sexually transmitted disease, and incorrect organism identification could have multiple ramifications including risk management issues, particularly if the urine specimen is from a child (potential child abuse).

Since there is no known cyst stage, transmission probably occurs in the trophic form. If ingested in a protecting substance such as milk, these organisms can survive passage through the stomach and small intestine in patients with achlorhydria. *P. hominis* cannot be transplanted into the vagina, the natural habitat of *T. vaginalis*. The incidence of this organism is relatively low, but it tends to be recovered more often than *E. hominis* or *R. intestinalis*. The infection is diagnosed more often in warm climates and in children. Because of the fecal-oral transmission route, preventive measures should emphasize improved hygienic and sanitary conditions.

## *Trichomonas tenax*

*T. tenax* was first recovered from tartar from the teeth. Its distribution is worldwide. There is no known cyst stage. Transmission is assumed to be by direct contact or use of contaminated dishes and glasses. Prevalence rates vary from 0 to 25%. Although *T. tenax* is considered a harmless commensal in the mouth, there are reports of respiratory infections and thoracic abscesses, particularly in patients with underlying cancers or other lung diseases.

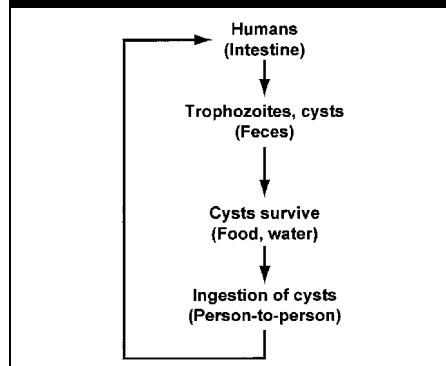
## PROTOZOA • Flagellates (Intestinal)

### *Enteromonas hominis*

### *Retortamonas intestinalis*

<b>Pathogenic</b>	No
<b>Disease</b>	None
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	None
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *E. hominis* and *R. intestinalis* in stool specimens. Microscopic examination of a direct saline wet mount may reveal motile trophozoites or nonmotile cyst forms. The concentration technique is helpful in demonstrating these organisms, but *the most important technique for the recovery and identification of protozoan organisms is the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

These organisms can be easily missed, even with multiple stool examinations and the permanent stained smear.

Both flagellates can be easily confused with other small flagellates.

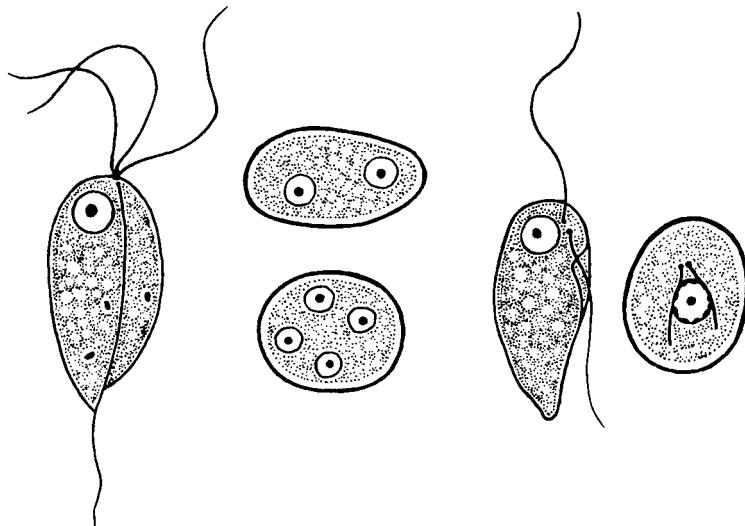
These organisms are rarely found in clinical specimens, probably because of the difficulties in accurate identification of small flagellates, even when the permanent stained smear is used. Infection is through ingestion of the cysts, and improved hygienic conditions would certainly prevent spread of the infection. *E. hominis* and *R. intestinalis* are considered nonpathogens; thus, no therapy is indicated.

### General Comments

Although many people worldwide may be infected with *E. hominis* or *R. intestinalis*, the organisms are not always seen, particularly if a permanent stained smear is not examined. *R. intestinalis* has been recovered from both warm and temperate areas of the world. It has also been found in certain groups such as mental hospital patients. Another challenge is correct identification and differentiation between these organisms and other intestinal protozoa that may be pathogenic (*G. lamblia*).

These organisms are currently classified with the protozoa (flagellates).

The presence of these organisms should always be reported, since nonpathogenic organisms are transmitted the same way as pathogens. The presence of these protozoa indicates that the patient has ingested something (food, water, etc.) contaminated with fecal material. If nonpathogenic protozoa are found, pathogens may also be found on additional examination of the specimen.



Images from left to right: *E. hominis* trophozoite and cysts; *R. intestinalis* trophozoite and cyst.

### Description (Trophozoite)

The standard O&P exam is recommended for recovery and identification of *E. hominis* and *R. intestinalis* in stool specimens. The trophozoites of *E. hominis* are somewhat pear shaped, measuring approximately 4 to 10  $\mu\text{m}$  by 3 to 6  $\mu\text{m}$ . There is no cytostome, and the flagella are rarely visible unless motile organisms are seen.

The trophozoites of *R. intestinalis* are elongate pyriform or ovoidal. They measure 4 to 9  $\mu\text{m}$  long by 3 to 4  $\mu\text{m}$  wide. This flagellate has a cytostome, which may be difficult to see, even in a permanent stained smear. Flagella are difficult to see unless there is obvious motility in a wet preparation. Morphology can be confirmed by using a permanent stained smear, particularly when the cytostome is visible.

As with most intestinal protozoa, the trophozoite form is usually seen in diarrheic or soft stools, while the cyst stage is seen in the more normal stool or when the patient is asymptomatic.

These trophozoites can be easily confused with those of *Chilomastix mesnili* and *Pentatrichomonas hominis*.

### Description (Cyst)

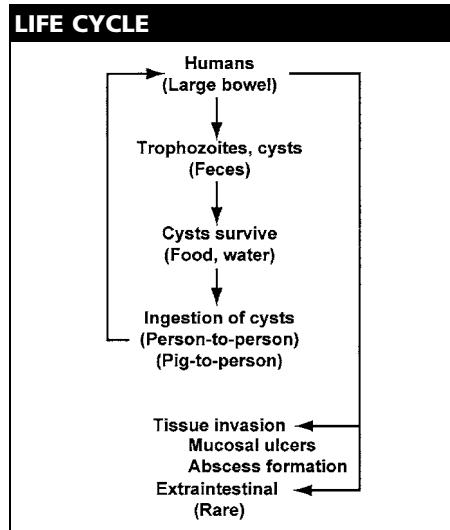
Trophozoites discharge their undigested food and begin to round up prior to precyst and cyst formation. The cyst of *E. hominis* measures approximately 6 to 10  $\mu\text{m}$  by 4 to 6  $\mu\text{m}$  and tends to be oval. There are two nuclei, and the cyst may mimic a two-nucleated *Endolimax nana* cyst. However, *E. nana* cysts containing two nuclei are quite rare in most clinical specimens. Also, *E. hominis* is not often reported, perhaps in part because of its small size and the difficulties in identification. Even on a permanent stained smear, this organism is often difficult to identify accurately.

The cysts of *R. intestinalis* are somewhat pear shaped, measuring 4 to 9  $\mu\text{m}$  long and 4 to 6  $\mu\text{m}$  wide. Both trophozoite and cyst have a single nucleus. These cysts can be confused with other small flagellate cysts, especially *Enteromonas hominis* cysts. The typical "bird's beak" structure of the fibrils within the cyst may not always be easy to see; these cysts occasionally mimic those of *Chilomastix mesnili*.

## PROTOZOA • Ciliates (Intestinal)

### Balantidium coli

<b>Pathogenic</b>	Yes
<b>Disease</b>	Balantidiasis
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	Intermittent diarrhea, abdominal pain, nausea, anorexia, malaise, fatigue, poor weight gain
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission, also pig-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables; prevention of human-pig contact, hygienic rearing of pigs



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *B. coli* in stool specimens. Microscopic examination of a direct saline wet mount may reveal motile trophozoites or cyst forms. The concentration technique is very useful in demonstrating these organisms, but *the most important technique for the recovery and identification of protozoan organisms is generally the permanent stained smear* (normally stained with trichrome or iron hematoxylin). A minimum of three specimens collected over not more than 10 days is often recommended.

However, in a permanent stained smear, these organisms take up so much stain that they can be confused with artifact material and/or helminth eggs. Organism identification is usually confirmed from microscopic examination of the concentration sediment.

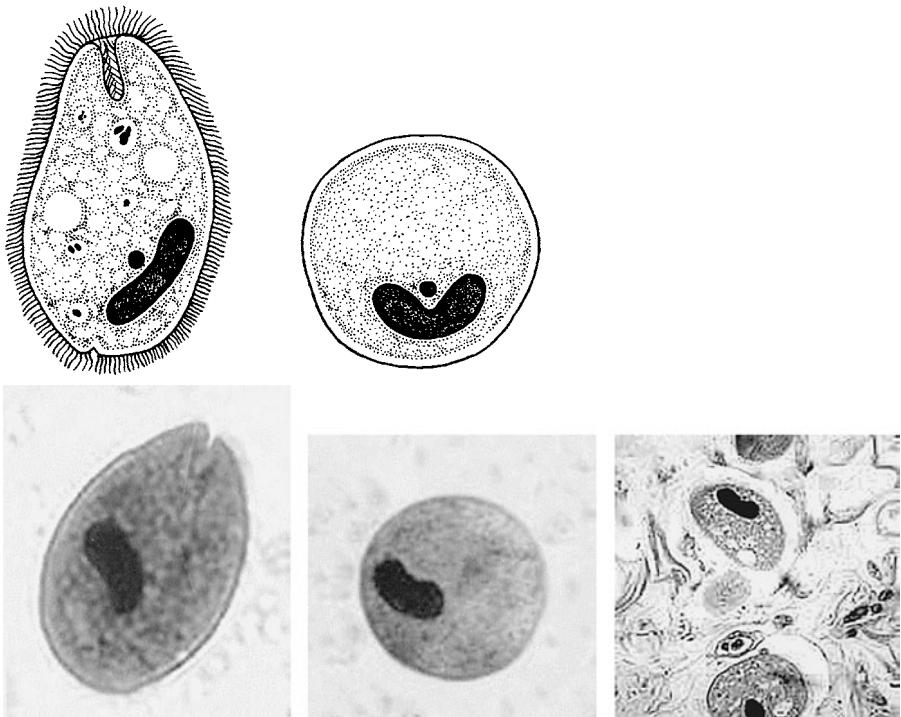
**Note:** In areas where pigs are the main domestic animal, the incidence of human infection can be quite high. Particularly susceptible are persons working as pig farmers or in slaughterhouses (28% infection rate in New Guinea). Human infection is fairly rare in temperate areas, although once the infection is established, it can develop into an epidemic, particularly where poor environmental sanitation and personal hygiene are found. This situation has been seen in mental hospitals in the United States.

### General Comments

*B. coli* is widely distributed in hogs, particularly in warm and temperate climates, and in monkeys in the tropics. Human infection is found in warmer climates, sporadically in cooler areas, and in institutionalized groups with low levels of personal hygiene. *B. coli* is rarely recovered in clinical specimens within the United States. This organism is currently classified with the protozoa (ciliates).

Some individuals with *B. coli* infections are asymptomatic, whereas others have symptoms of severe dysentery similar to those seen with amebiasis. Symptoms usually include diarrhea or dysentery, tenesmus, nausea, vomiting, anorexia, and headache. Insomnia, muscular weakness, and weight loss have also been reported. The diarrhea may persist for weeks to months prior to the development of dysentery. There may be tremendous fluid loss with a type of diarrhea similar to that seen in cholera or in some coccidial infections.

*B. coli* can invade tissue. It may penetrate the mucosa on contact, with cellular infiltration in the area of the developing ulcer. Some of the abscess formations may extend to the muscular layer. The ulcers may vary in shape, and the ulcer bed may be full of pus and necrotic debris. Although the number of cases is small, extraintestinal disease has been reported.



Images from left to right: (Top row) drawings of *B. coli* trophozoite and cyst; (bottom row) *B. coli* trophozoite, cyst, and trophozoites in intestinal tissue.

### Description (Trophozoite)

The trophozoite is quite large, oval, and covered with short cilia; it measures ca. 50 to 100  $\mu\text{m}$  long and 40 to 70  $\mu\text{m}$  wide. It can easily be seen in a wet preparation on lower power. The anterior end is somewhat pointed and has a cytostome; in contrast, the posterior end is broadly rounded. The cytoplasm contains many vacuoles with ingested bacteria and debris. There are two nuclei, a very large bean-shaped macronucleus and a smaller round micronucleus. The organisms normally live in the large intestine.

Trophozoites are occasionally found in tissue; however, dissemination from the intestine is rare. Although the number of cases is small, extraintestinal disease has been reported (peritonitis, urinary tract, inflammatory vaginitis).

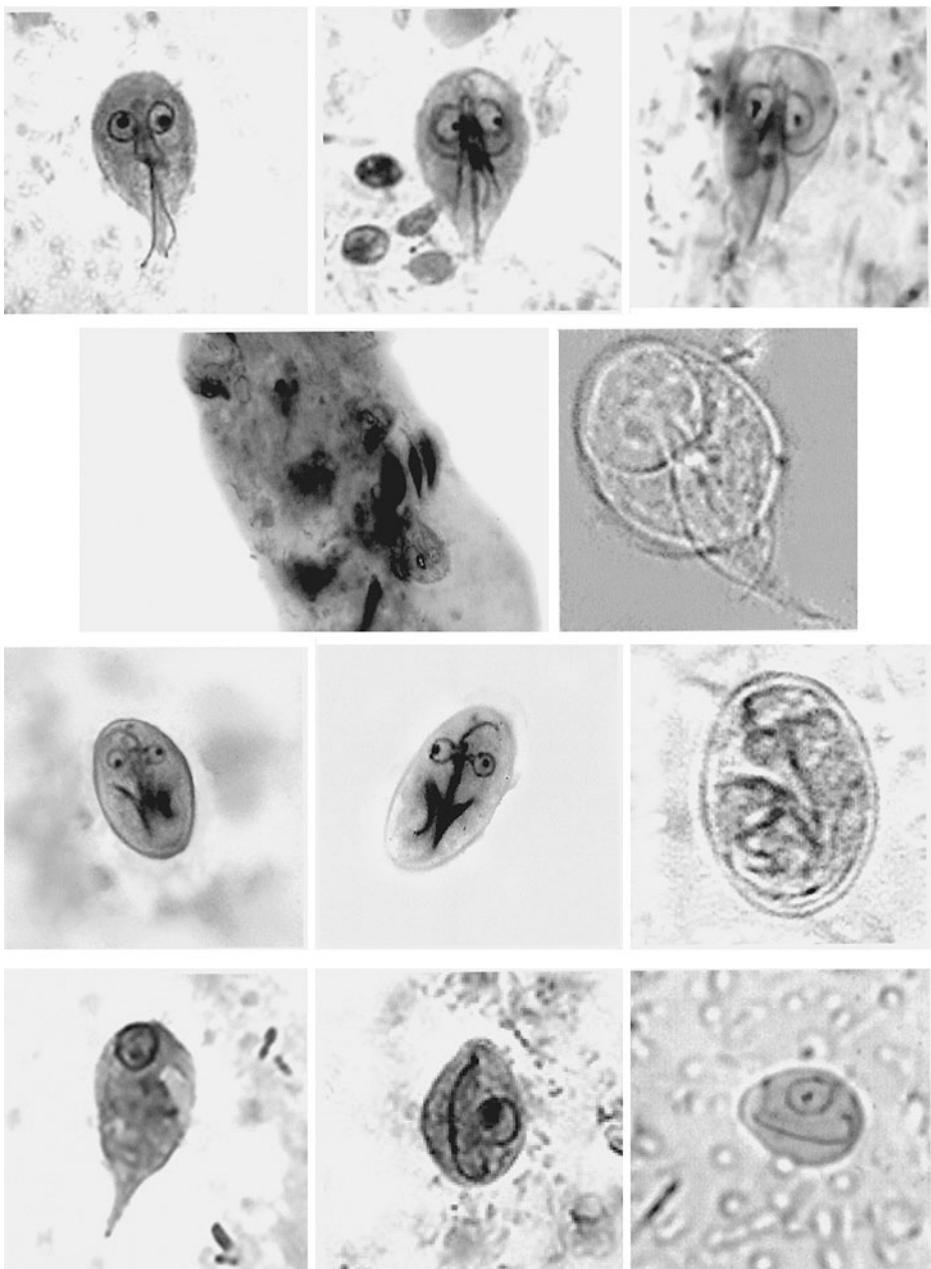
### Description (Cyst)

The cyst is formed as the trophozoite moves down the intestine. Nuclear division does not occur in the cyst; therefore, only two nuclei are present, the macronucleus and the micronucleus. The cysts measure 50 to 70  $\mu\text{m}$ .

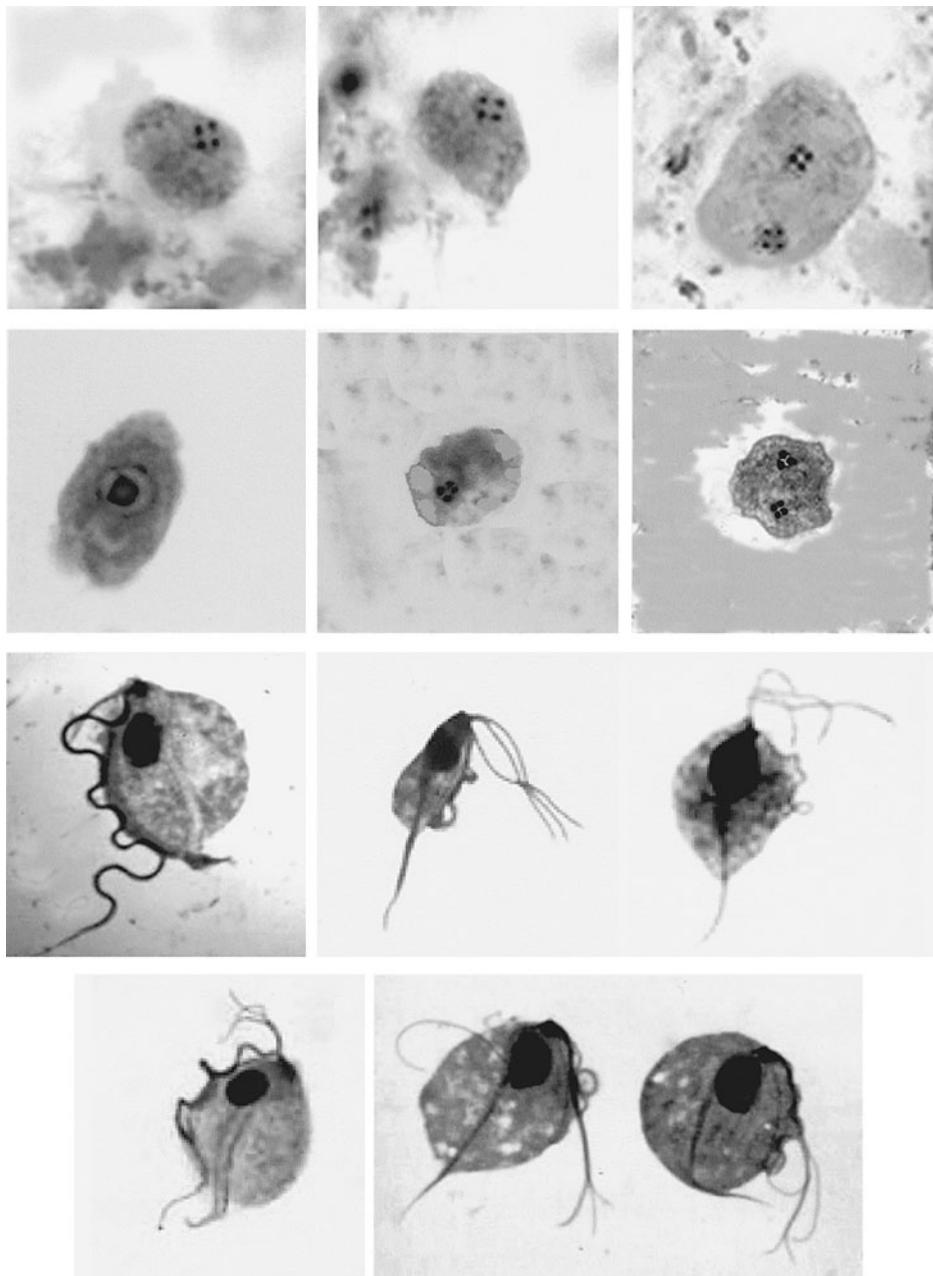
Cysts are never found in tissues, and the trophozoite form invades the intestinal tract on occasion. However, in a more formed stool, both forms can be found.

### Additional Information

The parasite is endemic in Yunan province in China, with infection rates of up to 4.24% in some villages. Apparently, the highest rate (up to 20% prevalence) has been seen in areas of Indonesia; there appears to be a very close association between pigs and humans in this area. Certainly human-to-human contact has been widely documented, particularly in psychiatric units within the United States and other countries.



**Plate 7.3** Row 1: *Giardia lamblia* trophozoites; row 2: *Giardia lamblia* trophozoites in mucus (left) and *Giardia lamblia* trophozoite (wet mount) (right); row 3: *Giardia lamblia* cysts (left and middle) and *Giardia lamblia* cyst (wet mount) (right); row 4 (left to right): *Chilomastix mesnili* trophozoite, *Chilomastix mesnili* cyst, and *Chilomastix mesnili* cyst (wet mount).



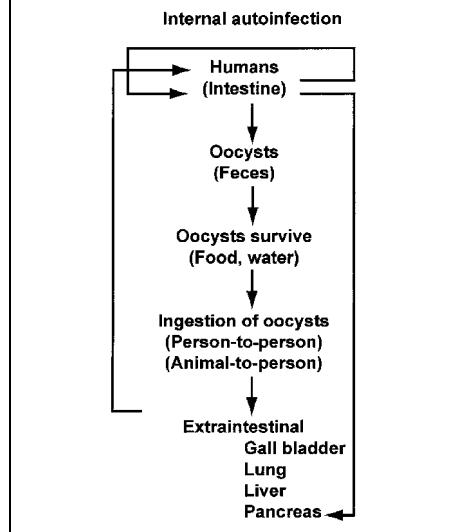
**Plate 7.4** Row 1: *Dientamoeba fragilis* trophozoites; row 2: *Dientamoeba fragilis* trophozoite (the nuclear chromatin has not yet completely fragmented; it can therefore mimic *Endolimax nana*) (left) and *Dientamoeba fragilis* trophozoites (middle and right); row 3 (left to right): *Pentatrichomonas hominis* trophozoite, *Trichomonas tenax* trophozoite, and *Trichomonas vaginalis* trophozoite; row 4: *Trichomonas vaginalis* trophozoites. *Trichomonas vaginalis*: See section on Protozoa, Flagellates (Other Body Sites).

## PROTOZOA • Coccidia (Intestinal)

### *Cryptosporidium* spp.

<b>Pathogenic</b>	Yes
<b>Disease</b>	Cryptosporidiosis
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine, disseminated infection in severely compromised patients
<b>Symptoms</b>	Nausea, low-grade fever, abdominal cramps, anorexia, and 5 to 10 watery, frothy bowel movements per day
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission, also animal-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, water testing, adequate washing of contaminated fruits and vegetables

### LIFE CYCLE



### Diagnosis

Oocysts recovered in clinical specimens are difficult to see without special staining techniques such as the modified acid-fast, Kinyoun's, and Giemsa methods or the newer direct FA or EIA methods. The four sporozoites may be seen within the oocyst wall in some of the organisms, although in freshly passed specimens they are not always visible. The standard O&P concentration is recommended ( $500 \times g$  for 10 min), with the concentrate being used for all smears and for the FA procedure. Shorter centrifugation at lower speeds (often mentioned in some procedures) may not guarantee recovery of the oocysts. Multiple stool specimens may have to be examined to diagnose the infection, particularly when dealing with formed stool specimens.

Uncentrifuged fresh, frozen, or fixed fecal material can be used for the antigen detection immunoassays (EIA, FA, rapid cartridges); except for FA, these procedures do not rely on visual identification of the oocysts but on antigen detection. The selection of fresh or frozen stool rather than fixed specimens will depend on testing parameters; currently, if the test reagents or kit format includes *Entamoeba histolytica/E. dispar* or *E. histolytica* alone, the test format requires fresh or frozen stool. If the test format includes *Cryptosporidium* and/or *Giardia*, fresh, frozen, or preserved stools can be used. The fecal immunoassays have excellent specificity and sensitivity and result in a significantly increased detection rate over conventional staining methods (modified acid-fast stains). Some of these reagents, particularly the combination DFA product used to identify both *Giardia lamblia* cysts and *Cryptosporidium* spp. oocysts, are being widely used in water testing and outbreak situations.

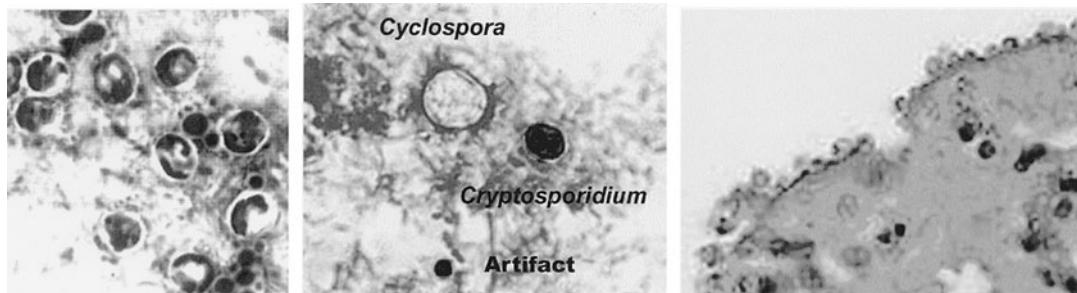
**Note:** Frozen specimens should not be used for FA; the freeze-thaw cycle destroys the oocysts.

### General Comments

*Cryptosporidium* developmental stages occur in an intracellular, extracytoplasmic location. Each stage is within a parasitophorous vacuole of host cell origin; however, the vacuole containing the organism is located at the microvillus surface of the host cell. The thin-walled autoinfective oocyst may explain why a small inoculum can lead to overwhelming infection in a susceptible host and why immunosuppressed patients may have persistent, life-threatening infections in the absence of documentation of repeated exposure to oocysts. The stages found on the microvillus surface are 1  $\mu m$ , and the oocysts recovered in stool specimens are 4 to 6  $\mu m$ .

Oocysts undergo sporogony while they are in the host cells and are already infective when passed in the stool. Approximately 20% of *Cryptosporidium* oocysts do not form the thick two-layered, environmentally resistant oocyst wall. The four sporozoites within this autoinfective stage are surrounded by a single-unit membrane. After release from a host cell, this membrane ruptures, and the invasive sporozoites penetrate the microvillus region of other cells within the intestine and reinitiate the life cycle.

The prepatent period from the time of ingestion of infective oocysts to completion of the life cycle with excretion of newly developed oocysts in the human is ca. 4 to 22 days. Characteristic antibody responses develop following infection, and persons with preexisting antibodies may be less likely to develop illness, particularly when infected with low oocyst doses.



**Images from left to right:** *Cryptosporidium* oocysts (4 to 6  $\mu\text{m}$ ) stained with modified acid-fast stain. These oocysts stain more consistently than those of *Cyclospora cayetanensis*. In some oocysts, the sporozoites can be seen; oocysts are infectious even if the sporozoites are not visible. In the middle are *C. cayetanensis* and *Cryptosporidium*, and an artifact can be seen (note that the *Cyclospora* oocyst did not retain the stain). On the right are developing *Cryptosporidium* at the microvillous surface (courtesy of Armed Forces Institute of Pathology).

## Description

In histology preparations, *Cryptosporidium* developmental stages are found at all levels of the intestinal tract, with the jejunum being the most heavily infected site. Routine H&E staining is sufficient to demonstrate these parasites. Using regular light microscopy, the organisms are visible as small (~1- to 3- $\mu\text{m}$ ) round structures aligned along the brush border. They are intracellular but extracytoplasmic and are found in parasitophorous vacuoles. Developmental stages are more difficult to identify without using transmission electron microscopy.

In severely compromised patients, *Cryptosporidium* occurs in other body sites, primarily the lungs, as disseminated infections. Within tissue, confusion with *Cyclospora cayetanensis* is unlikely since *Cyclospora* oocysts are approximately 8 to 10  $\mu\text{m}$  and the developmental stages occur within a vacuole at the luminal end of the enterocyte, rather than at the brush border. Developmental stages of *Isospora belli* also occur within the enterocyte, so they should not be confused with *Cryptosporidium*.

Microsporidia are present in ca. 30% of severely immunocompromised patients ( $\leq 100 \text{ CD4}^+$  cells) with cryptosporidiosis. The diagnostic procedures for identification of *Cryptosporidium* spp. are not appropriate for identification of microsporidial spores. Modified trichrome stains and optical brightening agents (calcofluor white) can be used for that purpose.

**Note:** *C. parvum* is found in animals and humans; *C. hominis* is found in humans. Differentiation is not possible from routine microscopy.

## Additional Information

In immunocompetent individuals, clinical symptoms include nausea, low-grade fever, abdominal cramps, anorexia, and 5 to 10 watery, frothy bowel movements per day, which may be followed by constipation. Some patients present with diarrhea, while others have relatively few symptoms, particularly later in the infection. In patients with the typical watery diarrhea, the stool specimen contains very little fecal material, mainly water and mucus flecks. Often the organisms are entrapped in the mucus. A patient with a normal immune system will have a self-limited infection.

In immunocompromised individuals, the duration and severity of diarrhea depend on the immune status of the patient. Most severely immunocompromised patients cannot self-cure, the illness becomes progressively worse, and the sequelae may be a major factor leading to death. In these patients, *Cryptosporidium* infections are not always confined to the gastrointestinal tract; additional symptoms (respiratory problems, cholecystitis, hepatitis, and pancreatitis) have been associated with extraintestinal infections.

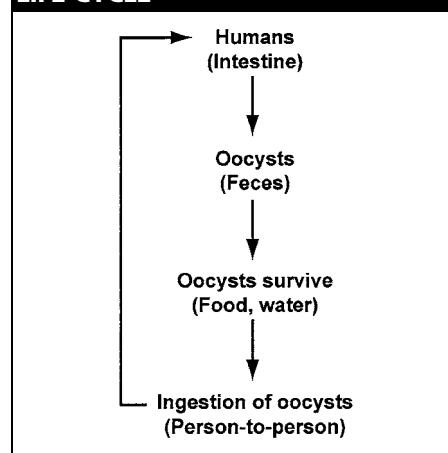
*Cryptosporidiosis* tends to be self-limiting in patients who have an intact immune system. In patients who are receiving immunosuppressive agents, one therapeutic option would be to discontinue the regimen. Other approaches with specific therapeutic drugs have been tried, but with inconclusive results. HAART has had a dramatic impact on cryptosporidiosis in AIDS patients, leading to an increased CD4 count. Resolution of the cryptosporidiosis diarrhea may be related to the enhanced CD4 count rather than any change in the viral load or any therapeutic impact of the drugs.

## PROTOZOA • Coccidia (Intestinal)

### *Cyclospora cayetanensis*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Cyclosporiasis
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	Nausea, low-grade fever, fatigue, anorexia, and up to seven bowel movements per day; relapses can occur
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission; animal-to-human not yet documented
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The organisms stain orange with safranin and are acid-fast variable, with some organisms staining deep red with a mottled appearance but no internal organization. Clean wet mounts show nonrefractile spheres that are acid-fast variable with the modified acid-fast stain; those that are unstained appear as glassy, wrinkled spheres. Modified acid-fast stains stain the oocysts light pink to deep red; some contain granules or have a bubbly appearance ("wrinkled cellophane"). When the modified acid-fast stain is used for *Cryptosporidium* and detects other similar but larger structures (approximately twice the size of *Cryptosporidium* oocysts), they may be *Cyclospora*. All acid-fast oocysts should be measured, particularly if they appear to be somewhat larger than those of *Cryptosporidium*. The oocysts autofluoresce strong green (450 to 490 nm excitation filter) or intense blue (365 nm excitation filter) under UV epifluorescence. During concentration (formalin/ethyl acetate) of stool specimens, centrifugation should be for 10 min at 500 × g to guarantee recovery of oocysts. The standard O&P concentration is recommended, with the concentrate being used for all smears and for the FA procedure.

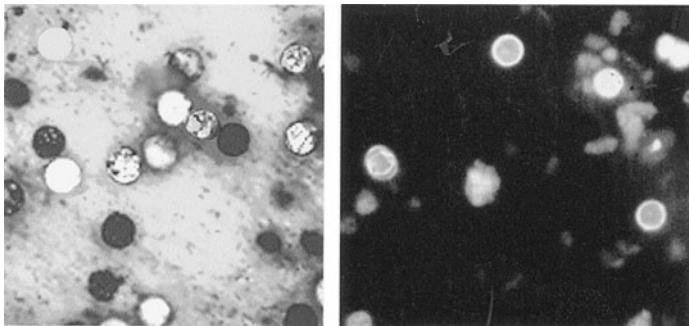
Apparently, *Cyclospora* oocysts have a much thicker oocyst wall than *Cryptosporidium* oocysts. Also, the oocyst contents of *Cyclospora* tend to be more granular.

### General Comments

The oocysts that had previously been recovered from human stool were immature, so the structure of the mature oocyst had not been seen. Unsporulated oocysts are passed in the stool; oocyst maturation takes approximately 5 to 13 days, so the mature stage may not have been seen in human specimens. The oocyst contains two sporocysts, each containing two sporozoites, a pattern which places these organisms in the coccidian genus *Cyclospora*. The species name comes from the university where it was initially studied (Universidad Peruana Cayetano Heredia).

In patients with *Cyclospora* in their stool specimens, parasites with coccidian characteristics have been found within the jejunal enterocytes. The entire life cycle can be completed within a single host. Information on potential reservoir hosts has yet to be defined.

Developmental stages of *C. cayetanensis* usually occur within epithelial cells of the jejunum and lower portion of the duodenum. *Cyclospora* infection reveals characteristics of a small bowel pathogen, including upper gastrointestinal symptoms, malabsorption of D-xylose, weight loss, and moderate to marked erythema of the distal duodenum. Histopathology in small bowel biopsy specimens reveals acute and chronic inflammation, partial villous atrophy, and crypt hyperplasia.



**Images from left to right:** The first image shows *C. cayetanensis* oocysts (8 to 10  $\mu\text{m}$ ) stained with modified acid-fast stain. There is a range of clear to deeply stained oocysts; there is a lot of variation with modified acid-fast staining (modified acid-fast variable). The second image shows autofluorescent oocysts on filters commonly used for calcofluor white staining.

## Additional Information

Individuals of all ages, including both immunocompetent and immunosuppressed, can become infected. In Peru, infections have shown some seasonal variation, with peaks during April to June. This pattern is similar to that seen with *Cryptosporidium* infections in Peru.

Transmission of *Cyclospora* is thought to be fecal-oral, although direct person-to-person transmission has not been well documented and may not be a factor, since sporulation takes days. Outbreaks linked to contaminated water and various types of fresh produce (raspberries, basil, baby lettuce leaves) have been reported.

There is generally 1 day of malaise and low-grade fever, with rapid onset of diarrhea of up to seven stools per day. There may also be fatigue, anorexia, vomiting, myalgia, and weight loss with remission of self-limiting diarrhea in 3 to 4 days, followed by relapses lasting from 4 to 7 weeks.

In patients with AIDS, symptoms may persist for 12 weeks; biliary disease has also been reported, as has diarrhea alternating with constipation (this is not uncommon in a number of protozoal gastrointestinal infections). Clinical clues include unexplained prolonged diarrheal illness during the summer in any patient and in persons returning from tropical areas. Most infected individuals had intermittent diarrhea for 2 to 3 weeks, and many complained of intense fatigue, as well as anorexia and myalgia, during the illness. The clinical presentation is similar to that of patients infected with *Cryptosporidium*.

The disease appears to be self-limiting within a few weeks. Trimethoprim-sulfamethoxazole (TMP-SMX) is the drug of choice; relief of symptoms has been seen 1 to 3 days posttreatment. AIDS patients may need higher doses and long-term maintenance treatment. However, recurrence of symptoms occurs within 1 to 3 months post-treatment in over 40% of patients.

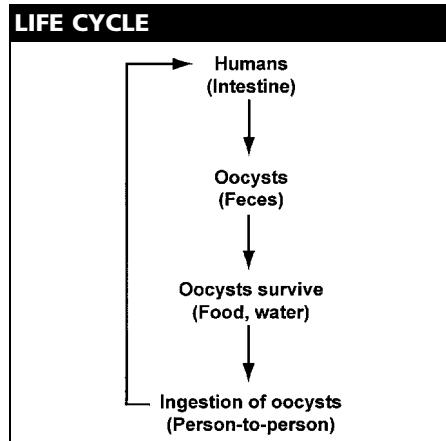
On modified acid-stained smears, the oocysts stain light pink to deep purple, and some contain granules or have a bubbly appearance. Those that do not stain may have a wrinkled appearance. A strong decolorizer should not be used; 1% sulfuric acid is recommended and also works well for modified acid-fast stains for *Cryptosporidium spp.* and/or 1% belli acid-fast stains. The original 3 to 5% sulfuric acid is usually too strong for *Cyclospora* and removes too much color. Even with the 1% acid decolorizer, some oocysts may appear clear or very pale.

In the modified safranin technique, the oocysts uniformly stain a brilliant reddish orange if fecal smears are heated in a microwave during staining. The stained slide can also be examined by epifluorescence microscopy first, and suspect oocysts can be confirmed by bright-field microscopy.

## PROTOZOA • Coccidia (Intestinal)

### *Isospora (Cystoisospora) belli*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Isosporiasis
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine
<b>Symptoms</b>	Diarrhea, which may last for months to years, weight loss, abdominal colic, and fever
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables



### Diagnosis

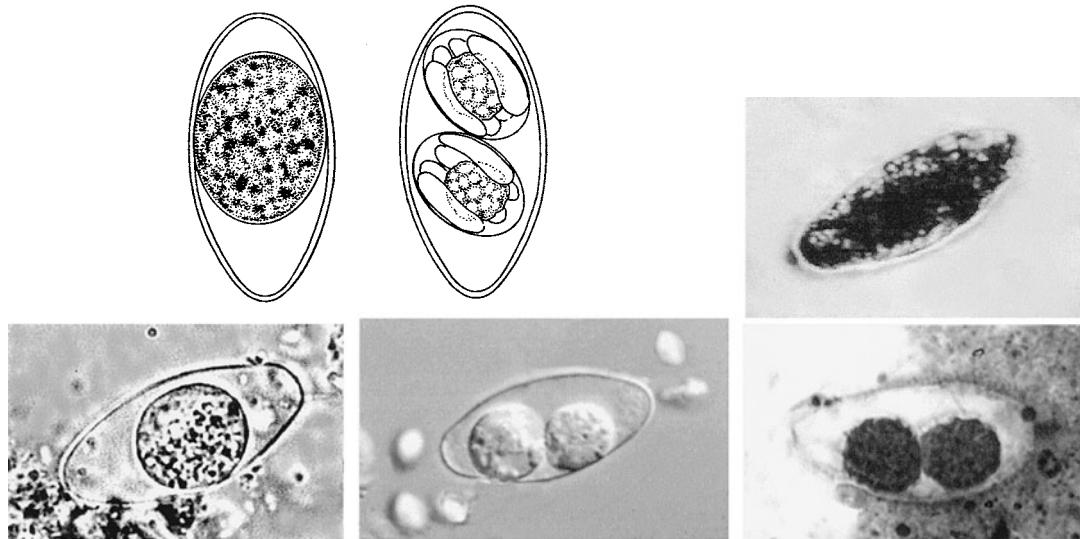
Examination of a fecal specimen for the oocysts is recommended. However, wet preparation examination of fresh material either as the direct smear or as concentrated material is recommended rather than the permanent stained smear. The oocysts are very pale and transparent and can easily be overlooked. They can also be very difficult to see if the concentration sediment is performed from PVA-preserved stool. The light level should be reduced, and additional contrast should be obtained with the microscope for optimal examination conditions. It is also possible to have a positive biopsy specimen and not recover the oocysts in the stool because of the small numbers of organisms present. These organisms are modified acid-fast positive and can also be demonstrated by using auramine-rhodamine stains. Organisms tentatively identified with auramine-rhodamine stains should be confirmed by wet smear examination or acid-fast stains, particularly if the stool contains other cells or excess artifact material (more normal stool consistency).

The patent period is not known but may be only 15 days. Chronic infections develop in some patients, and oocysts can be shed for several months to years. In one particular case, an immunocompetent individual had symptoms for 26 years and *I. belli* was recovered in stool a number of times over a 10-year period.

### General Comments

Certain tropical areas in the Western Hemisphere appear to contain some well-defined locations of endemic infections. These organisms can infect both adults and children, and intestinal involvement and symptoms are generally transient unless the patient is immunocompromised. *I. belli* has also been implicated in traveler's diarrhea.

Schizogonic and sporogonic stages in the life cycle of *I. belli* have been found in human intestinal mucosal biopsy specimens. Development in the intestine usually occurs within the epithelial cells of the distal duodenum and proximal jejunum. Eventually oocysts are passed in the stool; they are long and oval (20 to 33 µm by 10 to 19 µm). Usually the oocyst contains only one immature sporont, but two may also be present. Continued development occurs outside the body to form two mature sporocysts, each containing four sporozoites, which can be recovered from the fecal specimen. The sporulated oocyst is the infective stage that will excyst in the small intestine, releasing the sporozoites that penetrate the mucosal cells and initiate the life cycle. The life cycle stages (schizonts, merozoites, gametocytes, gametes, and oocysts) are structurally similar to those seen in the other coccidia.



Images from left to right: (Top row) Drawing of an immature oocyst, drawing of a mature oocyst containing two sporocysts, and an immature oocyst stained with modified acid-fast stain (note that the entire oocyst stains). (Bottom row) Immature oocyst, mature oocyst (wet mounts), and mature oocyst stained with modified acid-fast stain.

## Additional Information

*I. belli* is thought to be the only *Isospora* species that infects humans, and no other reservoir hosts are recognized for this infection. Transmission is through ingestion of water or food contaminated with mature, sporulated oocysts. Sexual transmission by direct oral contact with the anus or perineum has been postulated but is probably much less common. The oocysts are very resistant to environmental conditions and may remain viable for months if kept cool and moist. Diagnostic methods for laboratory examinations may tend to miss the organisms. Since transmission is via the infective oocysts, prevention centers on improved personal hygiene measures and sanitary conditions to eliminate possible fecal-oral transmission from contaminated food, water, and possibly environmental surfaces.

Oocysts passed during diarrhea contain the single developing sporont; oocysts passed in a more normal stool contain the two more mature sporocysts. The two sporocysts (each containing sporozoites) are seen within the oocyst wall.

Clinical symptoms include diarrhea, which may last for months to years, weight loss, abdominal colic, and fever; diarrhea is the main symptom. Bowel movements (usually 6 to 10 per day) are watery to soft, foamy, and offensive smelling, suggesting a malabsorption process. Eosinophilia is found in many patients, recurrences are quite common, and the disease is more severe in infants and young children.

Patients who are immunosuppressed, particularly those with AIDS, often present with profuse diarrhea associated with weakness, anorexia, and weight loss. In one patient with a well-documented long-term infection, a series of biopsies showed a markedly abnormal mucosa with short villi, hypertrophied crypts, and infiltration of

the lamina propria with eosinophils, neutrophils, and round cells. Charcot-Leyden crystals derived from eosinophils have also been found in the stools of infected patients. The diarrhea and other symptoms may continue in compromised patients, even those on immunosuppressive therapy when the regimen of therapy is discontinued. This infection has been found in homosexual men, all of whom were immunosuppressed and had diarrhea for several months.

Extraintestinal infections have occurred in AIDS patients. Microscopic findings associated with *I. belli* infection have been seen in the walls of both the small and large intestines, mesenteric and mediastinal lymph nodes, liver, and spleen. Finding the merozoites within the lymphatic channels documents a means of dissemination to lymph nodes and other tissues.

Effective eradication has been achieved by using co-trimoxazole, TMP-SMX, pyrimethamine-sulfadiazine, primaquine phosphate-nitrofurantoin, and primaquine phosphate-chloroquine phosphate. Ineffective drugs include dithiazanine, tetracycline, metronidazole, phanquone, and quinacrine hydrochloride. The drug of choice is TMP-SMX, which is classified as investigational for treatment of this infection.

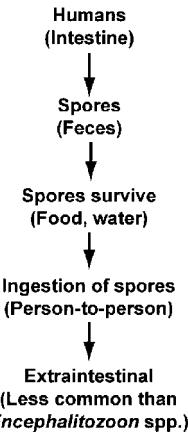
It has been recommended that physicians consider *I. belli* in AIDS patients with diarrhea who have immigrated from or traveled to Latin America, are Hispanics born in the United States, are young adults, or have not received prophylaxis with TMP-SMX for *Pneumocystis jiroveci*. It has also been recommended that AIDS patients who travel to Latin America and other developing countries be advised of the waterborne and food-borne transmission of *I. belli*; they should consider chemoprophylaxis.

## PROTOZOA • Microsporidia (Intestinal)

### *Enterocytozoon bieneusi*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Microsporidiosis
<b>Acquired</b>	Fecal-oral transmission; contaminated food and water
<b>Body site</b>	Intestine; dissemination can be seen in compromised patients
<b>Symptoms</b>	Intractable diarrhea, fever, malaise, and weight loss
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P concentration is recommended ( $500 \times g$  for 10 min), with the concentrate being used for all smears and for FA. Recommendations include using modified trichrome stains in which the chromotrope 2R component added to the stain is 10 times the concentration normally used in the routine trichrome stain for stool. Stool preparations must be very thin, the staining time is 90 min, and the slide must be examined at  $\times 1,000$  (or higher) magnification. Unfortunately, there are many objects within stool material that are oval, stain pinkish with trichrome, and measure ca.  $1.5$  to  $3 \mu\text{m}$ . If this stain is used to identify microsporidia in stool, positive control material should be available for comparison. Additional modifications include the use of heat and a shorter staining time. Pretreatment of fecal specimens (1:1) with 10% KOH may provide a better-quality smear with the modified trichrome stains.

Another approach involves chemofluorescent agents (optical brightening agents) such as calcofluor, Fungi-Fluor, or Uvitex 2B. These reagents are sensitive but non-specific; objects other than microsporidial spores also fluoresce. This is a particular problem when examining stool specimens; both false-positive and false-negative results have been seen.

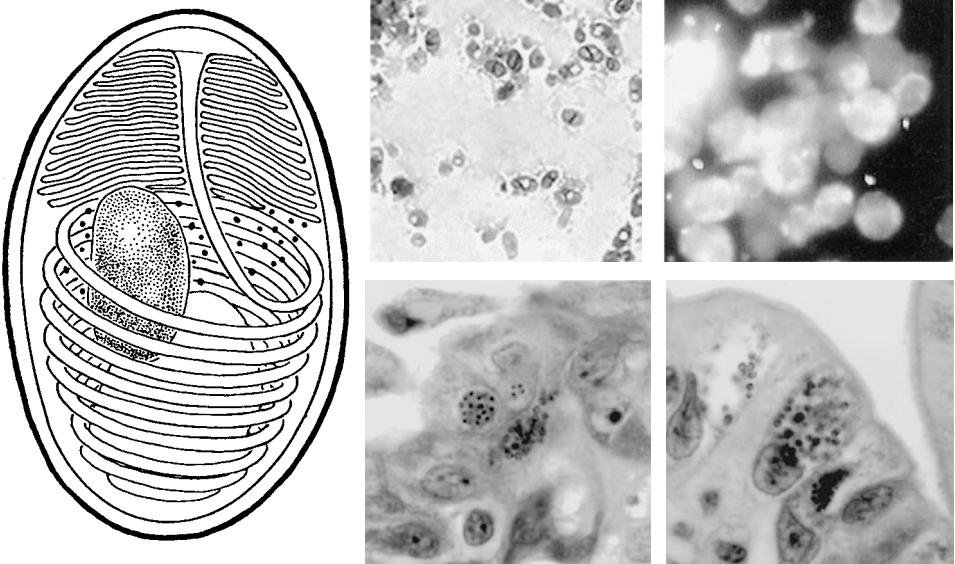
Fecal immunoassays for the microsporidia are under development.

### General Comments

Infection occurs with the introduction of infective sporoplasm through the polar tubule into the host cell. The microsporidia multiply extensively within the host cell cytoplasm; the life cycle includes repeated divisions by binary fission (merogony) or multiple fission (schizogony) and spore production (sporogony). Merogony and sporogony can occur in the same cell at the same time. During sporogony, a thick spore wall is formed, providing environmental protection for this infectious stage of the parasite. *E. bieneusi* spores are released into the intestinal lumen and are passed in the stool. They are environmentally resistant and can then be ingested by other hosts. There is also evidence for inhalation of spores and evidence in animals suggesting that human microsporidiosis may also be transmitted via the rectal route.

Seven genera have been isolated from humans: *Brachiola* (*Anncaliia*), *Encephalitozoon*, *Vittaforma*, *Pleistophora*, *Trachipleistophora*, *Enterocytozoon*, and *Microsporidium*, a catch-all genus for organisms not yet classified. Classification criteria include spore size, configuration of nuclei within the spores and developing forms, number of polar tubule coils within the spore, and relationship between the organism and host cell. These generic designations are frequently changed as more information becomes available.

*E. bieneusi* is the most common microsporidian found in humans, particularly in compromised patients. However, there are very few data regarding infections in immunocompetent hosts.



Images from left to right: The drawing illustrates the infective spore (1.5 to 2.5  $\mu\text{m}$ ) containing the coiled polar tubule. (Top left) Spores in stool, stained with Ryan modified trichrome blue (some of the spores show the horizontal "stripe" that indicates the presence of the polar tubule); (top right) calcofluor staining of spores in a urine sediment. (Bottom) Spore development in the human intestine. Note the small size of the spores in tissue.

## Additional Information

Microsporidiosis is an important emerging opportunistic infection in HIV-infected patients, and several hundred patients with chronic diarrhea have been seen worldwide. Up to 30% of patients infected with *Cryptosporidium* spp. may have concurrent infections with microsporidia. This emphasizes the importance of considering both organisms in compromised patients, particularly HIV patients, with diarrhea. The use of antiretroviral combination therapy has led to decreases in both cryptosporidiosis and microsporidiosis in this patient group. Microsporidia also cause disease in organ transplant recipients, children, travelers, contact lens wearers, and the elderly. Conjunctival and corneal epithelium infections occur in HIV patients, while corneal stroma and ulceration occur in immunocompetent individuals.

Symptoms include chronic intractable diarrhea, fever, malaise, and weight loss, similar to those of cryptosporidiosis or isosporiasis. These AIDS patients tend to have four to eight watery, nonbloody stools daily which can be accompanied by nausea and anorexia. There may be dehydration with mild hypokalemia and hypomagnesemia, as well as D-xylene and fat malabsorption. The patients tend to be severely immunodeficient, with a CD4 count always below 200 and often below 100. Dual infections with *E. bieneusi* and *E. intestinalis* have also been reported. Unfortunately, these infections do not respond to therapy with albendazole, unlike the infections with *Encephalitozoon* spp.

The microsporidia are obligate intracellular parasites that have been recognized in a variety of animals, particularly invertebrates. Typical sizes range from 1.5 to 5  $\mu\text{m}$  wide and 2 to 7  $\mu\text{m}$  long; organisms found in humans tend to be quite small (1.5 to 2  $\mu\text{m}$ ). Until the recent increased understanding of AIDS within the immunosuppressed population, awareness and understanding of human microsporidial infections have been marginal. Limited availability of EM has also affected the ability to recognize and diagnose these infections. However, the introduction of newer diagnostic methods has improved our diagnostic ability. The organisms are characterized by having spores containing a polar tubule, which is an extrusion mechanism for injecting the infective spore contents into host cells.

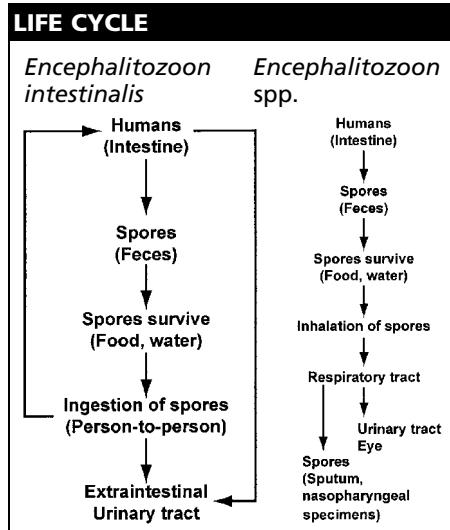
Microsporidial infections can be misdiagnosed in tissues and can be confused with *Cryptococcus neoformans* infections. Mucus granules in goblet cells can take up stain and be very confusing. Good preservation and thin tissue sections (1  $\mu\text{m}$ ) that have been resin embedded enhance the resolution of cellular detail. Demonstration of the coiled polar tube within spores is diagnostic for microsporidial infection.

## PROTOZOA • Microsporidia (Intestinal)

### *Encephalitozoon intestinalis*

### *Encephalitozoon* spp.

<b>Pathogenic</b>	Yes
<b>Disease</b>	Microsporidiosis
<b>Acquired</b>	Fecal-oral transmission, inhalation, fomites and direct inoculation; contaminated food and water
<b>Body site</b>	Intestine; dissemination to kidneys, lower airways, and biliary tract appears to occur via infected macrophages
<b>Symptoms</b>	Intractable diarrhea, fever, malaise, and weight loss
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables



### Diagnosis

The standard O&P concentration is recommended (500 × g for 10 min), with the concentrate being used for all smears and for FA. Recommendations include using modified trichrome stains in which the chromotrope 2R component added to the stain is 10 times the concentration normally used in the routine trichrome stain for stool. Stool preparations must be very thin, the staining time is 90 min, and the slide must be examined at ×1,000 (or higher) magnification. Unfortunately, there are many objects in stool material that are oval, stain pinkish with trichrome, and measure ca. 1.5 to 3 µm. If this stain is used to identify microsporidia in stool, positive control material should be available for comparison. Additional modifications include the use of heat and a shorter staining time. Pretreatment of fecal specimens (1:1) with 10% KOH may provide a better-quality smear with the modified trichrome stains.

Another approach involves the use of chemofluorescent agents (optical brightening agents) such as calcofluor, Fungi-Fluor, or Uvitex 2B. These reagents are sensitive but nonspecific; objects other than microsporidial spores also fluoresce. This is a particular problem when examining stool specimens; both false-positive and false-negative results have been seen.

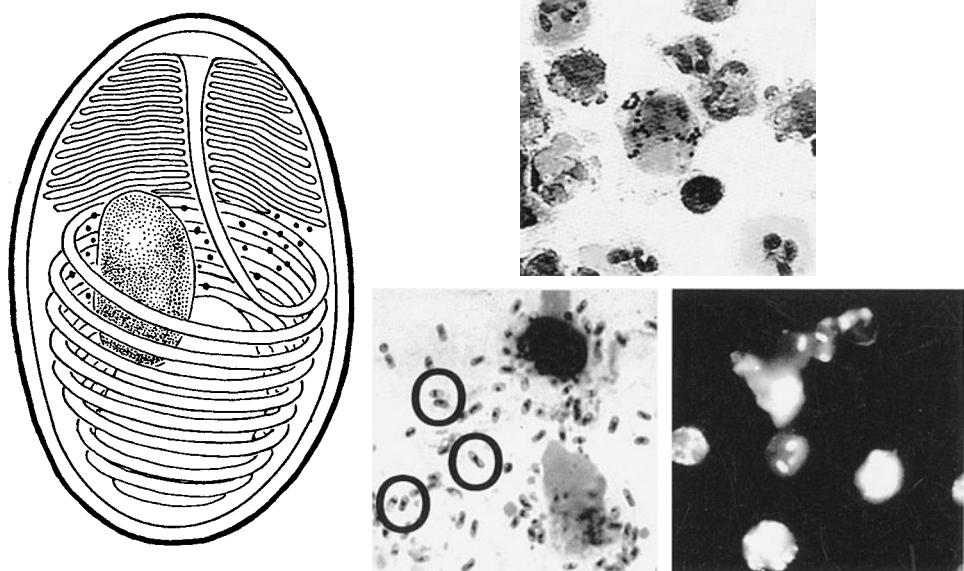
### General Comments

Infection occurs with the introduction of infective sporoplasm through the polar tubule into the host cell. The microsporidia multiply in the host cell cytoplasm; the life cycle includes repeated divisions by binary fission (merogony) or multiple fission (schizogony) and spore production (sporogony). Merogony and sporogony can occur in the same cell at the same time. During sporogony, a thick spore wall is formed, providing environmental protection for this infectious stage of the parasite.

Seven genera have been isolated from humans: *Brachiola* (*Anncaliia*), *Encephalitozoon*, *Vittaforma*, *Pleistophora*, *Trachipleistophora*, *Enterocytozoon*, and *Microsporidium*, a catch-all genus for organisms not yet classified. Classification criteria include spore size, configuration of nuclei within the spores and developing forms, number of polar tubule coils within the spore, and relationship between the organism and host cell.

*E. intestinalis* infects primarily small intestinal enterocytes, but infection does not remain confined to epithelial cells. It is also found in lamina propria macrophages, fibroblasts, and endothelial cells. Dissemination to the kidneys, lower airways, and biliary tract occurs via infected macrophages. Infections respond to therapy with albendazole, unlike infections with *E. bieneusi*.

Both *E. cuniculi* and *E. hellem* have been isolated from human infections, the first species from the CNS and the second from the eye.



**Images from left to right:** The drawing illustrates the infective spore (1.5 to 2.5  $\mu\text{m}$ ), containing the coiled polar tubule. (Top) Gram stain showing spores within a white blood cell; (bottom row left), nasopharyngeal aspirate with spores (Ryan blue modified trichrome stain); (bottom row right), urine sediment with direct FA reagent (to the genus *Encephalitozoon*).

## Description

Microsporidial infections can be misdiagnosed in tissues and can be confused with *Cryptococcus neoformans* infections. Mucus granules in goblet cells can take up stain and be very confusing. Good preservation and thin tissue sections (1  $\mu\text{m}$ ) that have been resin embedded enhance the resolution of cellular detail. Demonstration of the coiled polar tube within spores is diagnostic for microsporidial infection.

Conjunctival and corneal epithelium infections occur in HIV patients, while corneal stroma and ulceration occur in immunocompetent individuals.

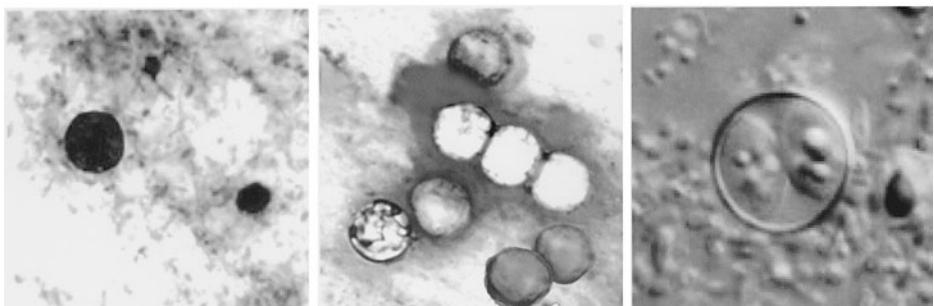
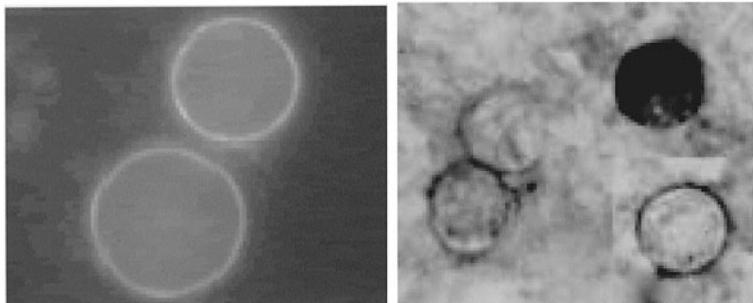
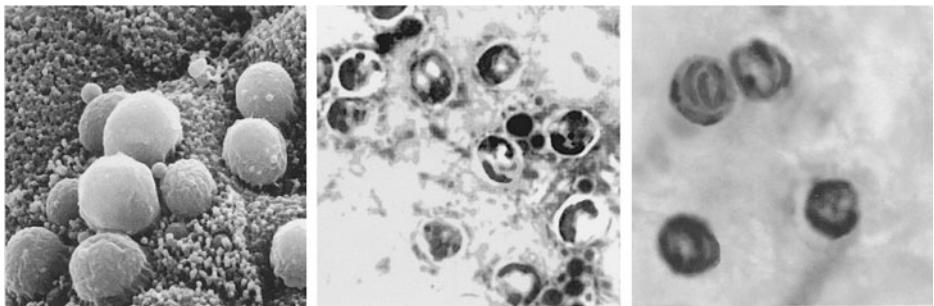
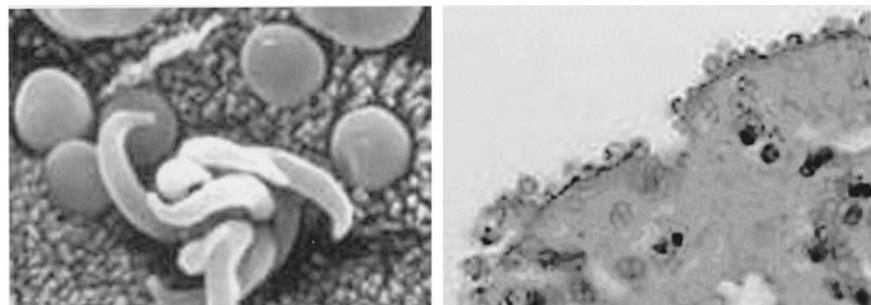
## Additional Information

*E. intestinalis* is not confined to epithelial cells, but is seen in macrophages in the lamina propria. Although the primary site appears to be the small bowel, organisms can disseminate to other sites, including duodenum, jejunum, ileum, colon, kidneys, liver, and gallbladder. They have also been identified in the lower airways. Concurrent infections with *E. bieneusi* have also been seen.

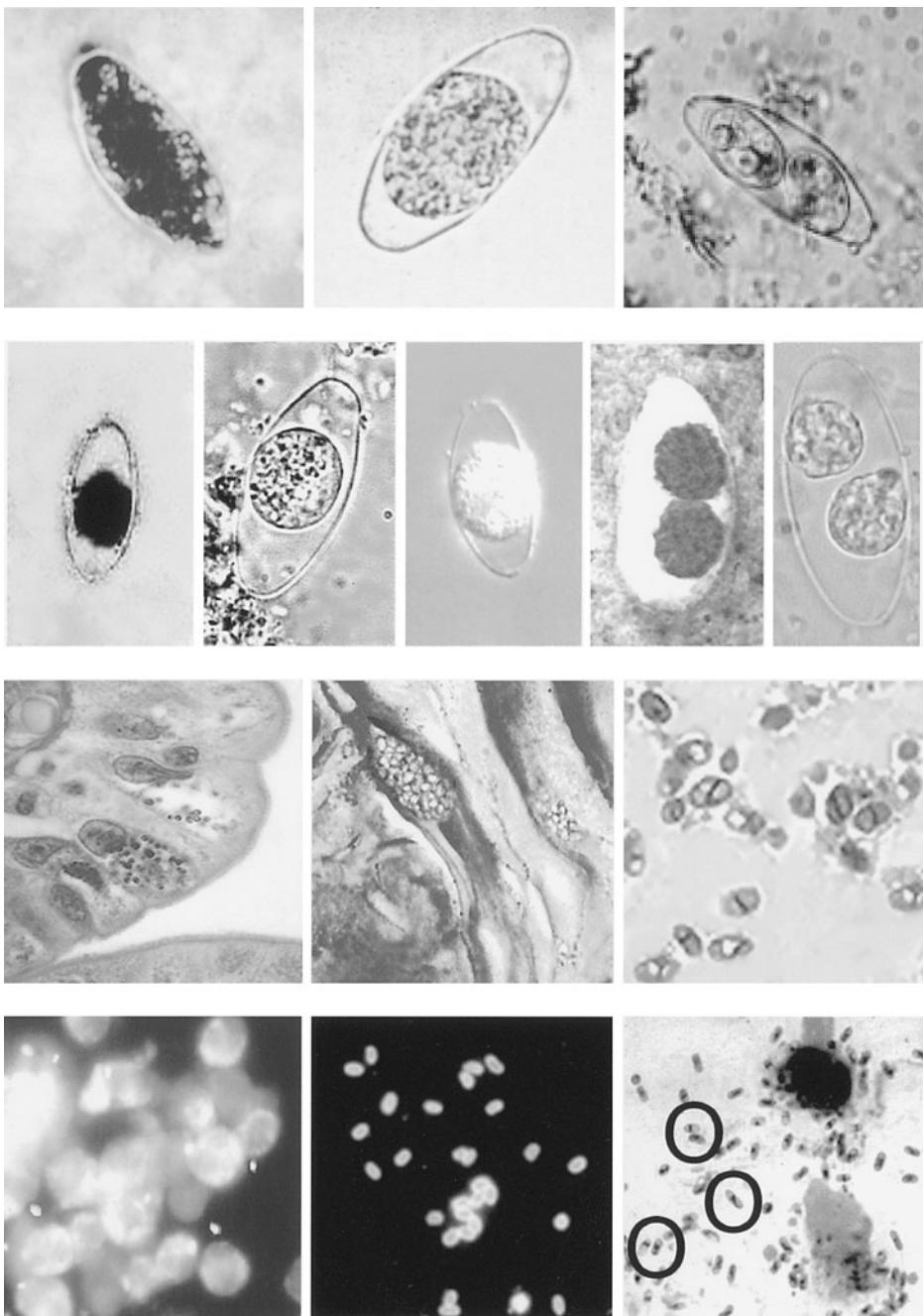
Currently, there are at least three *E. cuniculi* strains which may become more important in the epidemiology of human infections. Several *E. hellem* eye infections have been found in AIDS patients, as well as infections in the sinuses, conjunctivae, and nasal epithelium. In one case of disseminated *E. hellem* infection in an AIDS patient, autopsy revealed organisms in the eyes, urinary tract, and respiratory tract. The presence of numerous organisms within the lining epithelium of almost the entire length of the tracheobronchial tree suggests respiratory acquisition.

The microsporidia are obligate intracellular parasites that have been recognized in a variety of animals, particularly invertebrates. Typical sizes range from 1.5 to 5  $\mu\text{m}$  wide and 2 to 7  $\mu\text{m}$  long; organisms found in humans tend to be quite small (1.5 to 2  $\mu\text{m}$ ). Until recent increased understanding of AIDS within the immunosuppressed population, awareness and understanding of human infections have been marginal. Limited availability of EM has also affected the ability to recognize and diagnose these infections. However, the introduction of newer diagnostic methods has improved our ability to identify these parasites. The organisms are characterized by having spores containing a polar tubule, which is an extrusion mechanism for injecting the infective spore contents into host cells.

Serologic studies show that immunocompetent humans probably have persistent or chronic infections with microsporidia. This is also supported by the fact that microsporidiosis patients who are HIV seronegative show clinical resolution of their infections after a few weeks.



**Plate 7.5** Row 1: *Cryptosporidium* sporozoites (EM; courtesy of USDA) (left) and *Cryptosporidium* spp. on the surface of the intestinal tract epithelium (right); row 2: *Cryptosporidium* organisms on the surface of the intestinal tract (EM) (left) and *Cryptosporidium* oocysts stained with modified acid-fast stain (middle and right); row 3: *Cyclospora cayetanensis* autofluorescent oocysts (left) and *Cyclospora cayetanensis* oocysts stained with safranin (right); row 4: *Cyclospora cayetanensis* oocyst (the large object), *Cryptosporidium* oocyst (the medium-size object), and an artifact (the small dark-staining object at the top of the image) (left), *Cyclospora cayetanensis* oocysts (modified acid-fast variable staining) (center), and *Cyclospora cayetanensis* oocyst (mature) (right).



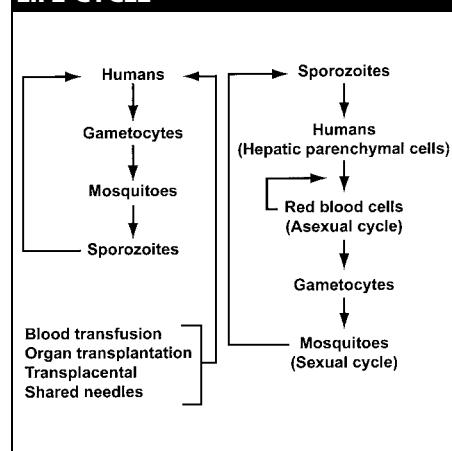
**Plate 7.6** Row 1: *Isospora belli* oocysts (from left to right: modified acid-fast stain, immature, and mature); row 2: *Isospora belli* oocysts (from left to right: modified acid-fast stain, wet mount, calcofluor, modified acid-fast stain, wet mount); row 3: microsporidial spores in intestinal tissue (routine H&E stain) (left), spores from an eye specimen (silver stain) (center), and spores (modified trichrome stain; note the horizontal lines representing the polar tubules) (right); row 4: spores in urine sediment (calcofluor) (left), spores using experimental DFA reagent (center), and spores (modified trichrome stain) (right).

## PROTOZOA • Sporozoa (Blood and Tissue)

### Plasmodium vivax

<b>Pathogenic</b>	Yes
<b>Disease</b>	Tertian malaria
<b>Acquired</b>	Bite of female anopheline mosquito; blood, shared needles, congenital infections
<b>Body site</b>	Liver, RBCs
<b>Symptoms</b>	Few; anemia, splenomegaly, paroxysm, (cold stage, fever, and sweats)
<b>Clinical specimen</b>	Blood, multiple draws (EDTA)
<b>Epidemiology</b>	May account for 80% of malaria in tropics, subtropics, and temperate zones; mosquito-to-human and human-to-human transmission
<b>Control</b>	Vector control, avoiding shared needles, checking blood supply; vaccine is possible in the near future

#### LIFE CYCLE



### Diagnosis

Although malaria is no longer endemic in the United States, it is life-threatening, and laboratory requests for blood smear examination and organism identification should be treated as STAT requests. Frequently, for a number of reasons, organism recovery and identification may be more difficult than the textbooks imply. It is very important that this fact be recognized, particularly when dealing with a possibly fatal infection with *P. falciparum*.

Both thick and thin blood films should be prepared on admission of the patient (clinic, emergency room, in-house), and at least 300 oil immersion fields should be examined on the thick and thin films before a negative report is issued. Since one set of negative films does not rule out malaria, additional blood specimens should be examined over 36 h. Although Giemsa stain is often used for parasitic blood work, the organisms can also be seen with other blood stains such as Wright's stain, a Wright-Giemsa stain, or any of the rapid blood stains. Blood collected with EDTA anticoagulant is acceptable; however, if the blood remains in the tube for any length of time, true stippling may not be visible within the infected RBCs (e.g., for *P. vivax*), organisms change their morphology, and some of the parasites actually disintegrate (after about 4 to 6 h). Also, the proper ratio between blood and anticoagulant is necessary for good organism morphology.

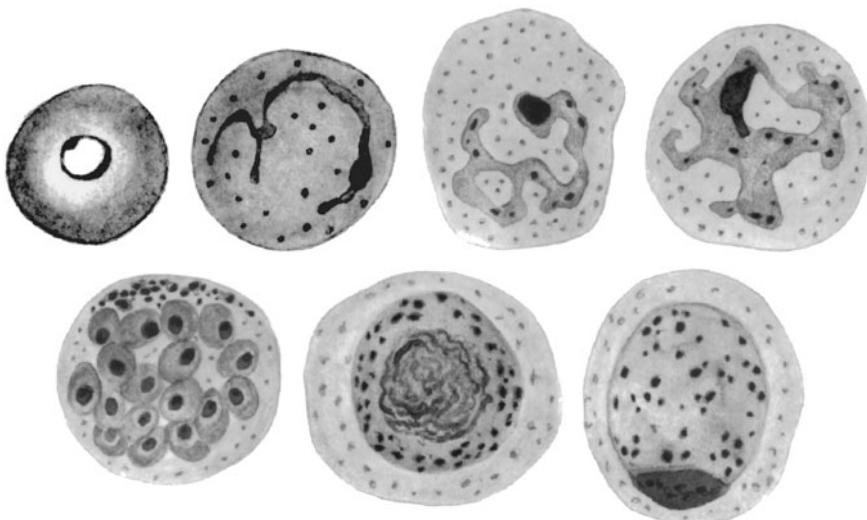
**Note:** Excess protein and hematin left over from the metabolism of hemoglobin combine to form malarial pigment; this is found in all four species. True stippling (evidence of RBC membrane damage; Schüffner's dots) is seen only in *P. vivax* and *P. ovale* malaria.

### General Comments

Within an hour, sporozoites from the mosquito are carried via the blood to the liver, where they penetrate parenchymal cells, initiating the preerythrocytic or primary exoerythrocytic cycle. The sporozoites become round or oval and begin dividing, resulting in many liver merozoites. The merozoites leave the liver and invade the RBCs, initiating the erythrocytic cycle. In *P. vivax* and *P. ovale*, a secondary or dormant schizogony occurs from organisms that remain quiescent in the liver until later; they are called hypnozoites. Delayed schizogony does not occur in *P. falciparum* or *P. malariae*.

If the RBC infection is not eliminated by the immune system or by therapy and the numbers in the RBCs begin to increase again with subsequent clinical symptoms, this is called a recrudescence; it can occur with all species. If the erythrocytic infection is eliminated and a relapse due to a new invasion of the RBCs from the liver occurs later, this is called a true relapse. It occurs only with *P. vivax* and *P. ovale*.

The merozoite (young trophozoite) is vacuolated, ring shaped, and uninucleate. Once the nucleus begins to divide, the trophozoite is called a developing schizont; the mature schizont contains merozoites which are released into the bloodstream. Merozoites invade RBCs, in which a new cycle of erythrocytic schizogony begins. After several erythrocytic generations, some of the merozoites undergo development into the male and female gametocytes.



Images from left to right: (Top row) Early ring form; the next three show developing trophozoites (note the parasite is very amoeboid and there are Schüffner's dots/stippling present); (bottom row) mature schizont containing ~16 to 18 merozoites, male microgametocyte, and female macrogamete. Also note the enlarged RBCs.

## Additional Information

*P. vivax* infects only the reticulocytes and the parasitemia is usually limited to 2 to 4% of the available RBCs. Splenomegaly occurs during the first few weeks, and the spleen becomes hard during a chronic infection. If therapy is given early, the spleen returns to normal size. Leukopenia is seen; leukocytosis may be present during the febrile episodes. Total plasma proteins are unchanged, although the albumin may be low and the globulin fraction may be elevated due to antibody development. Serum potassium may also be increased.

**Note:** When requests for malarial smears are received in the laboratory, the following patient history information should be provided to the laboratorian. (i) Where has the patient been and what was the date of return to the United States? (ii) Has malaria ever been diagnosed in the patient before? If so, what species was identified? (iii) What medication (prophylaxis or otherwise) has the patient received, and how often? When was the last dose taken? (iv) Has the patient ever received a blood transfusion? Is there a possibility of other needle transmission (drug user)? (v) When was the blood specimen drawn, and was the patient symptomatic at the time? Is there any evidence of a fever periodicity? The answers may help eliminate the possibility of infection with *P. falciparum*, usually the only species that can rapidly cause death.

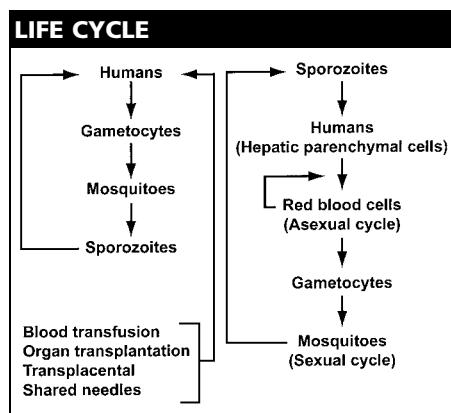
The primary clinical attack usually occurs 7 to 10 days after infection, although there are strain differences, with a much longer incubation period being possible. Symptoms such as headache, photophobia, muscle aches, anorexia, nausea, and sometimes vomiting may occur before organisms can be detected in the bloodstream. Alternatively, the parasites can be found in the bloodstream several days before symptoms appear.

During the first few days, the patient may not exhibit a typical paroxysm pattern but, rather, may have a steady low-grade fever or an irregular remittent fever pattern. Once the typical paroxysms begin, after an irregular periodicity, a regular 48-h cycle is established. An untreated primary attack may last from 3 weeks to 2 months or longer. The paroxysms become less severe and more irregular in frequency and then stop altogether. In 50% of patients, relapses may occur after weeks, months, or up to 5 years. Severe complications are rare in *P. vivax* infections, although coma and sudden death or other symptoms of cerebral involvement have been reported. Severe sequelae can be seen in cases of primaquine-tolerant or primaquine-resistant cases.

## PROTOZOA • Sporozoa (Blood and Tissue)

### Plasmodium falciparum

<b>Pathogenic</b>	Yes
<b>Disease</b>	Malignant tertian malaria
<b>Acquired</b>	Bite of female anopheline mosquito; blood, shared needles, congenital infections
<b>Body site</b>	Liver, RBCs
<b>Symptoms</b>	Few; anemia, splenomegaly, paroxysm (cold stage, fever, and sweats)
<b>Clinical specimen</b>	Blood, multiple draws (EDTA)
<b>Epidemiology</b>	Tropics, mosquito-to-human and human-to-human transmission
<b>Control</b>	Vector control, avoiding shared needles, checking blood supply; vaccine possible in the near future



### Diagnosis

Although malaria is no longer endemic in the United States, it is life-threatening, and laboratory requests for blood smear examination and organism identification should be treated as STAT requests. Frequently, for a number of reasons, organism recovery and identification may be more difficult than the textbooks imply. It is very important that this fact be recognized, particularly when dealing with a possibly fatal infection with *P. falciparum*.

Both thick and thin blood films should be prepared on *admission of the patient* (clinic, emergency room, in-house), and at least 300 oil immersion fields should be examined on the thick and thin films before a negative report is issued. Since one set of negative films does not rule out malaria, additional blood specimens should be examined over 36 h. Although Giemsa stain is used for parasitic blood work, the organisms can also be seen with other blood stains such as Wright's, Wright-Giemsa, or any of the rapid blood stains. Blood collected with EDTA anticoagulant is acceptable; however, if the blood remains in the tube for any length of time, true stippling may not be visible within the infected RBCs (e.g., for *P. vivax*), organisms change their morphology, and some of the parasites disintegrate (after about 4 to 6 h). Also, the proper ratio between blood and anticoagulant is necessary for good organism morphology.

**Note:** Excess protein and hematin left over from the metabolism of hemoglobin combine to form malarial pigment; this is found in all four species. True stippling (evidence of RBC membrane damage; Schüffner's dots) is seen only in *P. vivax* and *P. ovale* malaria.

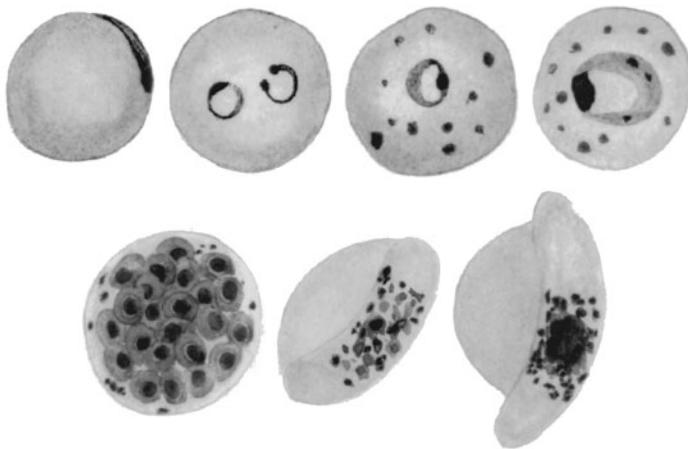
### General Comments

Within an hour, sporozoites from the mosquito are carried via the blood to the liver, where they penetrate parenchymal cells, initiating the preerythrocytic or primary exoerythrocytic cycle. The sporozoites become round or oval and begin dividing, resulting in many liver merozoites. The merozoites leave the liver and invade the RBCs, initiating the erythrocytic cycle. In *P. vivax* and *P. ovale*, a secondary or dormant schizogony occurs from organisms that remain quiescent in the liver until later; they are called hypnozoites. Delayed schizogony does not occur in *P. falciparum* or *P. malariae*.

If the RBC infection is not eliminated by the immune system or by therapy and the numbers in the RBCs begin to increase again with subsequent clinical symptoms, this is called a recrudescence; it can occur with all species. If the erythrocytic infection is eliminated and a relapse due to a new invasion of the RBCs from the liver occurs later, this is called a true relapse. It occurs only in *P. vivax* and *P. ovale*.

The merozoite (young trophozoite) is vacuolated, ring shaped, and uninucleate. Once the nucleus begins to divide, the trophozoite is called a developing schizont; the mature schizont contains merozoites which are released into the bloodstream. Merozoites invade RBCs, in which a new cycle of erythrocytic schizogony begins. After several erythrocytic generations, some of the merozoites undergo development into the male and female gametocytes.

Coincident infection with more than one species is more common than previously suspected. Dual *P. falciparum*-*P. vivax* infections are found, as are *P. falciparum*-*P. malariae* infections. *P. ovale* is common in areas of Africa with populations refractory to *P. vivax*, preventing coinfections. These species coexist in New Guinea.



**Images from left to right:** (Top row) Ring in the accolé or appliquéd form, double rings per cell, and developing rings (RBCs contain Maurer's clefts); (bottom row) mature schizont, male microgametocyte, and female macrogametocyte (note the crescent shape of gametocytes).

## Additional Information

*P. falciparum* tends to invade all ages of RBCs; the proportion of infected cells may exceed 50%. Schizogony occurs in the internal organs (spleen, liver, bone marrow, etc.) rather than in the circulating blood. Ischemia caused by the plugging of vessels within these organs by masses of parasitized RBCs produces various symptoms, depending on the organ involved. A decrease in the ability of the RBCs to change shape when passing through capillaries or the splenic filter may lead to plugging of the vessels.

**Note:** When requests for malarial smears are received in the laboratory, the following patient history information should be provided to the laboratorian. (i) Where has the patient been and what was the date of return to the United States? (ii) Has malaria ever been diagnosed in the patient before? If so, what species was identified? (iii) What medication (prophylaxis or otherwise) has the patient received, and how often? When was the last dose taken? (iv) Has the patient ever received a blood transfusion? Is there a possibility of other needle transmission (drug user)? (v) When was the blood specimen drawn, and was the patient symptomatic at the time? Is there any evidence of a fever periodicity? Answers to such questions may help eliminate the possibility of infection with *P. falciparum*, usually the only species that can rapidly cause death.

Accurate species diagnosis is essential for good patient management, since it may determine which drug(s) is indicated. Some patients do not yet have the crescent-shaped gametocytes in the blood. Low parasitemias with the delicate ring forms may be missed; oil immersion examination at  $\times 1,000$  is mandatory.

Onset of a *P. falciparum* malaria attack is 8 to 12 days after infection and is preceded by 3 to 4 days of vague symptoms such as aches, pains, headache, fatigue, anorexia, or nausea. The onset involves fever, severe headache, and nausea and vomiting, with occasional severe epigastric pain. There may be only a feeling of chilliness. *Periodicity of the cycle is not established during the early stages, and the presumptive diagnosis may be totally unrelated to a possible malaria infection.* If the fever does develop a synchronous cycle, it is usually a cycle of somewhat less than 48 h.

An untreated primary attack of *P. falciparum* malaria usually ends within 2 to 3 weeks. True relapses from the liver do not occur, and after a year recrudescences are rare.

Severe or fatal complications of *P. falciparum* malaria can occur at any time and are related to the plugging of vessels in the internal organs. *The symptoms depend on the organ(s) involved.* Severe complications may not correlate with the parasitemia seen in the peripheral blood. Disseminated intravascular coagulation is a rare complication of high parasite burden; vascular endothelial damage from endotoxins and bound parasitized blood cells may lead to clot formation in small vessels.

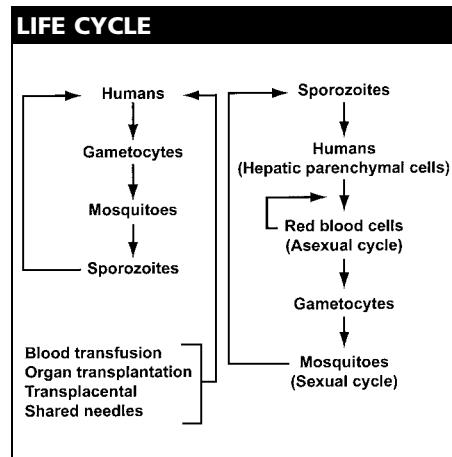
Cerebral malaria involving disorientation, violent episodes, severe headaches, and coma, is most often seen in *P. falciparum* malaria. There is no real correlation between the severity of symptoms and the peripheral blood parasitemia.

*Malaria is one of the few parasitic infections considered immediately life-threatening; and a patient with *P. falciparum* malaria is a medical emergency. Any laboratory providing the expertise to identify malarial parasites should do so on a 24-h basis, 7 days/week.*

## PROTOZOA • Sporozoa (Blood and Tissue)

### Plasmodium malariae

<b>Pathogenic</b>	Yes
<b>Disease</b>	Quartan malaria
<b>Acquired</b>	Bite of female anopheline mosquito; blood, shared needles, congenital infections
<b>Body site</b>	Liver, RBCs
<b>Symptoms</b>	Few; anemia, splenomegaly, paroxysm (cold stage, fever, and sweats)
<b>Clinical specimen</b>	Blood, multiple draws (EDTA)
<b>Epidemiology</b>	Sporadic distribution; mosquito-to-human and human-to-human transmission
<b>Control</b>	Vector control, avoiding shared needles, checking blood supply; vaccine is possible in the near future



### Diagnosis

Although malaria is no longer endemic in the United States, it is life-threatening, and laboratory requests for blood smear examination and organism identification should be treated as STAT requests. Frequently, for a number of reasons, organism recovery and identification may be more difficult than the textbooks imply. It is very important that this fact be recognized, particularly when dealing with a possibly fatal infection with *P. falciparum*.

Both thick and thin blood films should be prepared on admission of the patient (clinic, emergency room, in-house), and at least 300 oil immersion fields should be examined on the thick and thin films before a negative report is issued. Since one set of negative films does not rule out malaria, additional blood specimens should be examined over 36 h. Giemsa stain is recommended for all parasitic blood work; the organisms can also be seen with other blood stains such as Wright's stain. Blood collected with EDTA anticoagulant is acceptable; however, if the blood remains in the tube for any length of time, true stippling may not be visible within the infected RBCs (e.g., for *P. vivax*), organisms change their morphology, and some of the parasites disintegrate (after 4 to 6 h). Also, the proper ratio between blood and anticoagulant is necessary for good organism morphology.

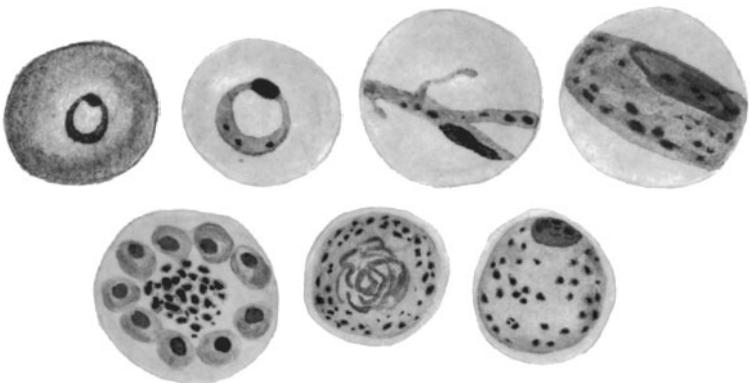
**Note:** Excess protein and hematin left over from the metabolism of hemoglobin combine to form malarial pigment; this is found in all four species. True stippling (evidence of RBC membrane damage; Schüffner's dots) is seen only in *P. vivax* and *P. ovale* malaria.

### General Comments

Within an hour, sporozoites from the mosquito are carried via the blood to the liver, where they penetrate parenchymal cells, initiating the preerythrocytic or primary exoerythrocytic cycle. The sporozoites become round or oval and begin dividing, resulting in many liver merozoites. The merozoites leave the liver and invade the RBCs, initiating the erythrocytic cycle. In *P. vivax* and *P. ovale*, a secondary or dormant schizogony occurs from organisms that remain quiescent in the liver until later; they are called hypnozoites. Delayed schizogony does not occur in *P. falciparum* or *P. malariae*.

If the RBC infection is not eliminated by the immune system or by therapy and the numbers in the RBCs begin to increase again with subsequent clinical symptoms, this is called a recrudescence; it can occur with all species. If the erythrocytic infection is eliminated and a relapse due to a new invasion of the RBCs from the liver occurs later, this is called a true relapse. It occurs only with *P. vivax* and *P. ovale*.

The merozoite (young trophozoite) is vacuolated, ring shaped, and uninucleate. Once the nucleus begins to divide, the trophozoite is called a developing schizont; the mature schizont contains merozoites which are released into the bloodstream. Merozoites invade RBCs, in which a new cycle of erythrocytic schizogony begins. After several erythrocytic generations, some of the merozoites undergo development into the male and female gametocytes.



**Images from left to right:** (Top row) Two developing ring forms and two typical band forms; (bottom row) mature schizont "rosette" formation, male microgamete, and female macrogamete.

## Additional Information

*P. malariae* invades primarily the older RBCs, so the number of infected cells is limited. Splenomegaly occurs during the first few weeks, and the spleen can become hard during a chronic infection. If therapy is given early, the spleen will return to normal size. Leukopenia is seen; leukocytosis may be present during the febrile episodes. Total plasma proteins are unchanged, although the albumin may be low and the globulin fraction may be elevated due to antibody development. Serum potassium may also be increased.

**Note:** When requests for malarial smears are received in the laboratory, the following patient history information should be provided to the laboratorian. (i) Where has the patient been and what was the date of return to the United States? (ii) Has malaria ever been diagnosed in the patient before? If so, what species? (iii) What medication (prophylaxis or otherwise) has the patient received, and how often? When was the last dose taken? (iv) Has the patient ever received a blood transfusion? Is there a possibility of other needle transmission (drug user)? (v) When was the blood specimen drawn, and was the patient symptomatic at the time? Is there any evidence of a fever periodicity? The answers may help eliminate the possibility of infection with *P. falciparum*, usually the only species that can rapidly cause death.

The incubation period between infection and symptoms may be much longer than that with *P. vivax* or *P. ovale* malaria, ranging from about 27 to 40 days. Parasites can be found in the bloodstream several days before the initial attack, and the prodromal symptoms may resemble those of *P. vivax* malaria. A regular periodicity is seen from the beginning, with a more severe paroxysm, including a longer cold stage and more severe symptoms during the hot stage. Collapse during the sweating phase is not uncommon.

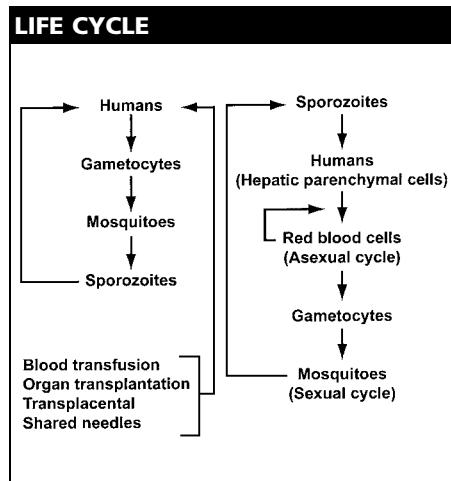
Proteinuria is common in *P. malariae* infections; in children it may be associated with clinical signs of the nephrotic syndrome. Kidney problems may result from deposition within the glomeruli of circulating antigen-antibody complexes in an antigen-excess situation in a chronic infection. Apparently, the nephrotic syndrome associated with *P. malariae* infections is unaffected by administration of steroids. A membranoproliferative type of glomerulonephritis with relatively sparse proliferation of endothelial and mesangial cells is the most common type of lesion seen in quartan malaria. Using immunofluorescence, granular deposits of IgM, IgG, and C3 are seen. Since chronic glomerular disease associated with *P. malariae* infections is usually not reversible with therapy, genetic and environmental factors may play a role in the nephrotic syndrome.

The infection may end with spontaneous recovery, or there may be a recrudescence or series of recrudescences over many years. These patients are left with a latent infection and persisting low-grade parasitemia for many years.

## PROTOZOA • Sporozoa (Blood and Tissue)

### Plasmodium ovale

<b>Pathogenic</b>	Yes
<b>Disease</b>	Ovale malaria
<b>Acquired</b>	Bite of female anopheline mosquito; blood, shared needles, congenital infections
<b>Body site</b>	Liver, RBCs
<b>Symptoms</b>	Few; anemia, splenomegaly, paroxysm (cold stage, fever, and sweats)
<b>Clinical specimen</b>	Blood, multiple draws (EDTA)
<b>Epidemiology</b>	Central West Africa and some South Pacific islands; mosquito-to-human and human-to-human transmission
<b>Control</b>	Vector control, avoiding shared needles, checking blood supply; vaccine is possible in the near future



### Diagnosis

Although malaria is no longer endemic within the United States, it is life-threatening, and laboratory requests for blood smear examination and organism identification should be treated as STAT requests. Frequently, for a number of reasons, organism recovery and identification may be more difficult than the textbooks imply. It is very important that this fact be recognized, particularly when dealing with a possibly fatal infection with *P. falciparum*.

Both thick and thin blood films should be prepared on admission of the patient (clinic, emergency room, in-house), and at least 300 oil immersion fields should be examined on the thick and thin films before a negative report is issued. Since one set of negative films does not rule out malaria, additional blood specimens should be examined over 36 h. Giemsa stain is recommended for all parasitic blood work; the organisms can also be seen with other blood stains such as Wright's stain. Blood collected with EDTA anticoagulant is acceptable; however, if the blood remains in the tube for any length of time, true stippling may not be visible within the infected RBCs (e.g., for *P. vivax*), organisms change their morphology, and some of the parasites will disintegrate (after 4 to 6 h). Also, the proper ratio between blood and anticoagulant is necessary for good organism morphology.

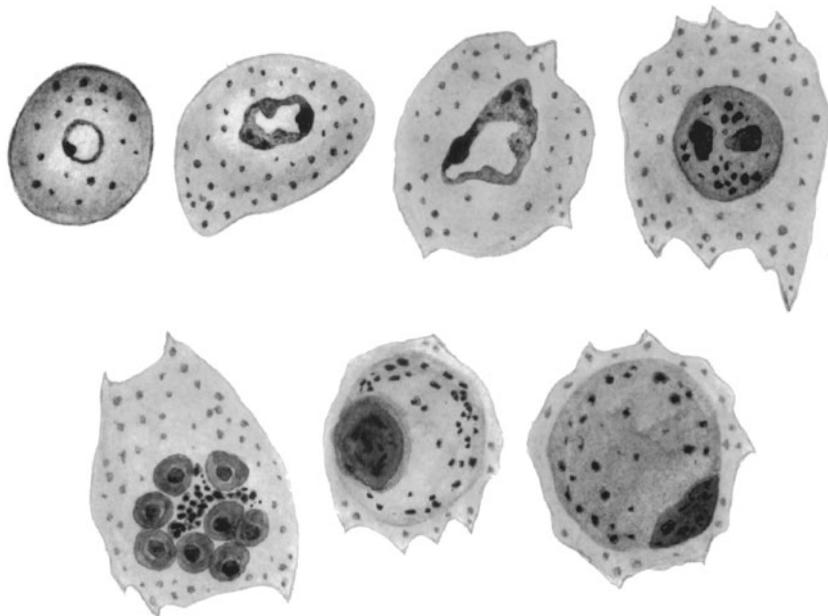
**Note:** Excess protein and hematin left over from the metabolism of hemoglobin combine to form malarial pigment; this is found in all four species. True stippling (evidence of RBC membrane damage; Schüffner's dots) is seen only in *P. vivax* and *P. ovale* malaria.

### General Comments

Within an hour, sporozoites from the mosquito are carried via the blood to the liver, where they penetrate parenchymal cells, initiating the preerythrocytic or primary exoerythrocytic cycle. The sporozoites become round or oval and begin dividing, resulting in many liver merozoites. The merozoites leave the liver and invade the RBCs, initiating the erythrocytic cycle. In *P. vivax* and *P. ovale*, a secondary or dormant schizogony occurs from organisms that remain quiescent in the liver until later; they are called hypnozoites. Delayed schizogony does not occur in *P. falciparum* or *P. malariae*.

If the RBC infection is not eliminated by the immune system or by therapy and the numbers in the RBCs begin to increase again with subsequent clinical symptoms, this is called a recrudescence; it can occur with all species. If the erythrocytic infection is eliminated and a relapse due to a new invasion of the RBCs from the liver occurs later, this is called a true relapse. It occurs only with *P. vivax* and *P. ovale*.

The merozoite (young trophozoite) is vacuolated, ring shaped, and uninucleate. Once the nucleus begins to divide, the trophozoite is called a developing schizont; the mature schizont contains merozoites which are released into the bloodstream. Merozoites invade RBCs, in which a new cycle of erythrocytic schizogony begins. After several erythrocytic generations, some of the merozoites undergo development into the male and female gametocytes.



Images from left to right: (Top row) Two developing ring forms and two developing trophozoites (note that all four images contain true stippling, Schüffner's dots; dots appear later in the cycle in *P. vivax*); (bottom row) mature schizont, male microgametocyte, and female macrogametocyte. Note the enlarged RBCs, oval shape, and the fimbriated edges of some of the RBCs.

## Additional Information

*P. ovale* infects only the reticulocytes, and the parasitemia is usually limited to 2 to 5% of available RBCs. Splenomegaly occurs during the first few weeks, and the spleen becomes hard during a chronic infection. If therapy is given early, the spleen will return to normal size. Leukopenia is seen; leukocytosis may be present during the febrile episodes. Total plasma proteins are unchanged, although the albumin may be low and the globulin fraction may be elevated due to antibody development. Serum potassium may also be increased.

**Note:** When requests for malarial smears are received in the laboratory, the following patient history information should be provided to the laboratorian. (i) Where has the patient been and what was the date of return to the United States? (ii) Has malaria ever been diagnosed in the patient before? If so, what species? (iii) What medication (prophylaxis or otherwise) has the patient received, and how often? When was the last dose taken? (iv) Has the patient ever received a blood transfusion? Is there a possibility of other needle transmission (drug user)? (v) When was the blood specimen drawn, and was the patient symptomatic at the time? Is there any evidence of a fever periodicity? Answers to such questions may help eliminate the possibility of infection with *P. falciparum*, usually the only species that can rapidly lead to death.

The primary clinical attack usually occurs 7 to 10 days after infection, although there are strain differences, with a much longer incubation period being possible. Symptoms such as headache, photophobia, muscle aches, anorexia, nausea, and vomiting may occur before organisms can be detected in the bloodstream. Alternatively, the parasites can be found in the bloodstream before symptoms appear.

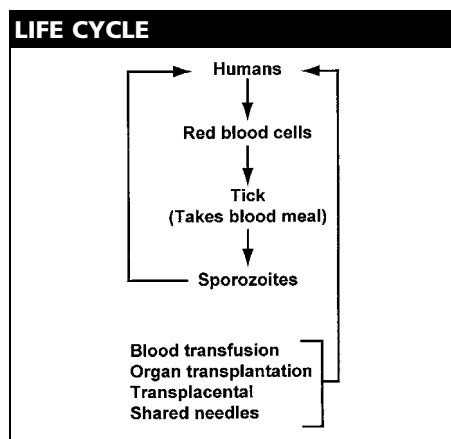
During the first few days, the patient may not exhibit a typical paroxysm pattern but, rather, may have a steady low-grade fever or an irregular remittent fever pattern. Once the typical paroxysms begin, after an irregular periodicity, a regular 48-h cycle is established. An untreated primary attack may last from 3 weeks to 2 months or longer. The paroxysms become less severe and more irregular and then stop altogether. In 50% of patients, relapses may occur after weeks, months, or up to 5 years. Severe complications are rare in *P. ovale* infections, although coma and sudden death or other symptoms of cerebral involvement can occur. *P. ovale* malaria is usually less severe than *P. vivax*, tends to relapse less frequently, and usually ends with spontaneous recovery, often after only 6 to 10 paroxysms. The incubation period is similar to *P. vivax* malaria and symptoms are less frequent and severe, with a lower fever and a lack of typical rigors.

The geographic range includes tropical Africa, the Middle East, Papua New Guinea, and Irian Jaya in Indonesia. However, infections in Southeast Asia may cause benign and relapsing malaria. In both Southeast Asia and Africa, two different types of *P. ovale* circulate in humans. Human infections with variant-type *P. ovale* are associated with higher parasitemias, with possible clinical relevance.

## PROTOZOA • Sporozoa (Blood and Tissue)

### Babesia spp.

<b>Pathogenic</b>	Yes
<b>Disease</b>	Babesiosis
<b>Acquired</b>	Bite of various ticks; blood, shared needles, congenital infections
<b>Body site</b>	RBCs
<b>Symptoms</b>	Few; general malaise followed by fever, shaking chills, profuse sweating, arthralgias, myalgias, fatigue, and weakness
<b>Clinical specimen</b>	Blood, multiple draws (EDTA)
<b>Epidemiology</b>	Sporadic distribution; tick-to-human and human-to-human transmission
<b>Control</b>	Vector control, avoiding shared needles, checking blood supply



### Diagnosis

Both thick and thin blood films should be prepared *on admission of the patient* (clinic, emergency room, in-house), and at least 300 oil immersion fields should be examined on the thick and thin films before a negative report is issued. Since one set of negative films does not rule out babesiosis, additional blood specimens collected over 36 h should be examined. Giemsa stain is recommended for all parasitic blood work; the organisms can also be seen with other blood stains such as Wright's stain. Blood collected with EDTA anticoagulant is recommended. Also, the proper ratio between blood and anticoagulant is necessary for good organism morphology.

Potential diagnostic problems with automated differential instruments have been reported. A case of babesiosis and two cases of malaria were missed when blood smears were examined by these methods, resulting in delayed therapy. The number of fields scanned by a technologist on instrument-read smears is quite small; thus, light parasitemia is almost sure to be missed. Although these instruments are not designed to detect intracellular blood parasites, routine use of the automated systems may pose serious diagnostic problems, particularly if the suspect diagnosis is not conveyed to the laboratory.

**Note:** Most documented human cases have a low parasitemia; thus, both thick and thin blood films stained with any of the blood stains must be examined. Although impractical for most laboratories, when the organisms cannot be demonstrated in a suspect patient, blood can be inoculated intraperitoneally into a hamster or gerbil. The organisms of some species are usually demonstrated within a few days, with the same morphology as that seen in humans.

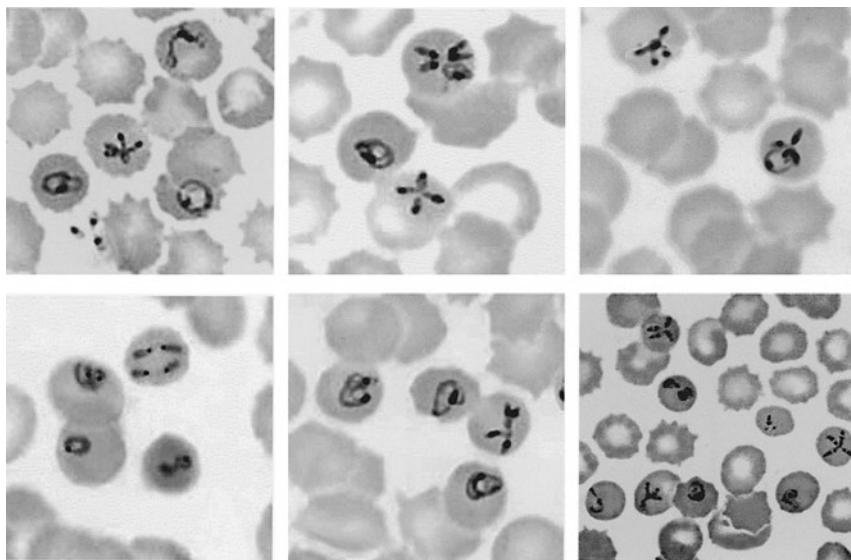
### General Comments

Infection is transmitted by several species of ticks in which the sexual multiplication cycle occurs. When a tick takes a blood meal, the infective forms are introduced into the human host. The organisms infect the RBCs, in which they appear as pleomorphic, ringlike structures. They resemble the early trophozoite (ring) forms of malarial parasites, particularly *P. falciparum*. The organisms measure 1.0 to 5.0  $\mu\text{m}$ , the RBCs are not enlarged or pale, and the cells do not contain stippling. Malarial pigment is never seen. The early form contains very little cytoplasm and has a very small nucleus. In mature forms, two or more chromatin dots may be seen. Occasionally a tetrad formation, referred to as a Maltese cross, is seen.

*Babesia* spp. are grouped into the small *Babesia* spp. (1.0 to 2.5  $\mu\text{m}$ ) and include *B. gibsoni*, *B. microti*, and *B. rodhaini*. The large *Babesia* spp. (2.5 to 5.0  $\mu\text{m}$ ) include *B. bovis*, *B. caballi*, and *B. canis*. These phenotypic classifications are, for the most part, consistent with genetic characterization based on nuclear small-subunit ribosomal DNA sequences. The small babesias are more closely related to *Theileria* spp. than are the larger organisms. The one exception is *B. divergens*, which appears small on blood smears but is genetically related to the large babesias. The two primary pathogens of humans are *B. microti* and *B. divergens*, along with the unnamed species WA1 (Washington), CA1 (California), and MO1 (Missouri).

Babesiosis is usually self-limiting; there are probably also asymptomatic and undiagnosed carriers; the mortality rate has been reported at about 5%. However, infections in Europe caused by *B. divergens* are far more serious, with a mortality rate of 42%. Fatal cases of *B. divergens* infection have been reported in France, Britain, Ireland, Spain, Sweden, Switzerland, the former Yugoslavia, and the former USSR.

**Note:** It is now well established that under blood-banking conditions (4°C for 30 days), *B. microti* can remain infective and transfusion-acquired infection with this parasite could occur during the normal storage time for blood.



**Images from left to right:** (Top row) *Babesia* rings (note the Maltese cross formation in some of the RBCs; also note the three rings outside of the RBC in the first image, a situation that occurs with *Babesia* but very rarely with *Plasmodium* spp.). (Bottom row) *Babesia* rings; the third image is photographed at a lower magnification.

## Additional Information

*Babesia* infections described in California and in other parts of the world are quite different from those seen in the northeastern United States, where the infection is most often subclinical. Infections in California and Europe tend to present as a fulminating, febrile, hemolytic disease affecting splenectomized or immunosuppressed individuals. Species other than *B. microti* or the bovine parasites may be involved.

A case of transfusion-induced babesiosis accompanied by disseminated intravascular coagulopathy was detected in 1982 in an elderly patient. Symptoms included fever, chills, nausea, arthralgias, and lethargy, which began after the patient received 2 units of packed RBCs during surgery. One of the donors had a high IFA titer against *B. microti*, but the infection was confirmed only by hamster inoculation.

The public health notice that was distributed to the Nantucket Board of Health indicated that *Babesia* parasites are transmitted from mice to humans primarily by the bite of the deer tick, *Ixodes scapularis*, and that persons with the infection may be asymptomatic. The notice indicated that prevention still relies on avoiding ticks or removing them promptly once detected, since there are apparently no fully effective tick repellents. If symptoms appear 1 to 2 weeks after a tick bite, a physician should be consulted.

In patients from Nantucket Island, MA, who were not splenectomized, symptoms began 10 to 20 days after a tick bite and continued for several weeks. There was general malaise followed by fever, shaking chills, profuse sweating, arthralgias, myalgias, fatigue, and weakness. Hepatosplenomegaly was present, and five patients had slightly elevated serum bilirubin and transaminase levels due to hemolytic anemia.

During summer 1980, six patients from Shelter Island and eastern Long Island were diagnosed as having *B. microti* babesiosis. Symptoms lasted for 19 to 24 days in five patients and included fever, shaking chills, dark urine, and headache, as well as anorexia, malaise, and lethargy. The splenectomized patient also had the most severe illness. Parasitemia could not be detected in one patient, was 5% in four of the six, and was 80 to 90% in the splenectomized patient. Hamster inoculation produced a patent parasitemia in all cases.

By the end of 1991, 13 cases of babesiosis had been reported in Connecticut; this was the largest number of human cases reported on the mainland United States. Information suggests that 12 infections were acquired via tick bites and 1 was acquired from a blood transfusion. Ages ranged from 61 to 95 for those with tick-acquired infections. Two patients died with active infections, and one died from chronic obstructive pulmonary disease. *B. microti* was isolated in Syrian hamsters given blood from 7 of 12 patients tested. Babesiosis is thus endemic in New Jersey and other areas of the northeast.

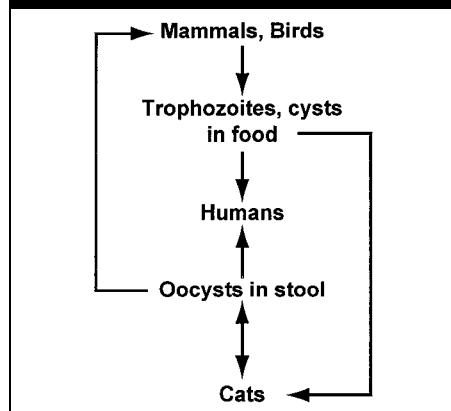
Serum of an 82-year-old splenectomized man from Washington reacted to *B. divergens* but not *B. microti* or WA1 antigens. This shows that unusual parasites may be detectable by blood film examination but not serologic or molecular testing for *B. microti* or WA1-type parasites. New organisms similar but not identical to some well-known *Babesia* spp. may be identified in patients in different parts of the world. Without manual examination of blood films, these organisms may be missed.

## PROTOZOA • Sporozoa (Blood and Tissue)

### Toxoplasma gondii

<b>Pathogenic</b>	Yes
<b>Disease</b>	Toxoplasmosis
<b>Acquired</b>	Ingestion of oocysts from cat feces; ingestion of rare or raw infected meats; congenital; transfusion; transplantation
<b>Body site</b>	Multiple tissues (muscle, brain)
<b>Symptoms</b>	Asymptomatic to severe (immuno-compromised, congenital)
<b>Clinical specimen</b>	Serum, serologic testing recommended
<b>Epidemiology</b>	Worldwide
<b>Control</b>	Hand hygiene, proper cooking of meat

#### LIFE CYCLE



### Diagnosis

Diagnosis is usually made by various serologic procedures. Other procedures include PCR; examination of biopsy specimens, buffy coat cells, or CSF; and organism isolation in tissue culture or in laboratory animals (rarely performed). It is difficult to demonstrate the parasite in lymph node biopsy specimens, and interpretation of the histologic appearance is controversial. However, because many individuals have been exposed to *T. gondii* and may have cysts within the tissues, recovery of organisms from tissue culture or animal inoculation may not indicate finding the etiologic agent of disease. Thus, serologic tests are often recommended as the diagnostic approach of choice; however, serologic diagnosis of toxoplasmosis is very complex. However, two situations in which organism detection may be significant are (i) tachyzoite-positive smears and/or tissue cultures from CSF and (ii) in patients with acute pulmonary disease, demonstration of tachyzoites in Giemsa-stained smears of bronchoalveolar lavage (BAL) fluid, some tachyzoites being extracellular and some intracellular.

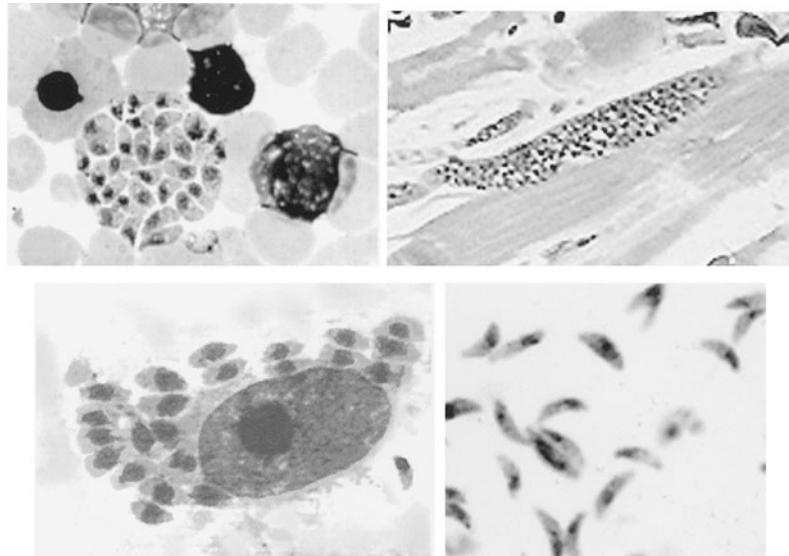
Cysts are formed in chronic infections, and organisms within the cyst wall are strongly periodic acid-Schiff positive. During the acute phase, there may be groups of tachyzoites that appear to be cysts; however, they are not strongly periodic acid-Schiff positive and have been termed pseudocysts.

### General Comments

There are three infectious stages of *T. gondii*: the tachyzoites (in groups or clones), the bradyzoites (in tissue cysts), and the sporozoites (in oocysts). Tachyzoites rapidly multiply in any cell of the intermediate host (many other animals and humans) and in nonintestinal epithelial cells of the definitive host (cats). Bradyzoites are found within the tissue cysts and multiply very slowly; the cyst may contain few to hundreds of organisms, and intramuscular cysts may reach 100  $\mu\text{m}$  in size. Although the tissue cysts may develop in visceral organs such as the lungs, liver, and kidneys, they are more prevalent in neural and muscular tissues, including the brain, eyes, and skeletal and cardiac muscle. Intact tissue cysts can persist for the life of the host and do not cause an inflammatory response.

When the tachyzoites are actively proliferating, they invade adjacent cells from the original infected cell as it ruptures. This process creates continually expanding focal lesions. Once the cysts are formed, the process becomes quiescent, with little or no multiplication and spread. In immunocompromised or immunodeficient patients, a cyst rupture or primary exposure to the organisms often leads to lesions. The organisms can be disseminated via the lymphatics and the bloodstream to other tissues. Disintegration of cysts may give rise to clinical encephalitis in the presence of apparently adequate immunity.

Toxoplasmosis is categorized into four groups: (i) disease acquired in immunocompetent patient; (ii) acquired or reactivated disease in immunosuppressed or immunodeficient patients; (iii) congenital; and (iv) ocular.



Images from left to right: (Top) *T. gondii* tachyzoites seen in bone marrow and bradyzoites seen in human tissue. (Bottom) Intracellular tachyzoites and extracellular tachyzoites from the mouse peritoneal cavity.

## Additional Information

In 90% of immunocompetent individuals, no clinical symptoms are seen during the acute infection. However, 10 to 20% of patients with acute infection may develop painless cervical lymphadenopathy, which is benign and self-limited, with symptoms resolving within weeks to months. Acute visceral manifestations are rarely seen.

In immunocompromised patients, underlying conditions that may influence disease include malignancies (such as Hodgkin's disease, non-Hodgkin's lymphomas, leukemias, and solid tumors), collagen vascular disease, organ transplantation, and AIDS. In immunocompromised patients, the CNS is primarily involved, with diffuse encephalopathy, meningoencephalitis, or cerebral mass lesions. More than 50% of these patients show altered mental state, motor impairment, seizures, abnormal reflexes, and other neurologic sequelae. Most patients receiving chemotherapy for toxoplasmosis will improve significantly or have complete remission. However, in those with AIDS, therapy must be continued for long periods to maintain a clinical response.

In transplant recipients, disease severity depends on previous exposure to *T. gondii* by donor and recipient, the type of organ transplanted, and the level of immunosuppression of the patient. Disease can be due to reactivation of a latent infection or an acute primary infection acquired directly from the transplanted organ. Stem cell transplant (SCT) recipients are particularly susceptible to severe toxoplasmosis, primarily due to reactivation of a latent infection. If SCT patients have a positive serology prior to transplantation, they are at risk for severe disseminated disease. Seronegative cardiac transplant recipients who receive an organ from a seropositive donor may develop toxoplasmic myocarditis; the symptoms may mimic organ rejection.

AIDS patients may develop disease when their CD4<sup>+</sup> T-lymphocyte count falls below 100,000/ml. Fever and malaise usually precede the first neurologic symptoms; headache, confusion, seizures, or other focal signs strongly suggest the diagnosis of toxoplasmosis.

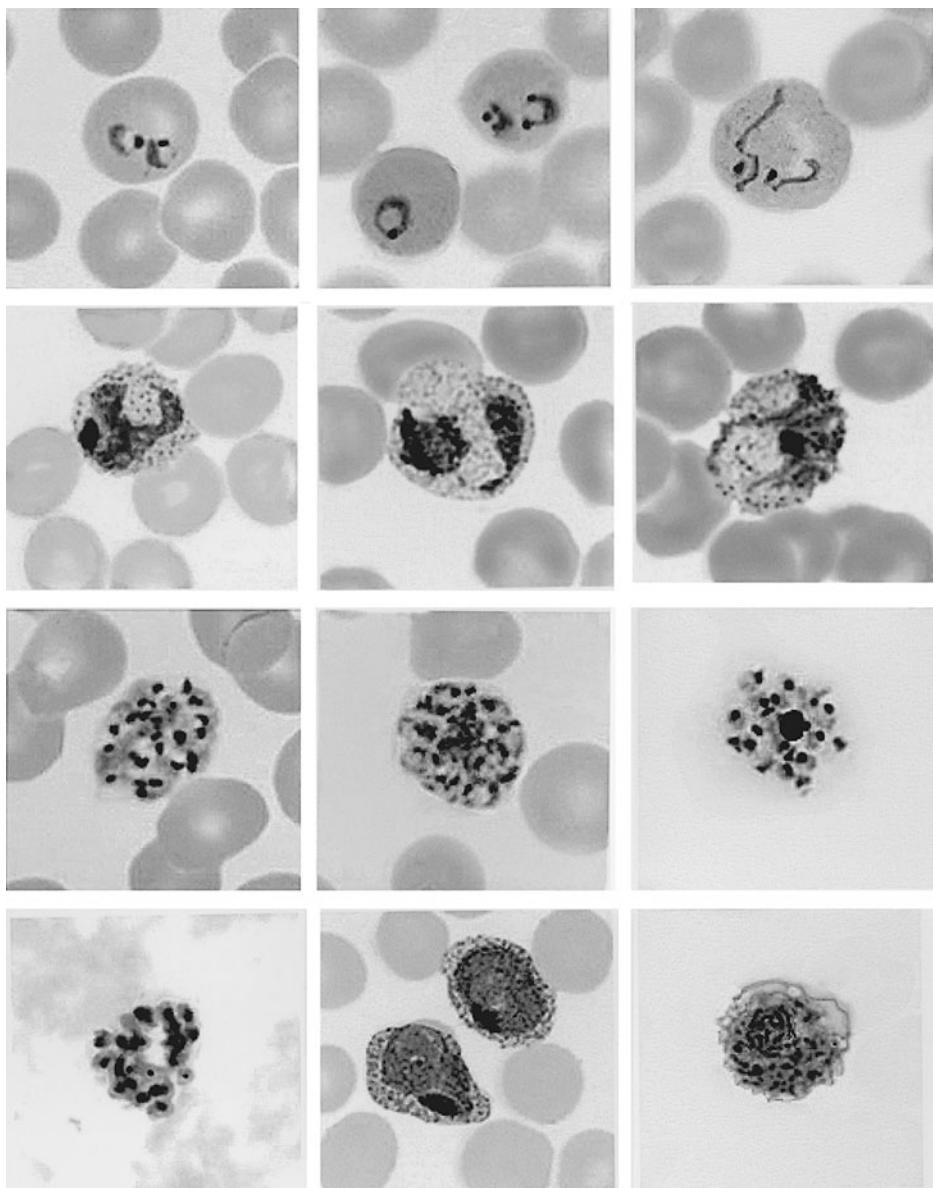
*Toxoplasma* encephalitis (TE) is fatal if untreated. Psychiatric manifestations of *T. gondii* are also seen in immunocompromised individuals with AIDS in whom latent infections have become reactivated. Altered mental status may occur in approximately 60% of patients, with symptoms including delusions, auditory hallucinations, and thought disorders.

Congenital infections result from the transfer of parasites from mother to the fetus when she acquires a primary infection during pregnancy. At birth or soon thereafter, symptoms in these infants may include retinchoroiditis, cerebral calcification, and occasionally hydrocephalus or microcephaly. Symptoms of congenital CNS involvement may not appear until several years later.

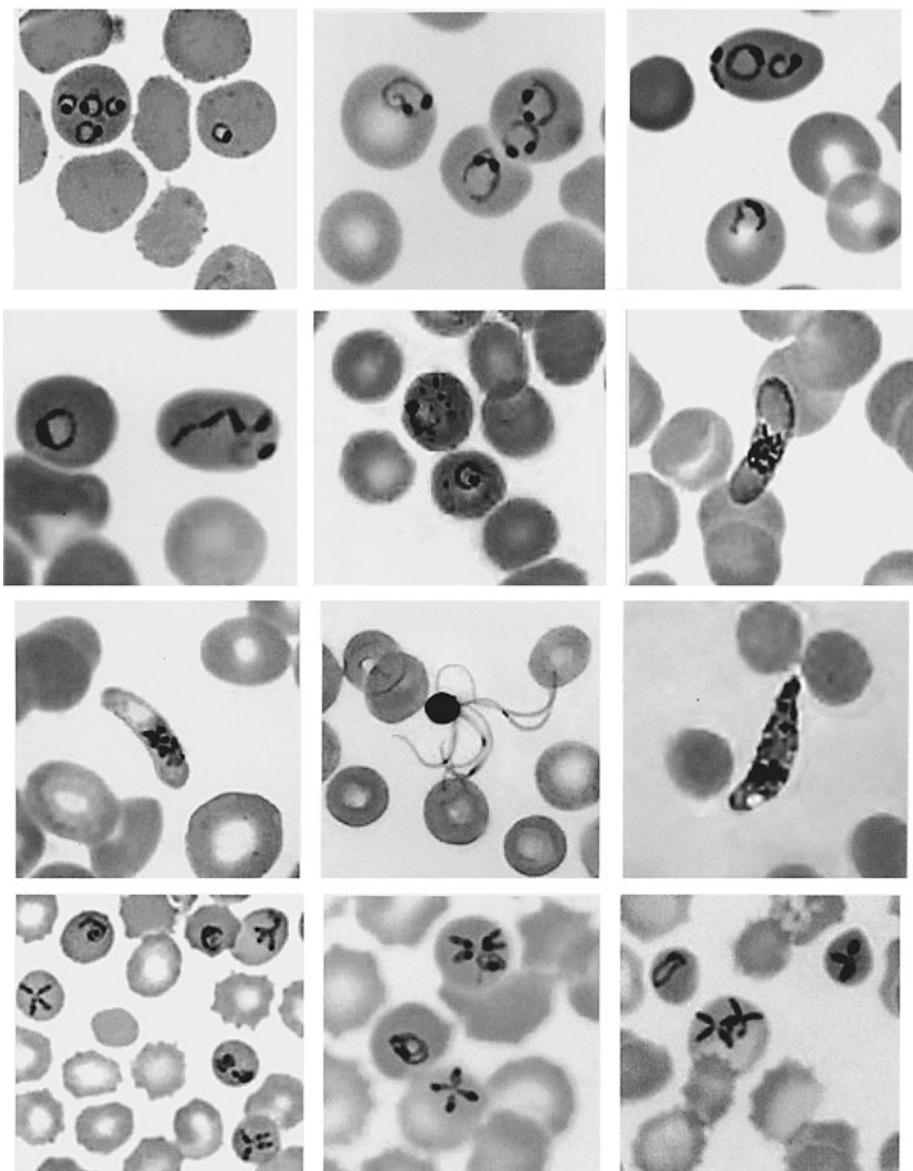
Congenital transmission can occur (rarely) even though the mother is immune; reinfection of the mother during pregnancy is possible, particularly if she is exposed to large numbers of infective cysts and/or oocysts.

Hydrocephalus, cerebral calcifications, and chorioretinitis resulting in mental retardation, epilepsy, and impaired vision represent the most severe form of the disease. Cerebral lesions may calcify, providing retrospective signs of congenital infection.

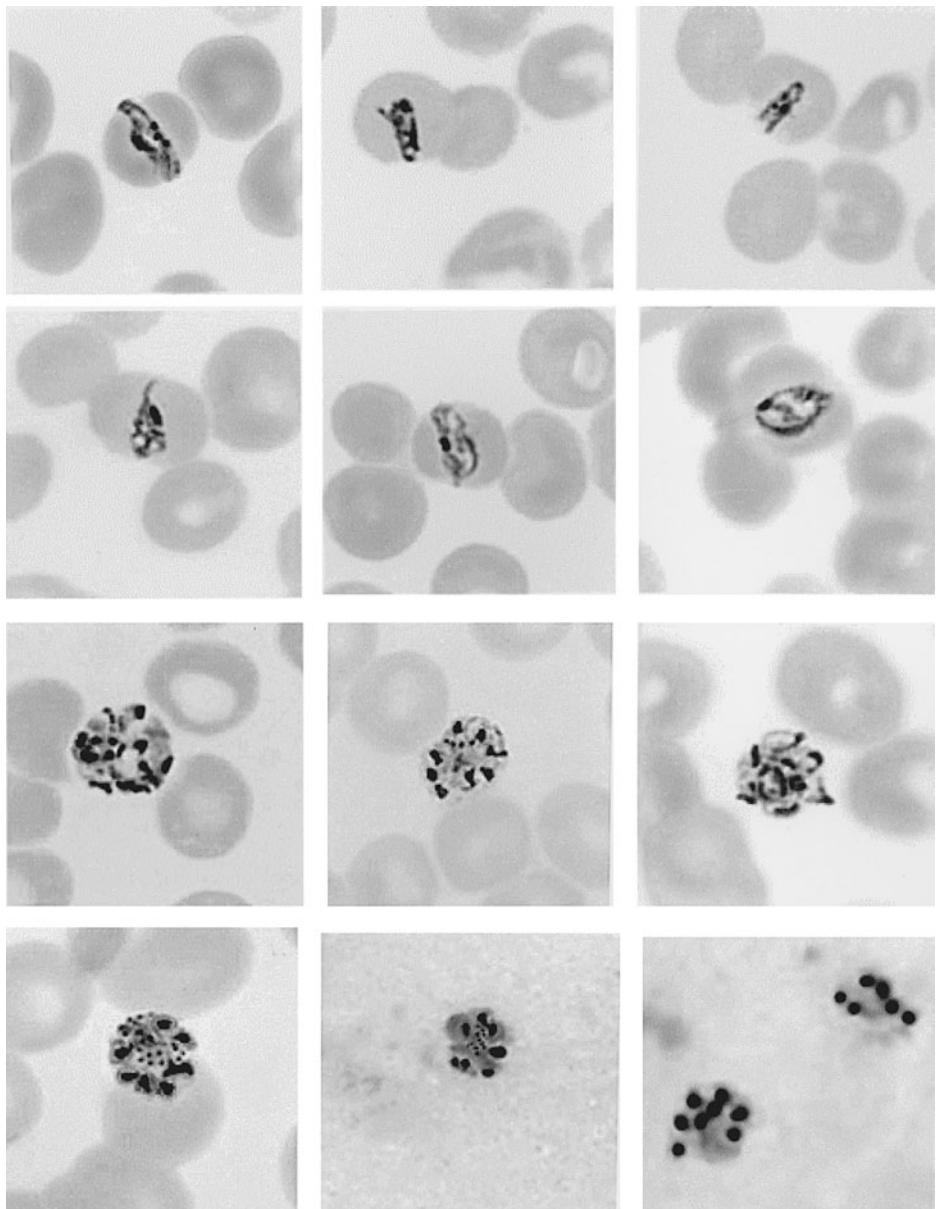
Chorioretinitis in immunocompetent patients is generally due to an earlier congenital infection. Patients may be asymptomatic until the second or third decade, at which time cysts may rupture with eye lesions developing. Chorioretinitis is usually bilateral in patients with congenital infections, and unilateral in patients with recently acquired infections.



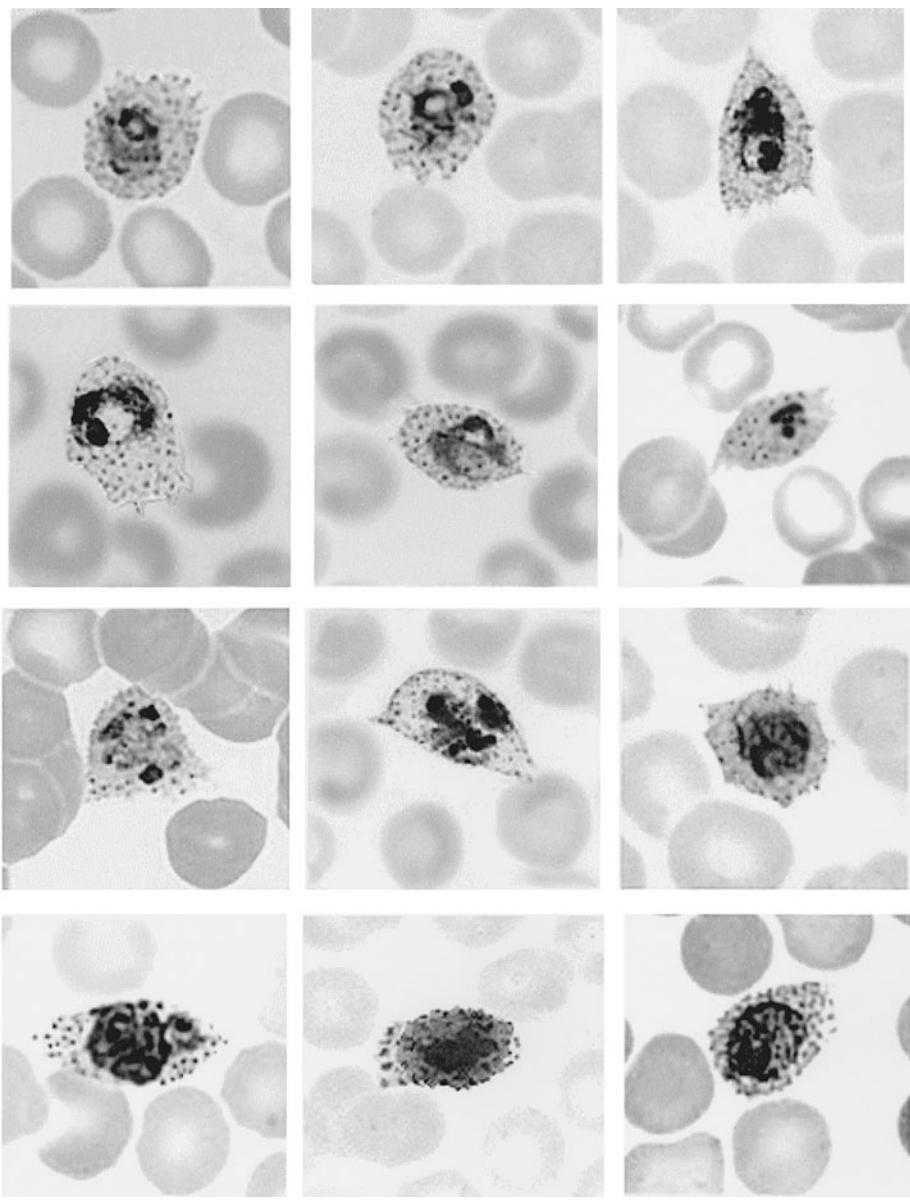
**Plate 7.7** *Plasmodium vivax*. Row 1: developing ring forms (in some RBCs there may be more than one ring; this is more commonly seen in *P. falciparum*, but does occur in *P. vivax* infections); row 2: developing trophozoites (note ameboid forms and Schüffner's dots); row 3: developing schizonts; row 4 (left to right): mature schizont, female macrogametocytes, and male microgametocyte.



**Plate 7.8** *Plasmodium falciparum* and *Babesia* spp. Row 1: developing ring forms of *P. falciparum* (note multiple rings per cell, "headphone" rings, and, in the third image, an appliqué form); row 2: ring forms (in the second image note the Maurer's clefts [not true stippling]) (left and middle) and crescent-shaped gametocyte (right); row 3: gametocyte, exflagellation of the male microgametocyte (can occur in any of the species of *Plasmodium* in a tube of EDTA blood if the cap is removed and the blood cools to room temperature; parasites assume they are in the mosquito) (middle), and ookinete (occurs in the mosquito cycle and can mimic a gametocyte) (right); row 4: various ring forms of *Babesia* spp. (some of the rings are in the "Maltese cross" configuration).



**Plate 7.9** *Plasmodium malariae*. Rows 1 and 2: developing trophozoites (note the band form configuration); row 3: developing schizonts; row 4: mature “rosette” schizont (the last two images are from thick blood films).



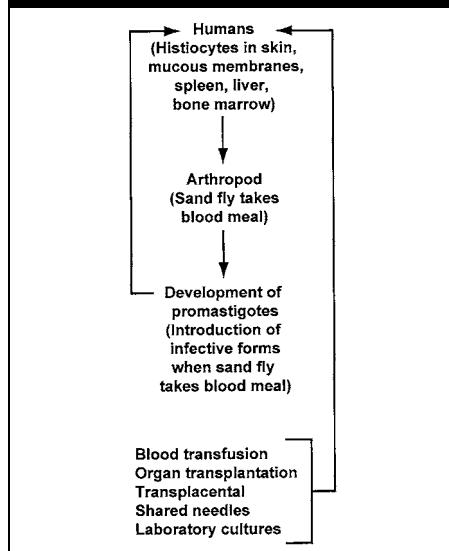
**Plate 7.10** *Plasmodium ovale*. Rows 1 and 2: developing trophozoites (the parasites are much less ameboid than *P. vivax*; note the presence of Schüffner's dots and the oval RBCs); row 3: developing schizonts; row 4: mature schizont and male and female gametocytes.

## PROTOZOA • Flagellates (Blood and Tissue)

### *Leishmania* spp.

<b>Pathogenic</b>	Yes
<b>Disease</b>	Leishmaniasis (cutaneous, mucocutaneous, visceral)
<b>Acquired</b>	Bite of sand flies; blood, shared needles, congenital infections
<b>Body site</b>	Reticuloendothelial system
<b>Symptoms</b>	Papule, ulcers (cutaneous); destruction of cartilage (mucocutaneous); splenomegaly and hepatomegaly (visceral)
<b>Clinical specimen</b>	<i>Cutaneous/mucocutaneous:</i> wall of the lesion (punch biopsy) <i>Visceral:</i> blood (EDTA), bone marrow aspirate
<b>Epidemiology</b>	Old and New World; sand fly-to-human and human-to-human transmission
<b>Control</b>	Vector control, avoiding shared needles, checking blood supply

### LIFE CYCLE



### Diagnosis

In areas of endemic infection, the diagnosis may be made on clinical grounds. The infection may go unrecognized in other areas. Definitive diagnosis depends on detecting either the amastigotes in clinical specimens or the promastigotes in culture. Cutaneous and mucocutaneous leishmaniasis may have to be differentiated from tropical ulcers, syphilis, yaws, leprosy, South American blastomycosis, sporotrichosis, pyogenic nodules or abscesses, furuncles, insect bites, cutaneous tuberculosis, and atypical mycobacterial infections of the skin.

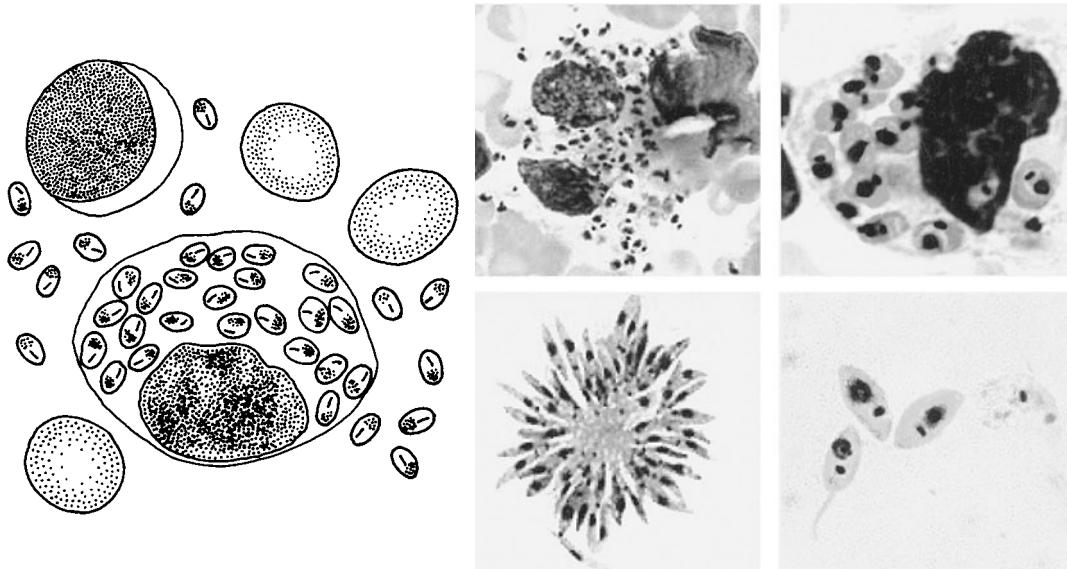
Detection of parasites in aspirates, scrapings, or biopsy tissues depends on organism distribution, the host immune response, bacterial infections in the lesions, and whether the specimen is from an active or healing lesion. Samples should be collected from the most active lesion. All lesions should be cleaned with 70% alcohol, and extraneous material should be removed to minimize the risk of bacterial or fungal contamination of subsequent cultures. Material collected from the center of a necrotic ulcer may reveal only pyogenic organisms. If specimens are collected from the margins by aspiration, scraping, or biopsy, local anesthesia may be used. Three biopsy specimens should be divided (for cultures, touch preparations, and histopathology). The core of tissue from a biopsy can be used to make imprints or touch preparations on a slide.

### General Comments

The parasite life cycle has two distinct phases. The organism is engulfed by reticuloendothelial cells (RE cells) of the mammalian host, where it can be found in the amastigote form (Leishman-Donovan body) within the phagocytic cell. Amastigote forms are small, round or oval bodies of 3 to 5  $\mu\text{m}$ ; the large nucleus and small kinetoplast can be seen with Giemsa or Wright's stain, and the short intracytoplasmic portion of the flagellum may also be seen. The amastigote multiplies by binary fission in the macrophage parasitophorous vacuole until the cell is destroyed; liberated parasites are phagocytized by other RE cells or ingested by the insect vector.

On ingestion during a blood meal by the phlebotomine sand fly vector, the amastigote transforms into the promastigote, a motile, slender organism (10 to 15  $\mu\text{m}$ ) with a single anterior flagellum. Promastigotes multiply by longitudinal fission in the insect gut, attaching to the gut wall by their flagella. Stages found in the vector vary from rounded or stumpy forms to elongated, highly motile metacyclic promastigotes. The metacyclic promastigotes migrate to the sand fly hypostome, where they are inoculated into humans during the next blood meal.

Leishmaniasis is diagnosed each year in the United States in immigrants from countries with endemic infection, military personnel, and travelers. There is potential for more infections in areas of endemic infection in Texas and Arizona. Organisms can remain latent for years; even when the potential exposure history is in the past, leishmaniasis should be considered, particularly in immunocompromised patients.



Images from left to right: Drawing of amastigotes in bone marrow (visceral leishmaniasis). (Top) Amastigotes in bone marrow. (Bottom) Promastigote stages from culture.

## Additional Information

Leishmaniasis is principally a zoonosis, although in certain areas it is endemic, with human-vector-human transmission. It is caused by a diverse group of agents of increasing public health importance (more than 400,000 new cases are reported annually). Approximately 350 million people are at risk of acquiring leishmaniasis, with 12 million currently infected. Depending on the species, *Leishmania* infection can result in cutaneous (CL), mucocutaneous (ML), or visceral (VL) disease.

From August 2002 to February 2004, over 500 confirmed cases of CL were found in military personnel serving in Afghanistan, Iraq, and Kuwait. Most were probably acquired in Iraq, with *L. major* being confirmed as the agent by isoenzyme electrophoresis. Based on data from Fort Campbell, KY, ca. 1% of troops returning from Iraq were diagnosed with CL, most by laboratory confirmation including PCR.

VL is very prevalent in AIDS patients in some areas, with many cases being subclinical. Subclinical VL occurs at any stage of HIV-1 infection, but symptomatic cases appear mainly when severe immunosuppression is present. HIV-*Leishmania* coinfection is increasingly common in the Mediterranean basin, especially in Spain, France, and Italy.

**Note:** Leishmaniasis should be suspected in individuals who resided in or traveled to areas where the disease is endemic. Diagnosis of VL would be supported by findings of remittent fevers, hypergammaglobulinemia with anemia, circulating immune complexes, rheumatoid factors, weight loss, leukemia, and hypersplenism. Differential diagnosis should include African trypanosomiasis, brucellosis, endocarditis, malaria, schistosomiasis, tuberculosis, typhoid, cirrhosis, leukemia, and lymphoma. Lesions caused by post-kala azar dermal leishmaniasis are confused with those of leprosy.

Reduction of reservoir hosts (rodents and dogs) and treatment of infected individuals have been successful in limiting transmission.

Cutaneous lesions are usually single, self-limiting papules, nodules, or ulcers, found mostly on the face and ears (60% of cases); they are painless and often heal spontaneously within a few months.

Early signs of ML are nasal inflammation and stuffiness. Metastatic spread to the nasal or oral mucosa may occur immediately or many years later. Mucosal disease is more prevalent in males than in females; patients are both serologically and skin test positive. There is progressive ulceration and erosion of the soft tissue and cartilage, leading to loss of the lips, soft parts of the nose, and soft palate. Mucosal lesions do not heal spontaneously; secondary bacterial infections are frequent and may be fatal. Aspiration pneumonia is a common complication. Mortality is low, but disfigurement can be substantial.

Symptoms of VL may be vague, while acute onset may resemble typhoid fever, malaria, acute Chagas' disease, amebic liver abscess, or other febrile diseases. Symptoms include fever, anorexia, malaise, weight loss, and often diarrhea. Fever may occur at irregular intervals; once infection is established, a double (dromedary) or triple fever peak may be seen daily. Clinical signs include nontender hepatomegaly and splenomegaly, lymphadenopathy, and occasional acute abdominal pain; darkening of facial, hand, foot, and abdominal skin (kala azar) is often seen in light-colored persons in India. Patients with kala azar have anemia (normocytic and normochromic unless there is an underlying iron deficiency), eosinopenia, neutropenia, thrombocytopenia and hypergammaglobulinemia (polyclonal B-cell activation).

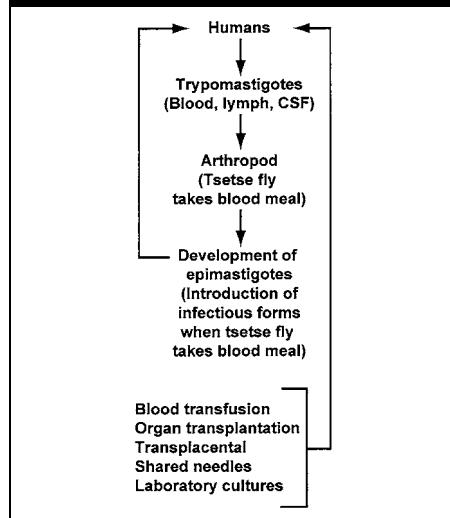
## PROTOZOA • Flagellates (Blood and Tissue)

### *Trypanosoma brucei gambiense* (West)

### *T. brucei rhodesiense* (East)

<b>Pathogenic</b>	Yes
<b>Disease</b>	African trypanosomiasis
<b>Acquired</b>	Tsetse fly; blood, shared needles, congenital infections
<b>Body site</b>	Blood, CNS
<b>Symptoms</b>	Acute febrile illness, leading to sleeping sickness (chronic CNS invasion)
<b>Clinical specimen</b>	Blood (buffy coat preparations, culture)
<b>Epidemiology</b>	Africa; vector-to-human and human-to-human transmission
<b>Control</b>	Vector control, avoiding shared needles, checking blood supply

#### LIFE CYCLE



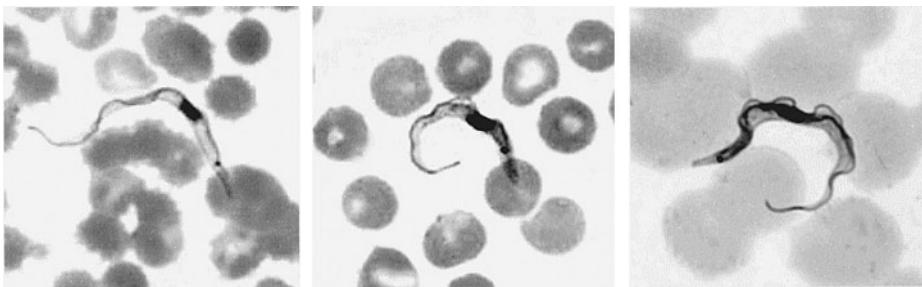
### Diagnosis

Symptoms include irregular fever, enlargement of the lymph nodes (particularly those of the posterior triangle of the neck, [Winterbottom's sign]), delayed sensation to pain (Kerandel's sign), and erythematous skin rashes. Definitive diagnosis depends on demonstration of trypomastigotes in blood, lymph node aspirates, sternum bone marrow, and CSF. There is a better chance of detecting organisms in body fluids in infections caused by *T. brucei rhodesiense* than *T. brucei gambiense*. Because of periodicity, parasite numbers in the blood may vary; therefore, multiple specimens should be collected and a number of techniques used to detect the trypomastigotes.

Health care personnel must use Standard Precautions when handling specimens from suspected cases of African trypanosomiasis because the trypomastigote is highly infectious. Blood can be collected from either finger stick or venipuncture (with EDTA). Multiple blood examinations should be performed before trypanosomiasis is ruled out. Parasites are found in large numbers in blood during the febrile period and in small numbers when the patient is afebrile. If CSF is examined, 5 ml or more should be collected. In addition to thin and thick blood films, a buffy coat concentration method is recommended. Parasites can be detected on thick blood smears when numbers are greater than 2,000/ml, and when they are greater than 100/ml with hematocrit capillary tube concentration. CSF examination must be done with centrifuged sediments.

### General Comments

In blood, the trypanosomes move rapidly among the RBCs. Trypomastigotes are 14 to 33  $\mu\text{m}$  long and 1.5 to 3.5  $\mu\text{m}$  wide. At the organism's posterior end is the kinetoplast and the remaining intracytoplasmic flagellum (axoneme), which may not be noticeable. The flagellum and undulating membrane arise from the kinetoplast. The flagellum runs along the edge of the undulating membrane until the undulating membrane merges with the trypanosome body at the organism's anterior end. At this point, the flagellum becomes free to extend beyond the body. Trypanosomal forms are ingested by the tsetse fly (*Glossina* spp.) when a blood meal is taken. Once the short, stumpy trypomastigote reaches the midgut of the fly, it transforms into a long, slender procyclic stage. The organisms multiply in the lumen of the midgut and hindgut of the fly. After about 2 weeks, they migrate to the salivary glands through the hypopharynx and salivary ducts, where they attach to the epithelial cells of the salivary ducts and then transform to their epimastigote forms. In the epimastigote forms, the nucleus is posterior to the kinetoplast, in contrast to the trypomastigote. Within the salivary gland, metacyclic (infective) forms develop from the epimastigotes in 2 to 5 days. With development of the metacyclic forms, the tsetse fly is infective and can introduce these forms into the puncture wound when the next blood meal is taken. The entire developmental cycle in the fly takes about 3 weeks. Once infected, the tsetse fly remains infected for life.



Images from left to right: *T. brucei gambiense* or *T. brucei rhodesiense* trypomastigotes (stained with any blood stain [Giemsa, Wright-Giemsa combination, any of the rapid blood stains]).

## Additional Information

African trypanosomiasis is limited to the tsetse fly belt of Central Africa, where it has been responsible for some of the most serious obstacles to economic and social development in Africa. Within this area, the vast majority of tsetse flies prefer animal blood, which limits the raising of livestock. Over 50 million people are at risk, and there are approximately 25,000 new cases per year. Human infections are caused by *T. brucei gambiense* and *T. brucei rhodesiense*.

The trypanosomal (trypomastigote) forms can be found in the blood, CSF, lymph node aspirates, and fluid aspirated from the trypanosomal chancre (if one forms at the site of the tsetse fly bite). The trypomastigote forms multiply by longitudinal binary fission. The forms range from long, slender-bodied organisms with a long flagellum (trypomastigote) ( $\geq 30 \mu\text{m}$  long) to short, fat, stumpy forms without a free flagellum (ca.  $15 \mu\text{m}$  long). The short, stumpy forms do not divide in the bloodstream but are the infective stage for the tsetse fly.

**Note:** Laboratory findings include anemia, granulocytopenia, reduction in platelets, increased sedimentation rate, polyclonal B-cell activation with a marked increase in serum IgM, heterophile and anti-DNA antibodies, rheumatoid factor, and circulating immune complexes. The sustained high IgM levels are a result of the parasite producing variable antigen types which allow the organism to evade the patient's defense system. *In an immunocompetent host, the absence of elevated serum IgM rules out trypanosomiasis.*

Metacyclic trypomastigote stages are introduced via the tsetse bite and set up a local inflammatory reaction. A nodule or chancre may develop after a few days and resolves spontaneously within 1 to 2 weeks; this is seen frequently in white Europeans but rarely in patients indigenous to the area. The trypomastigotes enter the bloodstream, causing a symptom-free low-grade parasitemia that may continue for months. This is stage I disease (systemic trypanosomiasis, no CNS involvement). Parasites may be difficult to detect, even on thick blood film examinations. The infection may self-cure during this period.

Symptoms include remittent, irregular fevers with night sweats. Headaches, malaise, and anorexia frequently accompany the fevers. Febrile periods alternate with periods of no fever. Many trypomastigotes are found in circulating blood during fevers; few are seen when fever is absent. Enlarged lymph nodes are soft, painless, and non-tender; enlarged posterior cervical nodes (Winterbottom's sign) are the most common. The spleen and liver are enlarged. With Gambian trypanosomiasis, the blood-lymphatic stage may last for years before sleeping sickness syndrome occurs. The Rhodesian form causes a more rapid fulminant disease; death may occur in months rather than years.

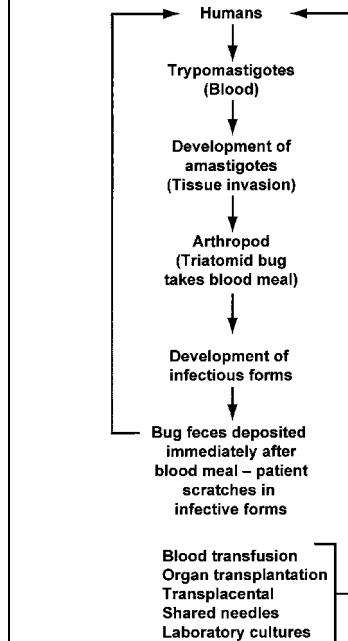
Trypomastigote invasion of the CNS causes sleeping sickness (stage II disease). Trypomastigotes are found primarily in the frontal lobes, pons, and medulla. Behavioral and personality changes occur during CNS invasion. Gambian trypanosomiasis is characterized by steady progressive meningoencephalitis, apathy, confusion, fatigue, coordination loss, and somnolence. In the terminal phase, the patient becomes emaciated, progressing to profound coma and death, usually from secondary infection.

## PROTOZOA • Flagellates (Blood and Tissue)

### *Trypanosoma cruzi*

<b>Pathogenic</b>	Yes
<b>Disease</b>	American trypanosomiasis
<b>Acquired</b>	Triatomid bug (feces); blood, shared needles, congenital infections, organ transplants
<b>Body site</b>	Blood, all tissues
<b>Symptoms</b>	Acute febrile illness, lymphadenopathy and myocarditis, chagoma or Romaña's sign; cardiomegaly, cardiac conduction defects, severe constipation, or dysphagia
<b>Clinical specimen</b>	Blood (buffy coat preparations, culture, xenodiagnosis)
<b>Epidemiology</b>	American continents; vector-to-human and human-to-human transmission
<b>Control</b>	Vector control, avoiding shared needles, checking blood supply and donor organs

### LIFE CYCLE



### Diagnosis

The differential diagnosis of acute Chagas' infection includes brucellosis, endocarditis, salmonellosis, schistosomiasis, toxoplasmosis, tuberculosis, connective tissue diseases, and leukemia. Chronic Chagas' disease with cardiomyopathy may be confused with endocarditis, ischemic heart disease, and rheumatic heart disease.

Trypomastigotes may be detected in the blood in young children; however, in chronic disease, this stage is rare or absent except during fever episodes. They may be detected by using thin and thick blood films or buffy coat concentration techniques (most sensitive). Any blood stains can be used for trypomastigote and amastigote stages. *T. rangeli* trypomastigote infections may have to be differentiated from those with *T. cruzi*. *T. cruzi* trypomastigotes are usually "C" or "U" shaped on fixed blood films and have a large oval kinetoplast at the posterior end. *T. rangeli* trypomastigotes have a smaller kinetoplast near the posterior end and do not have an amastigote stage.

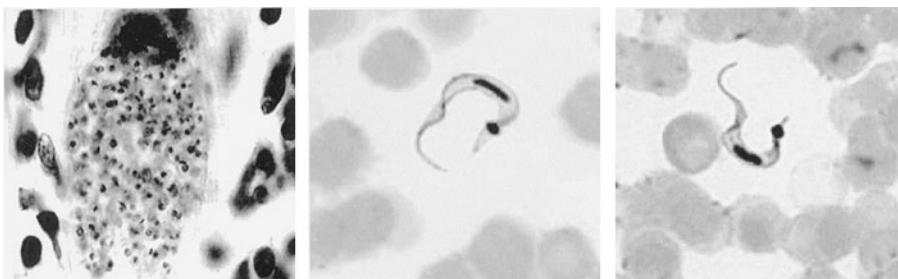
Aspirates from chagomas and enlarged lymph nodes can be examined for amastigotes and trypomastigotes. Histologic examination of biopsy specimens may also be done. Aspirates, blood, and tissues can be cultured. The medium of choice is Novy-MacNeal-Nicolle's medium. Cultures should be incubated at 25°C and observed for epimastigote stages for up to 30 days before being considered negative.

### Life Cycle

In humans, *T. cruzi* can be found in two forms, amastigotes and trypomastigotes. The trypomastigote does not divide in the blood but carries the infection to all parts of the body. The amastigote multiplies within virtually any cell, preferring cells of the reticuloendothelial system, cardiac muscle, skeletal muscle, smooth muscle, and neuromuscular.

The disease is transmitted to humans through the bite wound caused by reduviid bugs. Metacyclic trypomastigotes are released with feces during a blood meal, and the feces are rubbed or scratched into the bite wound or onto mucosal surfaces, an action stimulated by the allergic reaction to the insect's saliva. The metacyclic forms invade local tissues, transform to the amastigote, and multiply within the cells. Once inside a cell, the trypomastigote loses its flagellum and undulating membrane and divides by binary fission to form an amastigote. The amastigote continues to divide and eventually fills and destroys the infected cell. Both amastigote and trypomastigote forms are released from the cell.

Trypomastigotes are ingested by the reduviid bug during a blood meal. They transform into epimastigotes that multiply in the posterior portion of the midgut. Metacyclic trypomastigotes develop from the epimastigotes (10 days) and are passed in the feces to infect humans when rubbed into the insect's puncture wound or rubbed onto exposed mucous membranes.



Images from left to right: *T. cruzi* amastigotes in cardiac tissue, and typical trypomastigotes (note the large kinetoplast, much larger than that seen in the African sleeping sickness trypomastigotes).

## Additional Information

Chagas' disease is a zoonosis in the American continents and involves triatomid (reduviid) bugs living in close association with human reservoirs (dogs, cats, armadillos, opossums, raccoons, and rodents). There are ca. 100 million persons at risk of infection, of whom 16 million to 18 million are actually infected. There are ca. 50,000 deaths per year due to Chagas' disease. In some areas, ca. 10% of all adult deaths are due to Chagas' disease. Human infections occur mainly in rural areas where poor sanitary and socioeconomic conditions and poor housing provide excellent breeding places for reduviid bugs and allow maximum contact between the vector and humans. Reduviids in the United States have not adapted themselves to household habitation.

**Note:** The amastigote is indistinguishable from those found in leishmanial infections. It is 2 to 6  $\mu\text{m}$  in diameter and contains a large nucleus and rod-shaped kinetoplast that stains red or violet with Giemsa stain.

**Note:** Although it is less common than cardiac involvement, patients may have dilation of the digestive tract with or without cardiomyopathy. These symptoms are most often seen in the esophagus and colon as a result of neuronal destruction. Megaeosophagus characterized by dysphagia, chest pain, regurgitation, and malnutrition is related to loss of contractility of the lower esophagus. Hypersalivation may occur, leading to aspiration with repeated bouts of aspiration pneumonia. Megacolon results in constipation, abdominal pain, and inability to discharge feces. In some individuals, there may be acute obstruction leading to perforation, septicemia, and death.

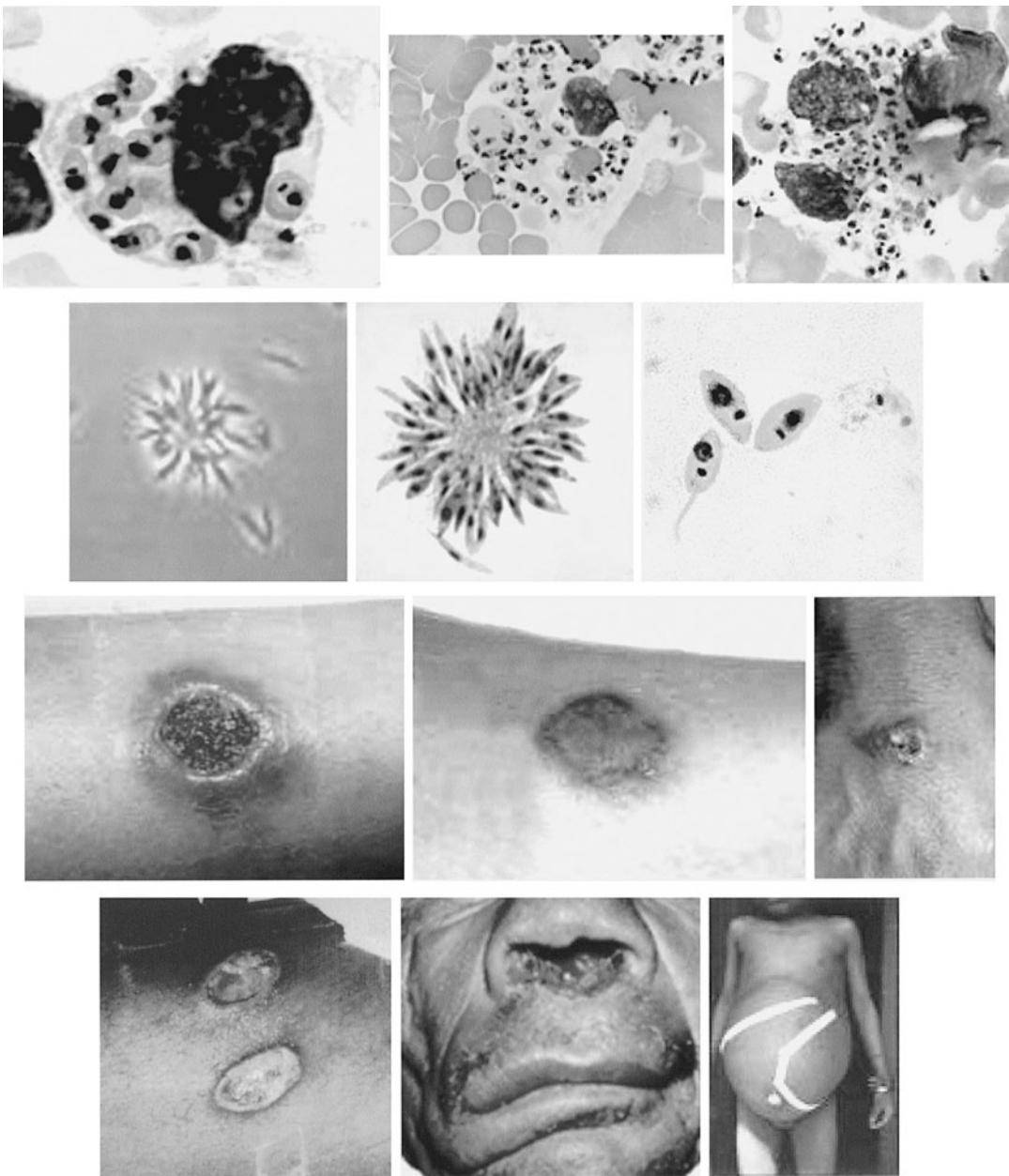
In children younger than 5, the disease is most severe; in older children and adults, it is more chronic. An erythematous painful nodule (chagoma) may form on the face; it may take 2 to 3 months to subside. Amastigotes

or trypomastigotes may be aspirated from the chagoma; if the route of inoculation is the ocular mucosa, edema of the eyelids and conjunctivitis may occur (Romaña's sign). The infective stages spread to the regional lymph nodes, which become enlarged, hard, and tender. Trypomastigotes appear in the blood about 10 days after infection and persist through the acute phase. These stages are rare or absent during the chronic phase.

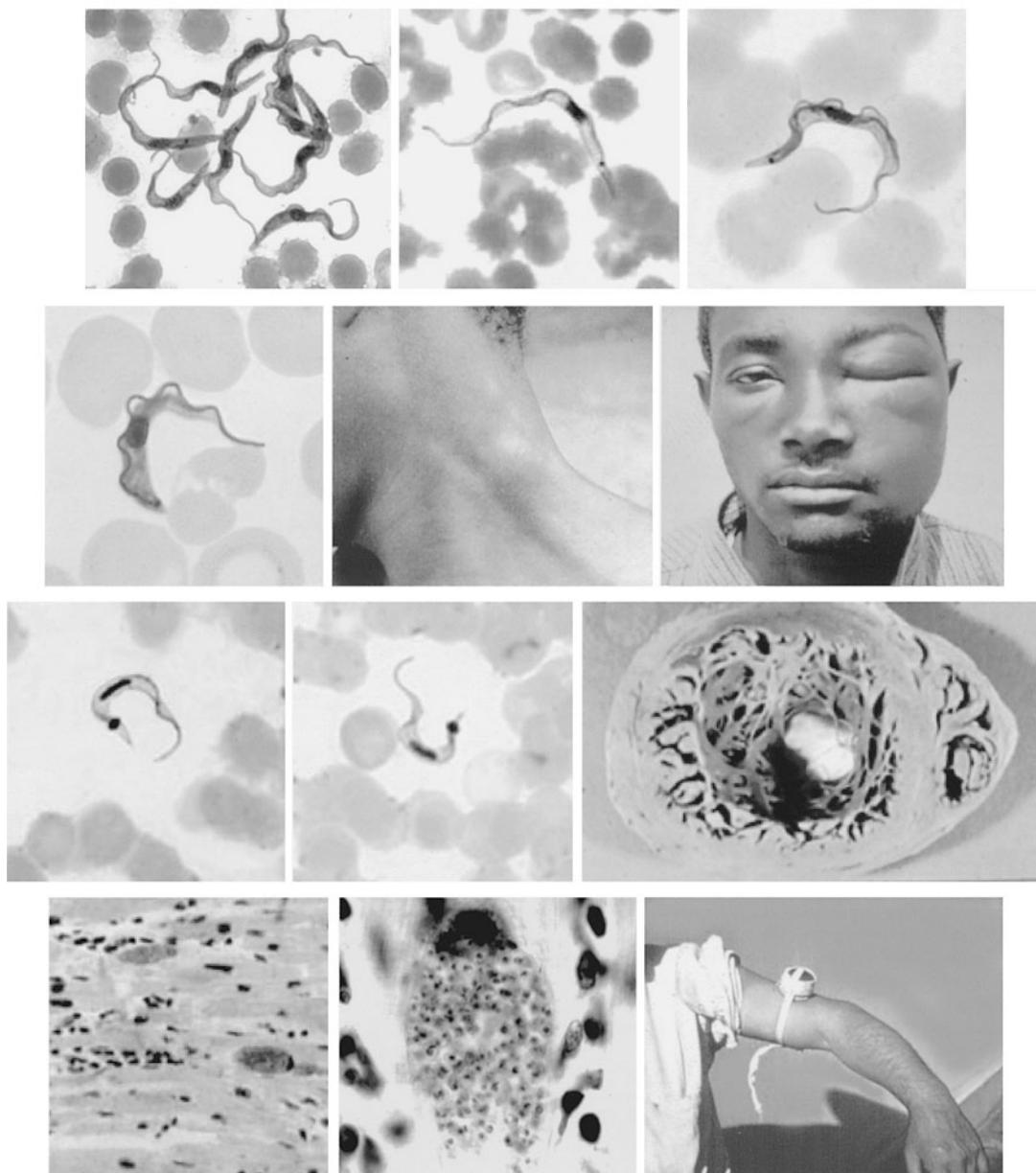
Acute infections are characterized by high fevers, which may be intermittent, remitting, or continuous; hepatosplenomegaly; myalgia; erythematous rash; acute myocarditis; lymphadenopathy; and subcutaneous edema of the face, legs, and feet. There may be meningoencephalitis, which has a very poor prognosis. Myocarditis is manifested by electrocardiographic changes, tachycardia, chest pain, and weakness. Amastigotes proliferate within and destroy the cardiac muscle cells, causing conduction defects and loss of heart contractility. Death may occur due to myocardial insufficiency.

Death may occur within a few weeks or months, or the patient may enter the chronic phase. This phase may be asymptomatic; organisms are seldom seen in peripheral blood, but transmission by blood transfusion is a serious problem in endemic areas. Also, recrudescence of *T. cruzi* infections in immunosuppressed patients, particularly transplantation patients, is a grave concern.

Chronic cardiomyopathy appears to fulfill many of the criteria for autoimmune diseases. However, validation of the target antigens must involve induction of cardiac lesions after immunization or passive transfer of antigen-specific T cells. Aberrant T-cell activation may also result in cardiac injury. Not all researchers agree that autoantibodies play a role in pathogenesis. Also, some propose a "combined" theory to explain the sequence of events leading to chronic myocarditis. The actual clinical course may vary from heart failure to a slow but progressive loss of cardiac function, with possible ventricular rupture and thromboemboli.



**Plate 7.11** Row 1 (left to right): *Leishmania* amastigotes within macrophage (note the nucleus and bar within each amastigote) (courtesy of the Centers for Disease Control and Prevention), *Leishmania donovani* in bone marrow and amastigotes in bone marrow; row 2 (left to right): *Leishmania donovani* promastigotes in culture (wet mount), promastigotes stained with a blood stain, and individual promastigotes (higher power); row 3 (left to right): *Leishmania* cutaneous lesion (active), *Leishmania* cutaneous lesion (healed), and *Leishmania* cutaneous lesion; row 4 (left to right): *Leishmania* cutaneous lesion (the bandage slipped, inoculating the skin; hence, there are now two lesions), *Leishmania* mucocutaneous lesions (note the lack of nasal septum), and *Leishmania donovani* (visceral leishmaniasis/kala azar, hepatomegaly and splenomegaly).



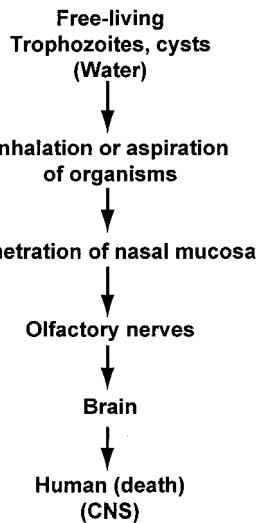
**Plate 7.12** Row 1: *Trypanosoma brucei gambiense* or *T. b. rhodesiense* (African trypomastigotes); row 2 (left to right): *Trypanosoma brucei gambiense* or *T. b. rhodesiense* (African trypomastigote), Winterbottom's sign (African trypanosomiasis, swollen lymph node at posterior cervical region), and Romaña's sign (Chagas' disease, edema of the eyelid); row 3: *Trypanosoma cruzi* trypomastigotes (left and middle) and heart showing cardiomyopathy (dilation and thinning of the apical myocardium and marked concentric muscular hypertrophy) (right) (from *A Pictorial Presentation of Parasites: a cooperative collection prepared and/or edited by H. Zaiman*); row 4 (left to right): *Trypanosoma cruzi* amastigotes in tissue, *Trypanosoma cruzi* amastigotes in tissue (higher magnification), and xenodiagnosis (trypanosome-free bugs are allowed to feed on individuals suspected of having Chagas' disease; if organisms are present in the blood meal, the parasites multiply and can be detected in the bug's intestinal contents, which should be examined for 3 months).

## PROTOZOA • Amebae (Other Body Sites)

### *Naegleria fowleri*

<b>Pathogenic</b>	Yes; rare but almost always fatal
<b>Disease</b>	Primary amebic meningoencephalitis (PAM)
<b>Acquired</b>	Contaminated water, olfactory epithelium, possible airborne exposure
<b>Body site</b>	CNS; hemorrhagic necrosis
<b>Symptoms</b>	Headache, nausea, vomiting, confusion, fever, stiff neck, seizures, coma
<b>Clinical specimen</b>	Brain biopsy specimen, CSF wet preparation, culture, IFA of tissue, PCR
<b>Epidemiology</b>	Worldwide
<b>Control</b>	Refrain from using homemade or outdated lens solutions; avoid water exposure (compromised patients)

#### LIFE CYCLE



### Diagnosis

A high index of suspicion is mandatory for early diagnosis. Most cases are associated with exposure to contaminated water through swimming or bathing. The rapidly fatal course of 3 to 6 days after the beginning of symptoms (with an incubation period of 1 day to 2 weeks) requires early diagnosis and immediate chemotherapy if the patient is to survive.

Analysis of CSF shows decreased glucose and increased protein levels. Leukocytes may range from several hundred to  $>20,000$  cells/ $\text{mm}^3$ . Gram stains and bacterial cultures of CSF are negative; however, the Gram stain background can incorrectly be identified as positive with bacteria, leading to a misdiagnosis and incorrect therapy.

In cases of presumptive pyogenic meningitis in which no bacteria are identified in the CSF, the CT appearance of basal arachnoiditis should alert the staff to the possibility of acute PAM.

Either CSF or sedimented CSF should be placed on a slide, under a coverslip, and observed for motile trophozoites; smears can also be stained with any of the blood stains. CSF, exudate, or tissue fragments can be examined by light microscopy or phase-contrast microscopy. It is very easy to confuse leukocytes and amebae, particularly when examining CSF in a counting chamber, hence the recommendation to use just a regular slide and coverslip. Motility may vary, so the main differential characteristic is the spherical nucleus with a large karyosome. Specimens should never be refrigerated prior to examination. When centrifuging the CSF, low speeds ( $250 \times g$ ) should be used so that the trophozoites are not damaged.

If *Acanthamoeba* is involved, cysts may also be seen in specimens from CNS infection. Unfortunately, most cases of PAM are diagnosed at autopsy; confirmation of these tissue findings must include culture and/or special staining with monoclonal reagents in IFA procedures. Organisms can also be cultured on nonnutritive agar plated with *Escherichia coli*.

In general, serologic tests have not been helpful in diagnosis. The disease progresses so rapidly that the patient is unable to mount an immune response.

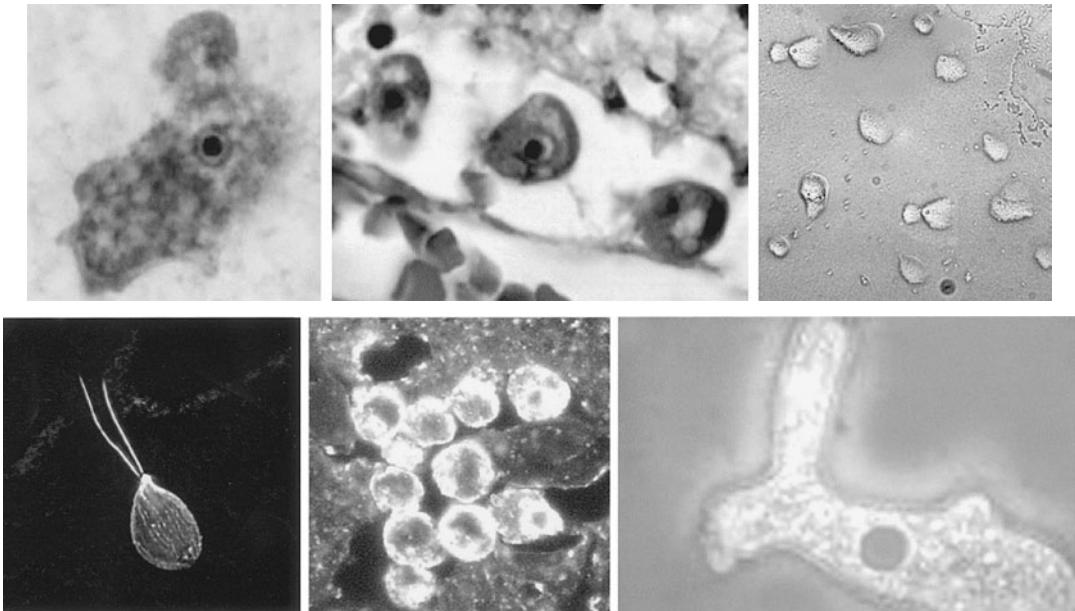
The risk of transmission of *N. fowleri* by donor organs has not been clarified, and no practical test is available to ensure that donor organs are organism free. Also, no prophylactic drug regimen to treat transplant recipients has been established.

### General Comments

PAM is an acute, suppurative infection of the brain and meninges. With extremely rare exceptions, the disease is rapidly fatal in humans.

The amebae may enter the nasal cavity by inhalation or aspiration of water, dust, or aerosols containing the trophozoites or cysts. They then penetrate the nasal mucosa and migrate via the olfactory nerves to the brain. The nasopharyngeal mucosa shows ulceration, and the olfactory nerves are inflamed and necrotic. Hemorrhagic necrosis is seen primarily in the olfactory bulbs and the base of the brain. Trophozoites can be found in the meninges, perivascular spaces, and sanguinopurulent exudates.

Early symptoms include vague upper respiratory distress, headache, lethargy, and occasionally olfactory problems. The acute phase includes sore throat, stuffy blocked or discharging nose, and severe headache. Progressive symptoms include pyrexia, vomiting, and stiffness of the neck. Mental confusion and coma usually occur approximately 3 to 5 days prior to death. The cause of death is usually cardiorespiratory arrest and pulmonary edema.



Images from left to right: (Top) *N. fowleri* trophozoite (stained), trophozoites in brain tissue, and trophozoites and cysts on the surface of an agar plate (growth on nonnutritive agar with bacterial overlay as a food source). (Bottom) Flagellated stage, indirect immunofluorescence of trophozoites in tissue (courtesy of CDC), and trophozoite (fresh, wet preparation).

## Description

The trophozoites can occur in two forms, amoeboid and flagellate. Motility can be observed in hanging-drop preparations from cultures of CSF; the amoeboid form (the only form recognized in humans) is elongate with a broad anterior end and tapered posterior end. The size ranges from 7 to 35  $\mu\text{m}$ ; the diameter of the rounded forms is usually 15  $\mu\text{m}$ . There is a large central karyosome and no peripheral nuclear chromatin. The cytoplasm is somewhat granular and contains vacuoles. The amoeboid-form organisms change to the transient, pear-shaped flagellate form when transferred from culture or teased from tissue into water and maintained at 27 to 37°C. The change may occur very quickly (within a few hours) or may take as long as 20 h. The flagellate form has two flagella at the broad end. Motility is typical, with either spinning or jerky movements. These flagellate forms do not divide, but when the flagella are lost, the amoeboid forms resume reproduction.

Cysts from nature and from agar cultures look the same and have a single nucleus almost identical to that seen in the trophozoite. They are generally round, measuring 7 to 15  $\mu\text{m}$ , and there is a thick double wall.

## Additional Information

Specific reminders on diagnosis include the following: Never refrigerate the specimen(s) prior to examination. Beware of the false-positive Gram stain, especially since PAM usually mimics bacterial meningitis. Trophozoites mimic leukocytes if put into a counting chamber. Motility is more likely to be seen if a drop of CSF is placed directly on a slide and a coverslip is added. Phase-contrast optics are recommended; however, if regular bright-field microscopy is used, low light is needed. The slide can also be warmed to 35°C to stimulate trophozoite motility. Organisms can be cultured on nonnutritive agar plated with *Escherichia coli*. Low-speed centrifugation is recommended for CSF ( $150 \times g$  for 5 min). Incubate the plate in room air at 35 to 37°C for CNS tissues and at 30°C for tissues from other sites. Using the low-power objective (10 $\times$ ), observe the plates daily for 7 days. *B. mandrillaris* does not grow on agar plates seeded with bacteria. Standard Precautions should be used when handling these specimens and cultures. Procedures should be performed in a biological safety cabinet.

**Note:** This is always considered a STAT diagnostic test request (specimen collection, processing, testing, and reporting).

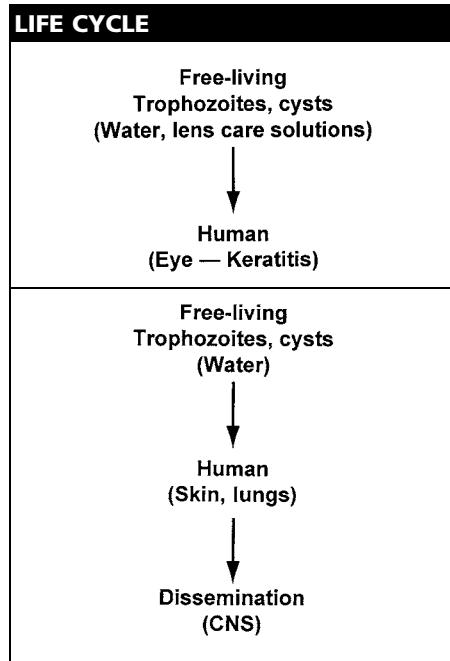
## PROTOZOA • Amebae (Other Body Sites)

### *Acanthamoeba* spp.

### *Balamuthia mandrillaris*

### *Sappinia diploidea*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Amebic keratitis, amebic encephalitis (granulomatous amebic encephalitis [GAE])
<b>Acquired</b>	Contaminated water for keratitis (not linked to <i>Balamuthia</i> , unknown for <i>Sappinia</i> )
<b>Body site</b>	Eyes, skin, lungs, CNS
<b>Symptoms</b>	<i>Eyes:</i> Keratitis, corneal ulceration, retinitis <i>Skin, lungs:</i> Nonhealing lesions, sinusitis <i>CNS:</i> Chronic, slow meningoencephalitis
<b>Clinical specimen</b>	<i>Eyes:</i> Corneal scrapings, opened lens solutions <i>CNS:</i> CSF
<b>Epidemiology</b>	Worldwide, primarily environment-to-human transmission
<b>Control</b>	Refrain from using homemade or outdated lens solutions; avoid water exposure (compromised patients)



### Diagnosis

Meningoencephalitis caused by *Acanthamoeba* spp. may present as an acute suppurative inflammation of the brain and meninges like that seen with *N. fowleri* infection. However, *Acanthamoeba* spp. generally cause a more chronic form of meningoencephalitis. GAE is characterized by confusion, dizziness, drowsiness, nausea, vomiting, headache, lethargy, stiff neck, seizures, and sometimes hemiparesis.

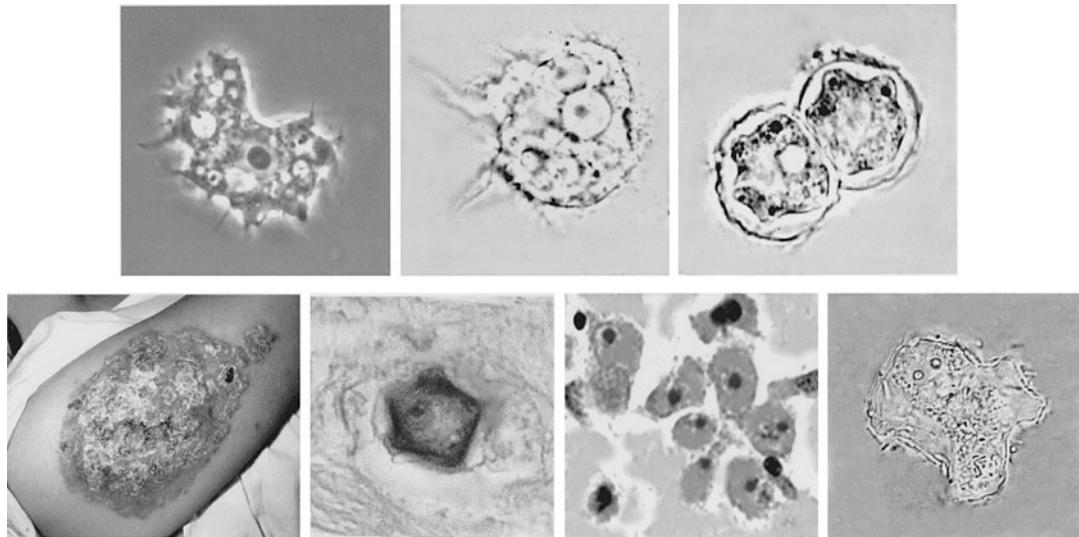
The differential diagnosis should include other space-occupying lesions of the CNS (tumor, abscess, fungal infection, etc.). Predisposing factors include Hodgkin's disease, diabetes, alcoholism, pregnancy, and steroid therapy. Organisms have also been found in the adrenal gland, brain, eyes, kidneys, liver, pancreas, skin, spleen, thyroid gland, and uterus.

A definite diagnosis could be made by demonstration of the amebae in the CSF or biopsy specimens. CSF or sedimented CSF should be placed on a slide, under a coverslip, and observed for motile trophozoites; smears can also be stained with Wright's or Giemsa stain. CSF, exudate, or tissue fragments can be examined by light microscopy or phase-contrast microscopy. It is very easy to confuse leukocytes and amebae, particularly when examining CSF by using a counting chamber, hence the recommendation to use just a regular slide and coverslip. Motility may vary, so the main differential characteristic is the spherical nucleus with a large karyosome.

Keratitis, uveitis, and corneal ulceration have been associated with *Acanthamoeba* spp. Infections have been seen in both hard- and soft-lens wearers. Particular attention has been paid to soft-lens disinfection systems, including homemade saline solutions. Cocontamination of lens systems with bacteria may be a prime factor in the development of amebic keratitis. The onset of *Acanthamoeba* corneal infection can vary tremendously; however, two factors often appear to be involved: trauma and contaminated water.

### General Comments

When corneal abrasions occur, the disease process is usually more rapid, with ulceration, corneal infiltration, iritis, scleritis, severe pain, hypopyon, and loss of vision. When this occurs in an individual who wears contact lenses, the onset is more gradual, but the results are often the same. A contact lens can act as a mechanical vector for transport of amebae from the storage case to the cornea. Another consideration involves the potential infection of the nasal cavity via lacrimal drainage, a condition that probably causes no problems in healthy individuals. It remains unknown whether these organisms in the nasopharynx of an immunocompromised individual increase the risk of GAE.



Images from left to right: (Top) *Acanthamoeba* trophozoites (note the spiky pseudopods) and cyst (note the double wall). (Bottom) *Acanthamoeba* skin lesion (courtesy of G. H. Healy), *Acanthamoeba* cyst in corneal tissue, *Balamuthia* trophozoites in brain tissue, and *Sappinia* trophozoite (note the double nuclei in upper left) (courtesy of G. Visvesvara).

### Description (Trophozoite)

Motile *Acanthamoeba* organisms have spine-like pseudopods; however, progressive movement is usually not very evident. There is a wide range (25 to 40  $\mu\text{m}$ ), with the average diameter of the trophozoites being 30  $\mu\text{m}$ . The nucleus has the typical large karyosome, like that in *N. fowleri*. This morphology can be seen on a wet preparation.

*B. mandrillaris* trophozoites are usually irregular in shape, and actively feeding amebae may measure 12 to 60  $\mu\text{m}$  long (normal, 30  $\mu\text{m}$ ). In tissue culture, broad pseudopodia are usually seen; however, as the monolayer cells are destroyed, the trophozoites develop fingerlike pseudopodia. These organisms do not grow using the seeded agar plate method.

*Sappinia* trophozoites measure 40 to 70  $\mu\text{m}$  and have a distinctive double nucleus. Transmission electron microscopy confirms that they contain two nuclei attached to each other by connecting perpendicular filaments. The trophozoites ingest host blood cells and stain brightly with Giemsa and periodic acid-Schiff stains.

**Warning:** It is important that all patients with unresponsive microbial keratitis, even those who do not wear contact lenses, be evaluated for possible *Acanthamoeba* infection.

### Description (Cyst)

*Acanthamoeba* cysts are usually round with a single nucleus; they also have the large karyosome seen in the trophozoite nucleus. The double wall is usually visible, with the slightly wrinkled outer cyst wall and a polyhedral inner cyst wall. This cyst morphology can be seen in organisms cultured on agar plates. Cyst formation occurs under adverse environmental conditions; cysts are resistant to biocides, chlorination, and antibiotics and can survive low temperatures (0 to 2°C).

*Balamuthia* cysts are usually spherical (6 to 30  $\mu\text{m}$  in diameter). Under electron microscopy, they are characterized by having three layers in the cyst wall: an outer wrinkled ectocyst, a middle structureless mesocyst, and an inner thin endocyst. Under light microscopy, they appear to have two walls, an outer irregular wall and an inner round wall.

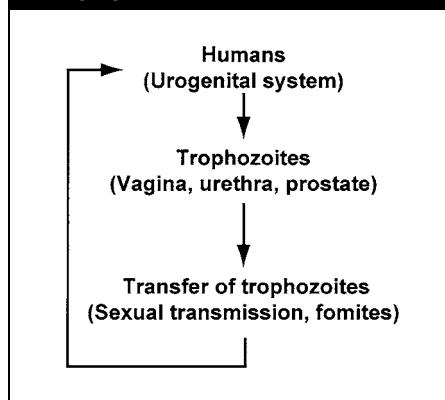
*Sappinia* cysts were not identified in the first human infection, but they cannot be excluded as being present in the human host. Based on recent data, it appears that *S. diploidea* may have a sessile, bicellular cyst.

## PROTOZOA • Flagellates (Other Body Sites)

### Trichomonas vaginalis

<b>Pathogenic</b>	Yes
<b>Disease</b>	Trichomoniasis
<b>Acquired</b>	Direct sexual contact; contaminated towels and underclothes (rare)
<b>Body site</b>	Urogenital tract
<b>Symptoms</b>	Vaginal pruritus, discharge, urethritis
<b>Clinical specimen</b>	Vaginal and urethral discharges and prostatic secretions
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Treat asymptomatic males, promote awareness of sexual transmission

#### LIFE CYCLE



### Diagnosis

Identification is usually based on examination of wet preparations of vaginal and urethral discharges and prostatic secretions. Several specimens may have to be examined to detect the organisms. The specimen should be diluted with a drop of saline and examined under low power with reduced illumination for the presence of actively moving organisms; urine sediment can be examined in the same way. As the jerky motility of the trophozoite diminishes, it may be possible to see the movement of the undulating membrane, particularly under high dry power. Specimens should never be refrigerated.

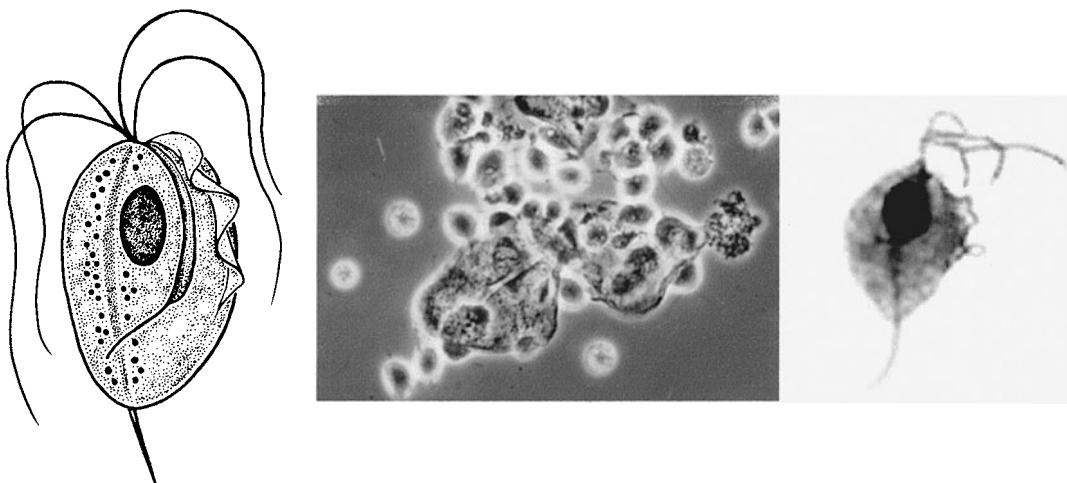
Since the morphology of nonpathogenic *P. hominis* from stool is very similar to that of pathogenic *T. vaginalis*, it is important to ensure that the specimen is not contaminated with fecal material.

Diagnostic tests other than wet preparations, such as permanent stains, fluorescent stains, culture, probes, and rapid tests (dipsticks), can also be used. Organisms may be difficult to recognize in permanent stains; however, if a dry smear is submitted to the laboratory, Giemsa or Papanicolaou stain can be used. Chronic *Trichomonas* infections may cause atypical cellular changes that can be misinterpreted, particularly on the Papanicolaou smear. Organisms are routinely missed on Gram stains. Diagnosis should be confirmed by observation of motile organisms either from the direct wet mount or from appropriate culture media. Results on the rapid dipstick tests are also excellent.

### General Comments

*T. vaginalis* has only the trophozoite stage in its life cycle, and it is very similar in morphology to other trichomonads. The trophozoite is 7 to 23  $\mu\text{m}$  long and 5 to 15  $\mu\text{m}$  wide. The axostyle is clearly visible, and the undulating membrane stops halfway down the side of the trophozoite. The nuclear chromatin is uniformly distributed, and there are abundant siderophil granules that are particularly evident around the axostyle. Normal body sites for these organisms include the vagina and prostate. Apparently, the organisms feed on the mucosal surface of the vagina, where bacteria and leukocytes are found. The preferred pH for good growth is slightly alkaline or acid, not the normal pH of the healthy vagina. Although the organisms can be recovered in urine, in urethral discharge, or after prostatic massage, the pH preference of the organisms in males has not been determined. Often the organisms are recovered in the spun urine sediment from both male and female patients. This organism, like the other trichomonads, divides by binary fission. There are no known cyst forms for this organism.

**Note:** If culture techniques are used, it is mandatory that the specimen be collected correctly, immediately inoculated into the proper medium, and properly incubated. If these requirements are not met, a false-negative result may be obtained. Although culture is more sensitive than wet mounts, this approach is not always used because of cost.



Images from left to right: Drawing of *T. vaginalis* (note that the undulating membrane stops about halfway down the organism), wet mount of organisms, and stained *T. vaginalis* trophozoite.

## Additional Information

Several techniques used concurrently may increase organism recovery. Both monoclonal antibodies and DNA probes for the detection of *T. vaginalis* are very effective. An EIA has been developed to detect *T. vaginalis* antigen from vaginal swabs. The predictive value of a positive test was >80% and that of a negative test was almost 100% (482 women in the study). A rapid latex agglutination test for diagnosing *T. vaginalis* is also available, and results for 395 women attending a genitourinary medicine clinic indicated a sensitivity of 95% for the latex agglutination test and the EIA compared with 74% for microscopy and 76% for culture in Oxoid media. Commercial products based on these methods should be very helpful in diagnosing this infection. Some of the new culture pouch techniques are more sensitive than the older culture methods.

This envelope (pouch) approach allows both immediate examination and culture in one self-contained system. In a group of 62 positive patients, wet mounts (direct or from the envelope) were equal in sensitivity (66%); however, values were 89% with *Trichomonas* medium no. 2 (Oxoid) and 97% with PEM-TV. This system is commercially available as the InPouch TV (Biomed Diagnostics, San Jose, CA), which serves as the specimen transport container, the growth chamber during incubation, and the "slide" during microscopy. Once inoculated, it requires no opening for examination, and positive growth will occur within 5 days.

Various dipsticks are also available (OSOM [Genzyme Diagnostics, Cambridge, MA] and XenoStrip-Tv [Xenotope Diagnostics, Inc., San Antonio, TX]).

For clinical settings in which vaginal specimens are not available and culture is not an option, urine-based PCR-EIA may be another option.

*T. vaginalis* is site specific and usually cannot survive outside the urogenital system. After introduction, proliferation begins, with resulting inflammation and many trophozoites in tissues and secretions. Vaginal secretions have been described as being liquid, greenish or yellowish, sometimes frothy, and foul smelling. As the infection becomes more chronic, the purulent discharge diminishes, with a decrease in the number of organisms. The normal incubation period is 4 to 28 days. The onset of symptoms such as vaginal or vulval pruritus and discharge is often sudden and occurs during or after menstruation as a result of the increased vaginal acidity. About 20% of women with vaginal trichomoniasis have dysuria, which may occur before any other symptoms. Infection in males may be latent, with essentially no symptoms, or may be present as self-limited, persistent, or recurring urethritis. *T. vaginalis* has been detected in 10 to 20% of subjects with nonspecific urethritis and in 20 to 30% of those whose sexual partners had vaginitis. There is also an association with increased HIV transmission and cervical dysplasia.

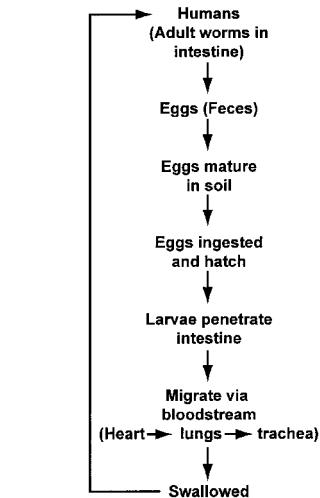
*T. vaginalis* has caused respiratory distress in a full-term, normal male infant after delivery. A wet preparation of thick, white sputum showed few leukocytes and motile flagellates, which were identified as *T. vaginalis*. This study supports previous data indicating that this organism may cause neonatal pneumonia.

## NEMATODES • Intestinal

### *Ascaris lumbricoides*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Ascariasis
<b>Acquired</b>	Fecal-oral transmission of infective eggs in contaminated food or water
<b>Body site</b>	Intestine, larvae in lungs
<b>Symptoms</b>	Pneumonitis, vague intestinal complaints or asymptomatic
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *A. lumbricoides* eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. Diagnosis in the larval migration phase of the infection is based on finding larvae in sputum or gastric washings. The typical Loeffler's syndrome is more likely in areas where transmission is highly seasonal. Diagnosis in the intestinal phase is based on finding eggs (unfertilized or fertilized) or adult worms in the stool. The eggs are most easily seen on a direct wet smear or a wet preparation of the concentration sediment.

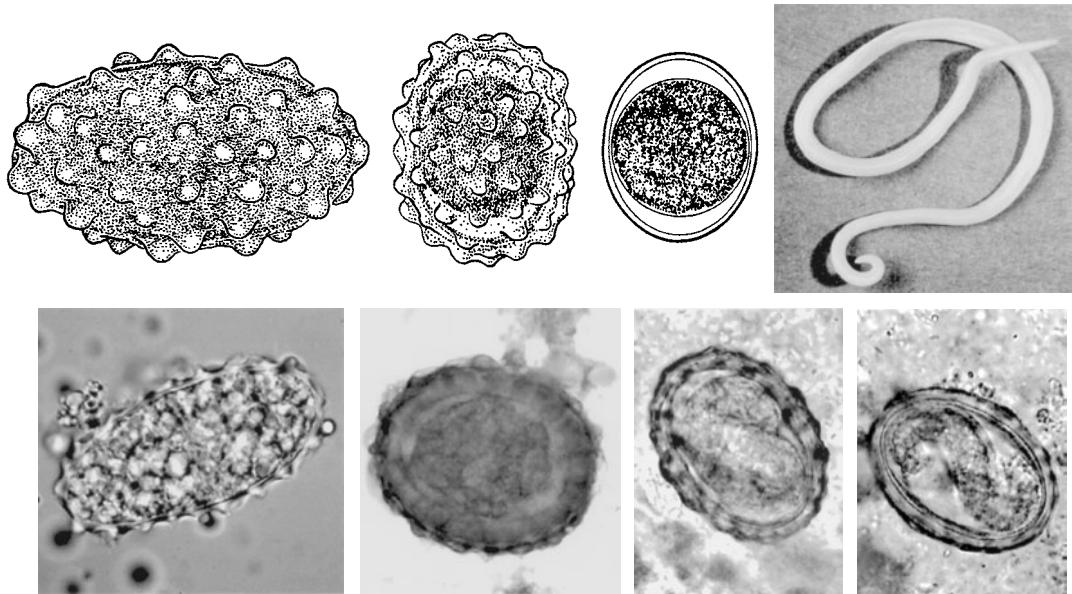
**Caution:** Unfertilized *Ascaris* eggs do not float in the zinc sulfate flotation concentration method (they are too heavy). Also, if too much iodine is added to the wet preparations, the eggs may look like very dark debris. Eggs may be very difficult to identify on a permanent stained smear because of stain retention and asymmetric shape.

Intestinal disease can often be diagnosed from radiographs of the gastrointestinal tract, where the worm intestinal tract may be visualized. This may be particularly obvious when two worms are lying parallel, like "trolley car lines." Other involved body sites may cause specific symptoms indicative of bowel obstruction, biliary or pancreatic duct blockage, appendicitis, or peritonitis. Therapy targets specific symptoms and involved areas.

### General Comments

The adult worms are cylindrical, with a tapering anterior end. They are the largest of the common nematode parasites of humans; females measure 20 to 35 cm long, and males are 15 to 31 cm long, with a curved posterior end. Also, the three well-developed lips are characteristic.

Human infection is acquired through ingestion of embryonated eggs from contaminated soil. On ingestion, the eggs hatch in the stomach and duodenum, where the larvae actively penetrate the intestinal wall; they are carried to the right heart via the hepatic portal circulation and then into the pulmonary circulation, where they are filtered out by the capillaries. After ca. 10 days in the lungs, they break into the alveoli, migrate via the bronchi until they reach the trachea and pharynx, and then are swallowed. They mature and mate in the intestine, and produce eggs, which are passed in the stool. The entire developmental process from egg ingestion to egg passage from the adult female takes from 8 to 12 weeks. During her life span, she may deposit a total of 27,000,000 eggs. Both unfertilized and fertilized eggs are passed. Often only female worms are recovered from the intestine. Fertilized eggs become infective within 2 weeks if they are in moist, warm soil, where they may remain viable for months or even years. Often both types of eggs are found in the same stool specimen. The total absence of fertilized eggs means that only female worms are present in the intestine.



Images from left to right: (Top) Unfertilized egg, fertilized egg, decorticate egg (lost the bumpy coat), and adult male worm. (Bottom) Unfertilized egg (note the very bumpy shell and somewhat elongated shape), fertilized egg, fertilized egg containing larva, and fertilized egg containing larva (the shell is less bumpy than most).

## Description

The fertilized egg is broadly oval, with a thick, mammillated coat, usually bile stained a golden brown; it measures up to 75  $\mu\text{m}$  long and 50  $\mu\text{m}$  wide.

Fertilized eggs may be heavy and may not float in the zinc sulfate flotation concentration procedure. When seen on a wet preparation, the eggs may resemble debris if too much iodine is used and the appearance is quite dark. Also, when these eggs are seen in a permanent stained smear, they may appear to be debris.

The mammillated (bumpy, tuberculated) coat appears less pronounced than that seen in the unfertilized eggs. In proficiency testing specimens, some fertilized eggs continue to mature and you may actually see motile larvae within the eggshell.

Unfertilized eggs are usually more oval, measure up to 90  $\mu\text{m}$  long, and may have a pronounced mammillated coat or an extremely minimal mammillated layer.

Unfertilized eggs may be heavy and do not float in the zinc sulfate flotation concentration procedure. On a wet preparation, they may resemble debris if too much iodine is used and the appearance is quite dark. In a permanent stained smear, they may appear to be debris; normally they stain very dark and no longer resemble helminth eggs. The mammillated shell may be quite pronounced and irregular, more so than that seen with the fertilized eggs. In some cases, the mammillated shell is somewhat minimal.

## Additional Information

The minimum prepatent period after ingestion of the infective eggs is 60 days. Infection may be aborted by spontaneous passage of adult worms within about a year.

Eggs are difficult to kill while they are in the soil, especially clay soil under favorable environmental conditions. In some countries where infections are common, mass population treatment plans have been used with great success, even in areas with high reinfection rates. The use of human feces, or "night soil," for fertilization of crops should be recognized as a potential hazard. Any vegetables or fruits from such fields cannot be eaten raw or unprocessed. Even with proper pretreatment of night soil, *Ascaris* eggs remain viable and infective more often than eggs of any other helminth species.

**Note:** In children, particularly those under 5, there may be severe nutritional impairment related to the worm burden. Direct effects include increased fecal nitrogen and fecal fat and impaired carbohydrate absorption, all of which would return to normal with elimination of the adult worms. Worms can also be spontaneously passed without any therapy.

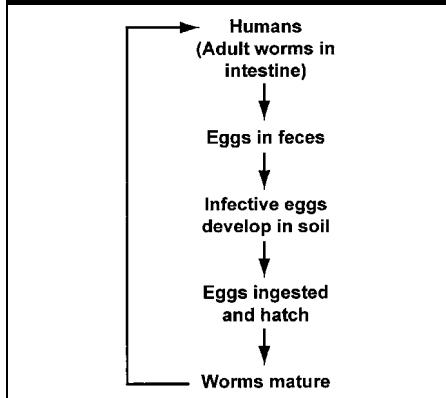
Developmental disabilities are a significant and frequently undetected health problem in developing countries; malnutrition associated with intestinal helminth infections may be an important contributory factor for these disabilities.

## NEMATODES • Intestinal

### *Trichuris trichiura*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Trichuriasis
<b>Acquired</b>	Ingestion of infective eggs from contaminated soil, food, or water
<b>Body site</b>	Intestine
<b>Symptoms</b>	Abdominal cramps, rectal tenesmus or rectal prolapse (children), or asymptomatic
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *T. trichiura* eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. Most whipworm infections can be easily diagnosed by finding these characteristic eggs in the stool. The eggs should be quantitated (rare, few, moderate, many), since light infections usually cause no problems and do not require therapy.

*T. trichiura* eggs submitted in stool preserved with PVA do not concentrate as well as those preserved in formalin. However, very small numbers of eggs that might be missed in a concentrate obtained from PVA-preserved stool material are not clinically significant. If the specimen was collected early in the infection when egg production was minimal, eggs would be seen in later specimens (if they were submitted because of patient symptoms).

Although dysentery with *T. trichiura* and dysentery with *Entamoeba histolytica* are very similar, whipworm dysentery is usually more chronic, associated with malnutrition, and likely to cause rectal prolapse. Recovery and identification of the eggs or protozoan trophozoites will differentiate the two infections. In severe infections, the adult worms are usually visible on the rectal mucosa.

The eggs can usually be identified from the permanent stained smear, but morphology is more easily seen in the wet smear preparations.

### General Comments

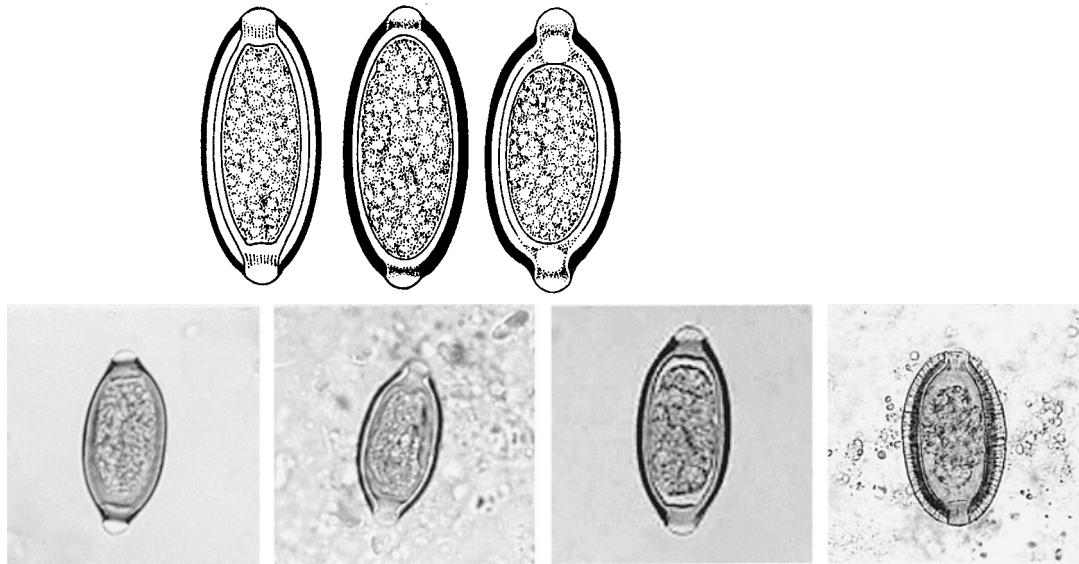
*T. trichiura* (whipworm) infection is more common in warm, moist areas of the world and is often seen in conjunction with *Ascaris* infections. Worm burdens vary considerably; individuals with few worms are unaffected by the presence of these parasites. Prevalence rates of 20 to 25% have been reported from the southern United States.

Whipworms are much larger than pinworms, being 35 to 50 mm long (female) and 30 to 45 mm long (male); the male has a 360° coil at the caudal extremity. Adult worms are rarely recovered from the stool since they are attached to the wall of the intestine.

Human infection is acquired through ingestion of fully embryonated eggs from the soil. The eggs hatch in the small intestine and eventually attach to the mucosa in the large intestine. The adults mature in about 3 months and begin egg production.

*Trichuris* dysentery syndrome develops in some children because they have a defect in antiparasite cell-mediated immunity. Probably both cellular and humoral responses are required to eliminate worms from the colon.

Differential diagnosis of chronic diarrhea includes celiac disease, inflammatory bowel disease, and irritable bowel syndrome. Heavily infected patients may present with a chronic dysentery-like syndrome leading to anemia and growth retardation. Diarrhea without blood and mucus may last for years. However, once blood is evident, medical intervention may be required; in some cases, the diagnosis requires colonoscopy, and prolonged therapy may be necessary to eliminate the parasites.



Images from left to right: (Top) Drawings of *T. trichiura* eggs. (Bottom) The first three images are typical eggs, with the barrel shape and polar plugs; the last egg is *Capillaria*, which has striations on the eggshell that are not found on the *Trichuris* eggshell.

### Description (Eggs)

The eggs are barrel shaped with clear, mucoid-appearing polar plugs. They are 50 to 54  $\mu\text{m}$  long and 22 to 23  $\mu\text{m}$  wide. They are passed in the unsegmented stage and require 10 to 14 days in moist soil for embryonation to occur.

Distorted eggs that are much larger than normal have been seen following therapy with mebendazole and with other drugs. This is not common but should be considered if distorted eggs are seen. There are also some reports in the literature that *T. vulpis* (dog whipworm) eggs have been recovered in human stools. These eggs tend to be larger (70 to 80  $\mu\text{m}$  long by 30 to 42  $\mu\text{m}$  wide) and have prominent but small polar plugs compared with those of *T. trichiura*.

These nematode eggs are probably the easiest to identify; the shape is very consistent in wet preparations and the eggs maintain their shape in permanent stained smears. However, on the permanent stained smear, they tend to stain dark and may be mistaken for debris.

### Description (Adults)

The head portion of the worm is very thin and is embedded in the mucosa, while the posterior end is much thicker and lies free in the lumen of the large intestine. The large posterior end has been described as the whip handle, while the thin anterior end is the whip itself, hence the name whipworm.

It is unusual to find adult worms in the stool unless the infection is quite severe with a very heavy worm burden. The majority of cases are diagnosed based on the presence of the typical eggs.

### Additional Information

Although eosinophils and Charcot-Leyden crystals are present in the stool in patients with dysentery, a peripheral eosinophilia on the differential smear is not always seen and the degree of eosinophilia may not correlate with the severity of infection (it rarely exceeds 15%). Heavy infections are rare in developed countries, as are complications requiring surgical intervention.

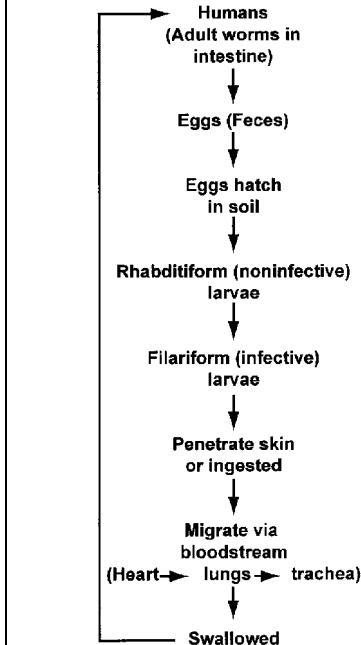
Inflammatory bowel disease, including Crohn's disease, probably occurs from a failure to down regulate a chronic Th1 intestinal inflammatory process. Induction of a Th2 immune response by intestinal helminths reduces the Th1 inflammatory process. Ulcerative colitis is more common in Western industrialized countries than in underdeveloped countries, particularly those where helminth infections are common. People with helminth infections exhibit altered immunologic antigen responses. Helminths prevent or alleviate colitis through the induction of regulatory T cells and modulatory cytokines. The use of *T. suis* in the therapy of ulcerative colitis has been controversial for years. However, studies have shown improvement in 43.3% of patients with ova treatment compared with 16.7% who received placebo.

## NEMATODES • Intestinal

### *Necator americanus* *Ancylostoma duodenale* (Hookworms)

<b>Pathogenic</b>	Yes
<b>Disease</b>	No specific designation
<b>Acquired</b>	Fecal-oral transmission of infective eggs from contaminated soil, food, or water
<b>Body site</b>	Intestine, larvae in lungs
<b>Symptoms</b>	Pneumonitis, vague intestinal complaints or asymptomatic
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of hookworm eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. In the larval migration phase of the infection, diagnosis can rarely be made by finding the larvae in sputum or in gastric washings.

Diagnosis in the intestinal phase is based on finding the eggs (8- to 16-cell stage of development) in the stool. Adult worms are rarely seen. The eggs are most easily seen on a direct wet smear or a wet preparation of the concentration sediment.

**Caution:** These eggs have a thin shell; when iodine is added to the wet preparation, there will be a clear space between the eggshell and developing embryo. Eggs may be very difficult to identify on a permanent stained smear because of stain retention and potential collapse.

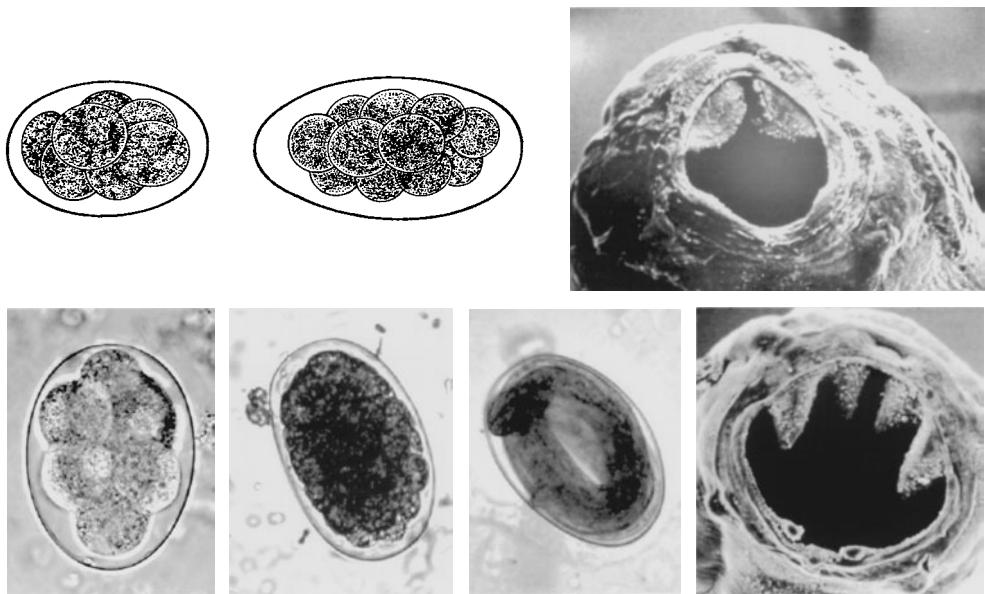
If the stool specimen is stored at room temperature (no preservative) for more than 24 h, the larvae continue to mature and hatch. These larvae must be differentiated from *Strongyloides* larvae, since therapy is often quite different for the two infections.

In chronic infections, the main clinical finding is iron deficiency anemia (microcytic, hypochromic) with pallor, edema of the face and feet, listlessness, and hemoglobin levels of 5 g/dl or less. There may be cardiomegaly and both mental and physical retardation.

### General Comments

Infection is acquired through skin penetration of filariform larvae from the soil. During skin penetration, infective hookworm larvae encounter hyaluronic acid; hookworm hyaluronidase activity has now been confirmed and can facilitate passage of the infective larvae through the epidermis and dermis during larval migration. After skin penetration, the larvae are carried first by the venules to the right heart and then to the lungs. Larvae invade the alveoli, migrate via the bronchi until they reach the trachea and pharynx, and are swallowed, bringing them to the small intestine, where they reside. They attach to the mucosa via a temporary mouth structure, mature sexually, and finally develop the permanent characteristic mouth structure that they use to attach to the mucosa. Any pneumonitis due to migrating larvae depends on the burden. These larvae do not cause the same sensitization level seen with *Ascaris* or *Strongyloides* infection.

Symptoms from the intestinal phase of infection are caused by (i) necrosis of the intestinal tissue within the adult worm mouth and (ii) blood loss by direct ingestion of blood by the worms and continued blood loss from the original attachment site possibly as a result of anti-coagulant secreted by the worm. Patients with acute infections may experience fatigue, nausea, vomiting, abdominal pain, diarrhea with black to red stools, weakness, and pallor. Heavy worm burdens in young children may have serious sequelae, including death.



Images from left to right: (Top) Drawing of a hookworm egg (*N. americanus* or *A. duodenale*), drawing of a *Trichostrongylus* egg (it appears longer, with one end more pointed than the hookworm egg), and the mouth parts of adult *Necator* (note the cutting plates). (Bottom) Three typical hookworm eggs (the third contains a larval worm; this finding suggests that fresh, unpreserved stool was left at room temperature for some time before being examined or being placed in fixative; if this egg hatches, the rhabditiform larva would have to be differentiated from that of *S. stercoralis*) (left and middle) and the mouth parts (teeth) of *Ancylostoma* (right).

## Description (Eggs)

The eggs are usually in the early cleavage stage when passed in the stool. They are oval (ca. 60 µm long by 40 µm wide) with broadly rounded ends. They have a clear space between the developing embryo and the thin eggshell; this feature can be easily seen in the wet preparation stained with D'Antoni's iodine.

Egg survival and larval development are maximum in moist, shady, warm soil (sandy loam), where larvae hatch from the eggs within 1 to 2 days. The infective filariform larvae develop within 5 to 8 days and may remain viable in the soil for several weeks.

Egg counts of 5/mg of stool are rarely clinically significant, counts of more than 20/mg are usually associated with symptoms, and counts of 50/mg or more represent very heavy worm burdens. For the most part, egg counts are rarely performed in routine clinical laboratories.

## Description (Adults)

Adult males are 7 to 11 mm long by 0.4 to 0.5 mm wide. *Ancylostoma* worms are larger than *Necator* worms. Adults are rarely seen, since they remain firmly attached to the intestinal mucosa via well-developed mouth parts (teeth in *A. duodenale* and cutting plates in *N. americanus*).

Females begin to deposit eggs 5 months or more after initial infection. If mature filariform larvae of *A. duodenale* are swallowed, they can develop into mature worms in the intestine without migrating through the lungs.

## Additional Information

Eosinophilia is common, usually develops 25 to 35 days after exposure, and peaks about 1 (*N. americanus*) to 2 (*A. duodenale*) months later. Eosinophilia may reach approximately 18%.

Hookworms cause gastrointestinal blood loss. Significant loss can occur with *A. duodenale*, but its impact on the iron status of populations is no greater than for *N. americanus*.

Hookworm-mediated immunosuppression affects vaccine development. Unless preexisting infections are cured, vaccine-induced immunity may be affected. Also, if the vaccine is only partially effective, vaccine recipients may be susceptible to reinfection.

Helminth infection may protect against asthma and malaria; however, it may increase susceptibility for HIV-AIDS or tuberculosis. People with hookworm infection are also more likely to be infected with *A. lumbricoides* and *T. trichiura*, findings that can only partially be explained by overlapping endemic areas and potential exposure.

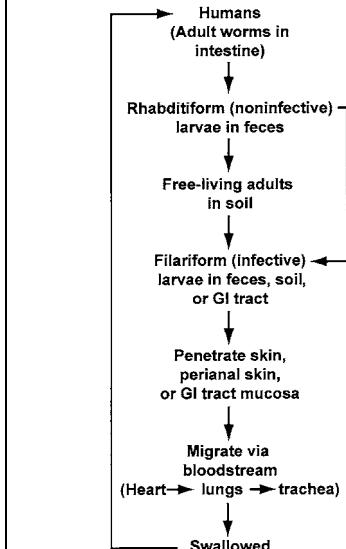
*A. duodenale* is found primarily in southern Europe, the north coast of Africa, northern India, northern China, and Japan. *N. americanus* is found throughout the southern United States, the Caribbean, Central America, northern South America, central and southern Africa, southern Asia, Melanesia, and Polynesia. In some areas such as northern Ghana, both hookworms are present; mixed infections have been confirmed by PCR.

## NEMATODES • Intestinal

### *Strongyloides stercoralis*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Strongyloidiasis
<b>Acquired</b>	Fecal-oral transmission of infective filariform larvae from contaminated soil, food, or water
<b>Body site</b>	Intestine, larvae in lungs
<b>Symptoms</b>	Pneumonitis, vague intestinal complaints or asymptomatic
<b>Clinical specimen</b>	<i>Intestinal:</i> Stool <i>Disseminated:</i> Sputum, other tissues
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *Strongyloides* larvae in stool specimens, primarily from the wet preparation examination of the concentration sediment. In the larval migration phase of the infection, diagnosis is occasionally made by finding the larvae in sputum or in gastric washings. During the intestinal phase, the diagnosis is based on finding rhabditiform larvae in the stool. Adult worms and eggs are rarely seen, except in a very heavy infection. The larvae are most easily seen on a direct wet smear or a wet preparation of the concentration sediment.

**Caution:** These noninfective rhabditiform larvae can transform to the infective filariform larvae, both of which are found in the stool, and must be differentiated from hookworm larvae, since therapy is often different.

The efficacy of sampling of duodenal contents is controversial. Other techniques include the Entero-Test capsule, special concentration techniques (Baermann), and larva culture (Harada-Mori, petri dish).

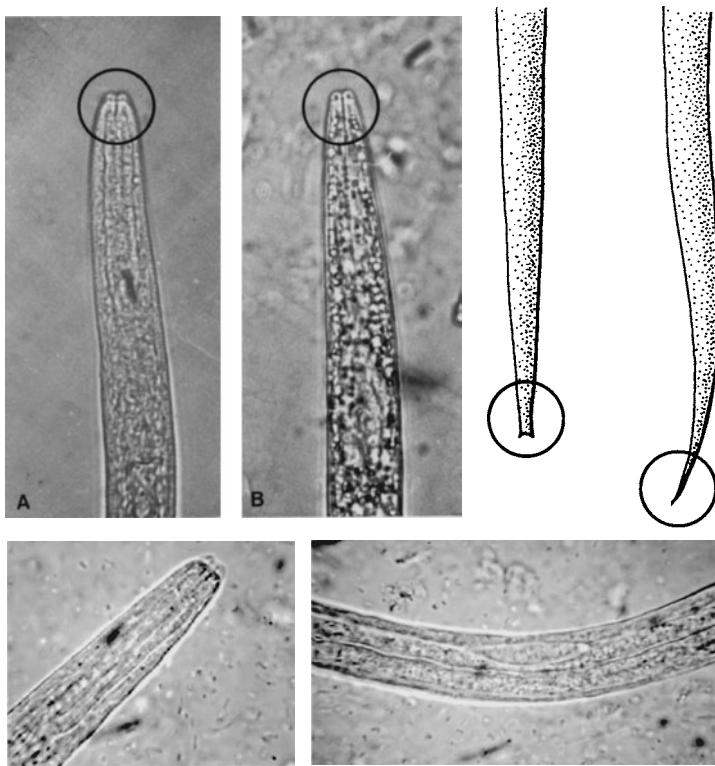
Agar plate cultures are recommended and are more sensitive than some other available diagnostic methods; stool is placed on agar plates, which are sealed to prevent accidental infections and held for 2 days at room temperature. As the larvae crawl over the agar, they carry bacteria with them, creating visible tracks over the agar. The plates are examined under the microscope for confirmation of larvae, the surface of the agar is then washed with 10% formalin, and larval identification is confirmed via wet examination of the sediment from the formalin washings.

### General Comments

Human infection is acquired by skin penetration of the filariform larvae (infective larvae) from the soil. After penetration of the skin, the larvae are carried via the cutaneous blood vessels to the lungs, where they break out of the pulmonary capillaries into the alveoli. They migrate via the respiratory tree to the trachea and pharynx, are swallowed, and enter the mucosa in the duodenum and upper jejunum. Development usually takes about 2 weeks; the females then begin egg production. The eggs usually hatch, and the rhabditiform larvae (noninfective larvae) pass out of the intestinal tract in the feces onto the soil, where they develop into free-living male and female worms, eventually producing infective filariform larvae (egg, noninfective larvae, and infective larvae). In temperate climates, the free-living male and female worms do not develop; however, the rhabditiform larvae develop into the filariform (infective) larvae, which are ready to infect the next host through skin penetration.

The pulmonary route is one of several possible pathways to the duodenum, regardless of whether the larvae penetrated the skin or the intestine, and may not be as universally applicable as was once thought.

Initial skin penetration usually causes very little reaction, although there may be some pruritus and erythema. Pulmonary symptoms, intestinal pain, sepsis, and meningitis with intestinal bacterial flora can also be seen, particularly in immunocompromised patients with disseminated disease (hyperinfection).



Images from left to right: (Top) Short mouth opening of *Strongyloides* rhabditiform larva, longer mouth opening of hookworm rhabditiform larva, drawing of slit in tail of *Strongyloides* filariform larva, and pointed tail of hookworm filariform larva. (Bottom) Short mouth (buccal) opening and packet of genital primordial cells of the *Strongyloides* rhabditiform larva.

## Description

The eggs are oval and thin shelled and are 50 to 58  $\mu\text{m}$  long by 30 to 34  $\mu\text{m}$  wide (generally a bit smaller than hookworm eggs).

The rhabditiform larvae that pass out in the stool are up to 380  $\mu\text{m}$  long by 20  $\mu\text{m}$  wide, with a muscular esophagus (club-shaped anterior, then a restriction, and a posterior bulb). There is a genital primordium packet of cells, which is fairly obvious and can be seen about two-thirds of the way back from the anterior end. A key morphologic difference between these larvae and those of hookworm is the length of the mouth opening (buccal capsule). The opening in the rhabditiform larvae of *S. stercoralis* is very short (only a few micrometers), while the mouth opening in hookworm rhabditiform larvae is approximately three times as long. These differences can be seen by examining the larvae under the microscope using the low (10 $\times$ ) or high dry (40 $\times$ ) objectives. The filariform larvae are long and slender (up to 630  $\mu\text{m}$  long by 16  $\mu\text{m}$  wide) and may remain viable in soil or water for several days.

## Additional Information

When autoinfection occurs, some rhabditiform larvae in the intestine develop into filariform larvae while passing through the bowel. These larvae can reinfect the host by

(i) invading the intestinal mucosa, traveling via the portal system to the lungs, and returning to the intestine or (ii) being passed out in the feces and penetrating the host on reaching the perianal or perineal skin.

Filarial larval migration through the lungs may stimulate symptoms; some patients are asymptomatic, while others may have pneumonia. With a heavy infective dose or in the *hyperinfection syndrome*, individuals often develop cough, shortness of breath, wheezing, fever, and transient pulmonary infiltrates (Loeffler's syndrome). Larvae may be found in the sputum.

Immunocompromised patients with disseminated strongyloidiasis may have sepsis and meningitis, and larvae can be found in almost every body site (reported at autopsy). In these cases, eggs and both types of larvae (rhabditiform, filariform) can be found in the stool specimen.

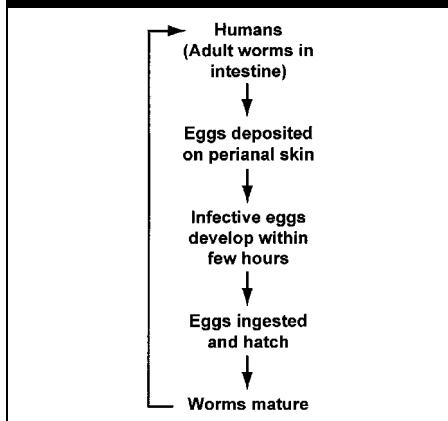
Debilitated or immunocompromised patients should always be suspected of having strongyloidiasis, particularly if there are unexplained bouts of diarrhea and abdominal pain, repeated episodes of sepsis or meningitis with intestinal bacteria, or unexplained eosinophilia. However, similar prevalences of infection have been seen in AIDS and non-AIDS patients. Diagnosing intestinal parasites in HIV-AIDS patients is necessary, especially in chronic alcoholics and those not on antiretroviral treatment. It is also important to check prehematopoietic stem cell transplantation patients in endemic areas.

## NEMATODES • Intestinal

### *Enterobius vermicularis*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Enterobiasis
<b>Acquired</b>	Fecal-oral transmission of infective eggs via contaminated food or water or on fingers and hands
<b>Body site</b>	Intestine
<b>Symptoms</b>	Anal itching may be the only symptom; nervousness, insomnia, nightmares; occasional vaginal discharge
<b>Clinical specimen</b>	Scotch tape anal swab
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of hands

#### LIFE CYCLE



#### Diagnosis

Diagnosis depends on finding eggs or adult worms. This is normally done by sampling the perianal and perineal skin with cellulose tape (Scotch tape), which is applied sticky side down to the skin. The tape is transferred to a glass slide and examined under the microscope for eggs or adult worms.

Since the female worms migrate on a sporadic basis, a series of four to six consecutive tapes may be necessary to demonstrate the infection. The samples are taken late in the evening, when the patient has been sleeping for several hours, or first thing in the morning before the patient takes a shower or goes to the bathroom.

Adult worms may be found on or under the surface of the stool specimen, particularly in children. Eggs are occasionally recovered in stool, but this is an incidental finding.

Treatment often includes counseling for the parents, who may be very upset at learning that their children have "worms." They may not realize how prevalent the infection is, particularly in children, and the fact that many children will never have any symptoms or sequelae from the infection.

Since this infection is so common and transmission is so easy (anus-to-mouth contamination, soiled nightclothes, airborne eggs, and contaminated furniture, toys, and other objects), prevention is marginal.

#### General Comments

*E. vermicularis* is thought to cause the world's most common human parasitic infection. The infection is more prevalent in cool and temperate zones where people tend to bathe less often and change their underclothes less frequently. Prevalence in children can be high.

Infection is initiated by the ingestion of infective eggs, which hatch in the intestine (cecal region), where they develop into adult worms. It probably takes about 1 month for the female to mature and begin egg production. After fertilizing the female worms, the males usually die and may be passed out in the stool. Almost the entire body of gravid females is filled with eggs. At this point, the female migrates down the colon and out the anus, where the eggs are deposited on the perianal and perineal skin.

Occasionally the female worm migrates into the vagina. After egg deposition, the female worm probably returns to the intestine. Occasionally when the bolus of stool passes out of the anus, adult worms are found on the surface. Adult worms may also be picked up on the Scotch tape preparations used to diagnose this infection. Although egg deposition usually does not occur in the intestine, some eggs may be recovered in the stool. The eggs are fully embryonated and infective within a few hours. Transmission is often attributed to ingestion of infective eggs by nail biting and inadequate hand washing, but airborne eggs can also be inhaled and ingested.

The most striking symptom is pruritus, which is caused by migration of female worms from the anus onto the perianal skin to deposit eggs. The sometimes intense itching results in scratching and occasional scarification. In most infected people this is the only symptom, and many individuals remain asymptomatic. Eosinophilia may or may not be present.

The degree of infection varies tremendously. As many as 5,000 worms have been removed from a single patient, but most cases average less than 1 migrating worm per evening. Women are symptomatic three times as often as men and young people more frequently than older people.



**Images:** The photograph on the left shows an adult pinworm full of eggs. The photograph on the right shows the typical eggs (football shaped with one side somewhat flattened). These eggs are often embryonated, containing larval forms. (Photomicrograph of adult worm by Zane Price [from L. S. Garcia, *Diagnostic Medical Parasitology*, 5th ed., ASM Press, Washington, DC, 2007].)

### Description (Eggs)

The eggs have been described as footballs with one side flattened. They are oval, compressed laterally, and flattened on one side and measure 50 to 60  $\mu\text{m}$  long by 20 to 30  $\mu\text{m}$  wide.

### Description (Adults)

The female worm is 8 to 13 mm long by 0.3 to 0.5 mm wide and has a pointed tail (hence the name pinworm). The male is much smaller, measuring 2 to 5 mm long by 0.1 to 0.2 mm wide, and has a curved caudal end.

The adult female is often white (or light in color), and the pointed tail is very obvious. There may be multiple worms on or below the surface of the stool specimen (fresh specimen); however, the adult worms are not seen that frequently, even on the Scotch tape preparations.

The adult female worms are full of eggs and often "explode" when they migrate outside of the body and are beginning to dry out. It has often been said that "You have pinworms now, had them as a child, or will get them again when you have children." It may be the world's most common parasitic infection, regardless of geographic area and/or socioeconomic class.

### Additional Information

Although tissue invasion has been attributed to the pinworm, it is uncommon. Enterobiasis is an uncommon cause of acute appendicitis in U.S. children; however, it may be associated with acute appendicitis, chronic appendicitis, ruptured appendicitis, or with no significant symptoms.

Other uncommon ectopic sites have included the peritoneal cavity, lung, liver, urinary tract, and natal cleft. Pathologic examination usually shows chronic granulomatous inflammation with or without central necrosis, which is surrounded by polymorphonuclear neutrophilic leukocytes, eosinophils, and fibroblasts. Macrophages, giant cells, epithelioid cells, and Charcot-Leyden crystals may also be present. During a case of suspected transverse colon carcinoma, histologic examination of the mass revealed eggs of *E. vermicularis* embedded in granulomatous tissue in the submucosa of the colon; no malignancy was found. Apparently, this is the first report of enterobiasis presenting as colon carcinoma.

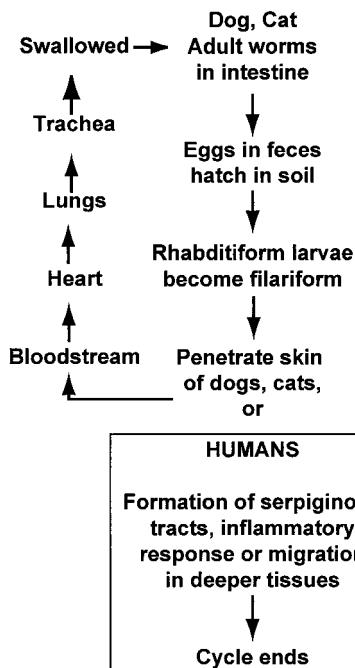
The eggs are infectious within a few hours and *under most circumstances, total prevention is neither realistic nor possible.*

## NEMATODES • Tissue

### *Ancylostoma braziliense* *Ancylostoma caninum* (Dog and Cat Hookworms)

<b>Pathogenic</b>	Yes
<b>Disease</b>	Cutaneous larva migrans
<b>Acquired</b>	Skin penetration by filariform (infective) larvae from contaminated soil, food, or water
<b>Body site</b>	Skin (serpiginous tracts)
<b>Symptoms</b>	Inflammatory response or migration of larvae in deeper tissues; intense itching
<b>Clinical specimen</b>	None; visual inspection of characteristic linear tunnels or tracts in the skin
<b>Epidemiology</b>	Worldwide, primarily dog/cat-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, covering of all sandboxes where pets may defecate and children play

#### LIFE CYCLE



### Diagnosis

Diagnosis is usually based on the characteristic linear tunnels or tracks and a history of possible exposure. Biopsy is not recommended. However, newer PCR methods for detection and identification of larvae in human tissues may improve the test results. There may be elevated eosinophilia (peripheral or sputum).

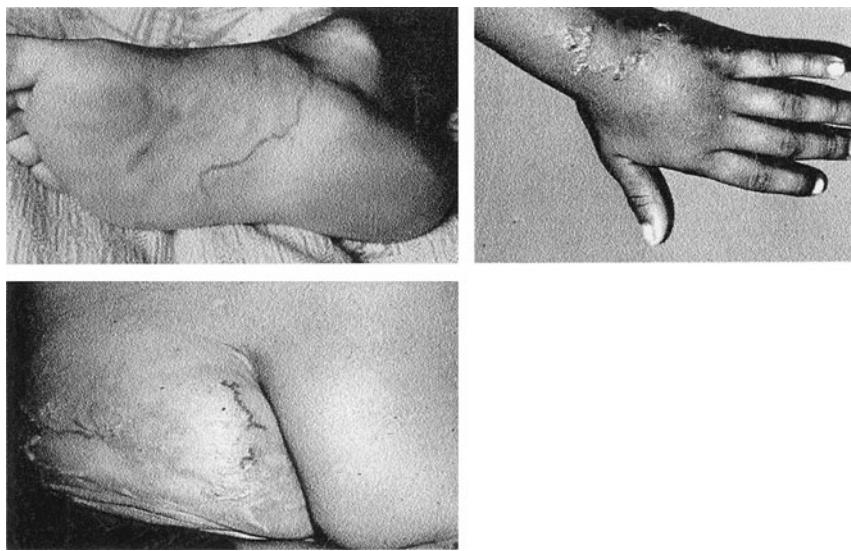
Patients with a relevant travel history may have travel-related skin diseases such as CLM as well as more common entities. To prevent and manage skin-related morbidity during travel, international travelers should avoid direct contact with sand, soil, and animals.

In 2006, the director of a children's aquatic sports day camp notified the Miami-Dade County Health Department (MDCHD) of three campers who had received a diagnosis of CLM, or "creeping eruption," a skin condition typically caused by *Ancylostoma* larvae. The investigation identified exposure to cat feces in a playground sandbox as the likely source of infection. Although CLM outbreaks are rarely reported to the Florida Department of Health, CLM is a potential health hazard in Florida. This disease cluster highlights the importance of appropriate environmental hygiene practices and education in preventing CLM.

### General Comments

Human infection is acquired through skin penetration by infective larvae from the soil. These larvae can also cause infection when ingested. When larvae penetrate the skin, they produce pruritic papules, which after several days become linear tracks that are elevated and vesicular. Movement by the larvae in the tunnel may extend the track several centimeters each day. Secondary infections often occur as a result of intense scratching of the tracks.

In hookworm folliculitis, the histologic picture is characterized by an eosinophilic folliculitis due to an inflammatory reaction to the presence of larvae trapped within the follicular canal. Only a few cases of hookworm folliculitis have been reported in the literature; however, this presentation should be recognized as one of the less typical presentations of CLM.



**Images from left to right:** (Top) Linear tracts on the bottom of the foot and linear tracts on the hand. (Bottom) Linear tracts on the buttocks of a child (who had sat down in a sandbox containing sand contaminated with dog/cat hookworm larvae). (From *A Pictorial Presentation of Parasites*: a cooperative collection prepared by H. Zaiman.)

## Additional Information

Larvae that first enter the skin and cause creeping eruption may later migrate to the deeper tissues (lungs), leading to pneumonitis with larval recovery in the sputum. Peripheral eosinophilia, as well as many eosinophils and Charcot-Leyden crystals in the sputum, may also be present.

Primary eosinophilic gastrointestinal disorders (eosinophilic esophagitis, eosinophilic gastritis, eosinophilic gastroenteritis, eosinophilic enteritis, and eosinophilic colitis) selectively affect the gastrointestinal tract with eosinophil-rich inflammation in the absence of known causes of eosinophilia, including parasitic infections, drug reactions, and malignancy. Eosinophils are important components of the gastrointestinal mucosal immune system and these disorders involve IgE-mediated and delayed Th2-type responses.

Segmental eosinophilic inflammation of the gastrointestinal tract may be isolated or part of a multisystem problem. An increasing number of cases have been reported in Northern Queensland, Australia. All of the patients were Caucasians with a wide age range and no previous illness, who had severe abdominal pain, occasional diarrhea, weight loss, and dark stools; all cases were associated with eosinophilia and elevated serum IgE levels.

In one patient, a single adult *A. caninum* worm was found in a segment of inflamed ileum. Human hookworms do not occur in urban Australia, and no hookworm eggs were being passed in the stool. All the patients were closely associated with dogs, most of which had hookworms. Also, all patients treated with anthelmintic agents showed a return to normal peripheral blood eosinophil counts.

The similarities among the reported cases implicate *A. caninum* as the cause of eosinophilic enteritis (EE). It has been speculated that *A. caninum* causes human EE by inducing allergic responses to its secretions, including cysteine proteinases, which are involved in pathogenesis in other parasites. Immunologic studies also suggest that this parasite is a major cause of EE and peripheral blood eosinophilia. Although there are other causes, this disease entity may become more commonly recognized in other areas of the world, confirming the causative agent as the common dog hookworm.

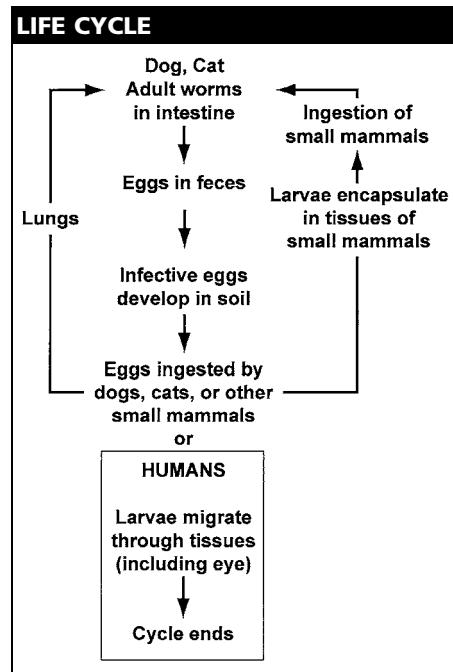
## NEMATODES • Tissue

### *Toxocara canis*

### *Toxocara cati*

### (Dog and Cat Ascarid Worms)

<b>Pathogenic</b>	Yes
<b>Disease</b>	Visceral larva migrans (VLM) Ocular larva migrans (OLM)
<b>Acquired</b>	Egg ingestion from the soil via contaminated food or water
<b>Body site</b>	Tissues, including the eyes
<b>Symptoms</b>	Fever, hepatomegaly, hyperglobulinemia, pulmonary infiltrates, cough, neurologic disturbances, endophthalmitis
<b>Clinical specimen</b>	Serum (EIA)
<b>Epidemiology</b>	Worldwide, primarily dogs/cats-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, covering all sandboxes where pets may defecate and children play; deworming pets



### Diagnosis

VLM symptoms caused by *Toxocara* spp. must be differentiated from those caused by other tissue-migrating helminths (ascariids, hookworms, filariae, *Strongyloides* spp., and *Trichinella* spp.), and other hypereosinophilic syndromes. OLM can be confused with retinoblastoma, ocular tumors, developmental anomalies, exudative retinitis, trauma, and other childhood eye problems. It should be considered in any child with unilateral vision loss and strabismus who has raised, unilateral, whitish or gray lesions in the fundus. Peripheral eosinophilia may be absent. VLM should be suspected in pediatric patients with unexplained febrile illness and eosinophilia, especially if there is a history of pica and if hepatosplenomegaly and multisystem disease occur.

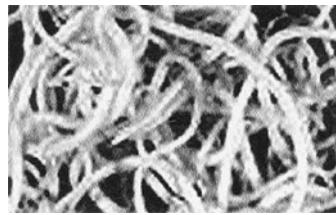
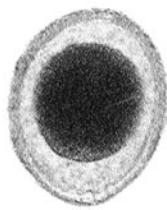
The diagnosis can be confirmed only by identification of larvae in autopsy or biopsy specimens. However, if children have *Ascaris* or *Trichuris* infections, toxocariasis might be considered, since all three infections are transmitted via ingestion of contaminated soil. Serologic testing is the most appropriate approach. EIA is recommended; it is highly specific and shows no cross-reactions with sera from patients infected with other common human parasites. The diagnostic titers vary between VLM throughout the body and OLM. Titers of 1:32 and 1:8 are diagnostic for VLM and OLM, respectively.

### Life Cycle

Humans acquire infection by ingesting infective eggs of *T. canis* or *T. cati*. Pups are often infected by vertical transfer of larvae from their dams transplacentally or lactogenically, and can begin shedding eggs by 2 weeks of age. In cats, lactogenic but not transplacental transmission occurs. Young kittens and pups recover at 3 to 6 months of age. Infections in older animals are acquired by ingestion of infective eggs from soil or ingestion of larvae in infected rodents, birds, or other paratenic hosts. Eggs are shed in the feces and take about 2 to 3 weeks to mature and become infective.

After the eggs are accidentally ingested by a human, larvae hatch in the small intestine, penetrating the intestinal mucosa and migrating to the liver. During migration, the larvae do not mature, even if they make their way back into the intestine. The larvae are usually <0.5 mm long and 20 µm wide. Information also implicates ingestion of uncooked meats as a potential cause of human toxocariasis.

Most infections are probably asymptomatic. VLM occurs mostly in younger children (ca. 3 years), while OLM is more likely in older children (ca. 8 years). This does not tend to be the case with *Baylisascaris procyonis* (raccoon roundworm), where most cases of VLM, NLM, and OLM occur in very young children.



Images from left to right: *T. canis* (left) and *T. cati* eggs (middle two images); Adult *Toxocara* worms (right). Notice the similarity of these eggs to those of *A. lumbricoides*.

## Additional Information

*Toxocara* spp. cause zoonotic infections worldwide which may be much more common than previously thought. Infection rates in dogs are 2 to 90%, with the highest rates in pups via transmission from their dams. The overall incidence of infected dogs older than 6 months is probably less than 10%.

Clinical symptoms depend on the number of migrating larvae and the tissue(s) involved. Infections range from asymptomatic to severe disease. Larvae often remain in the liver and/or lungs, where they become encapsulated in dense fibrous tissue. Other larvae continue to migrate, causing inflammation and granuloma formation. The most outstanding feature is a high peripheral eosinophilia, which may reach 90%. The overall severity of the clinical picture depends on the initial dose of infective eggs. As few as 200 *T. canis* larvae in small children may produce a peripheral eosinophilia of 20 to 40% for more than a year, with no other symptoms. Patients with 50% eosinophilia usually have symptoms, which might include fever, hepatomegaly, hyperglobulinemia, pulmonary infiltrates, cough, neurologic disturbances, and endophthalmitis. Although rare, CNS involvement can cause seizures, neuropsychiatric symptoms, or encephalopathy.

The relationship between asthma and covert toxocariasis remains unclear; however, data indicate a seroprevalence of anti-*T. canis* antigen (E/S antigen) of 26.3% in asthmatic patients and 4.5% in the controls. This suggests that asthmatic patients with anti-*Toxocara* IgE and IgG may have suffered a covert infection with *Toxocara* spp.

Evidence suggests that ocular disease can occur in the absence of systemic involvement and vice versa for VLM. Although these facts may be explained by possible *Toxocara* strain differences, VLM may reflect the consequences of the host inflammatory response to waves of migrating larvae, while OLM may occur in individuals who have not become sensitized.

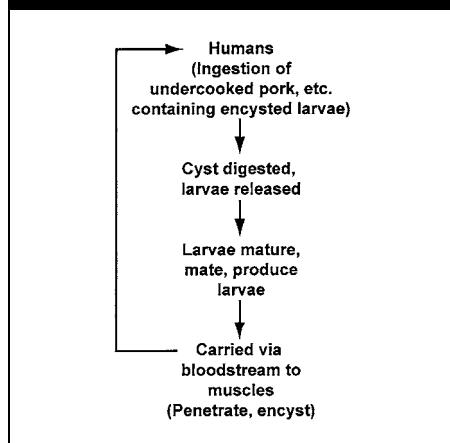
Although many cases of VLM are diagnosed by serologic testing, toxocariasis has generally been defined as an infection with *Toxocara* spp., with no attempt to identify the species involved. Using preabsorbed sera, the ability to distinguish between the two species should be helpful in further biological, epidemiologic, and clinical studies. Although the currently recommended serologic test is EIA, a measurable titer does not always represent current infection. A small percentage of the U.S. population (2.8%) exhibits a positive titer that reflects the prevalence of asymptomatic toxocariasis.

The probability of hepatic toxocariasis can be evaluated using imaging techniques and ultrasonography. Findings include focal ill-defined hepatic lesions, hepatosplenomegaly, biliary dilatation, sludge, and periportal lymph node enlargement.

The following preventive measures are recommended: regularly deworming dogs and cats, beginning at 2 weeks of age; removing cat and dog feces in places around homes and children's playgrounds; covering children's sandboxes when not in use; regular hand washing after handling soil and before eating; and teaching children not to put dirty objects into their mouths.

***Trichinella spiralis***

<b>Pathogenic</b>	Yes
<b>Disease</b>	Trichinosis
<b>Acquired</b>	Ingestion of infective raw or poorly cooked meat (pork, bear, walrus)
<b>Body site</b>	Intestine, striated muscle
<b>Symptoms</b>	Diarrhea (first 24 h), muscle pain, fever; depends on tissues infected
<b>Clinical specimen</b>	<i>Intestinal:</i> Stool (rarely requested) <i>Muscle:</i> Biopsy, routine histology <i>Serum:</i> EIA, bentonite flocculation
<b>Epidemiology</b>	Worldwide, primarily animal-to-human transmission
<b>Control</b>	Adequate cooking of meat, use of cooked rather than raw garbage for pigs

**LIFE CYCLE****Diagnosis**

The first clue may be a history of possible ingestion of raw or rare pork or other infected meat. There may also be other individuals from the same group with similar symptoms. Trichinosis should always be included in the differential diagnosis of any patient with periorbital edema, fever, myositis, and eosinophilia, regardless of whether a complete history of raw or poorly cooked pork consumption is available. If present, subconjunctival and subungual splinter hemorrhages also add support to such a presumptive diagnosis.

Muscle biopsy (gastrocnemius, deltoid, and biceps) specimens may be examined by compressing the tissue between two slides and checking the preparation under low power (10× objective). This method does not become positive until 2 to 3 weeks after the onset of the illness.

Serologic tests are also very helpful, the standard two being EIA and bentonite flocculation (BF), which are recommended for trichinosis. EIA is used for routine screening, and all EIA-positive specimens are tested by BF for confirmation. A positive reaction with both tests indicates infection with *T. spiralis* within the last few years. Titers tend to peak in the second or third months postinfection and then decline over a few years.

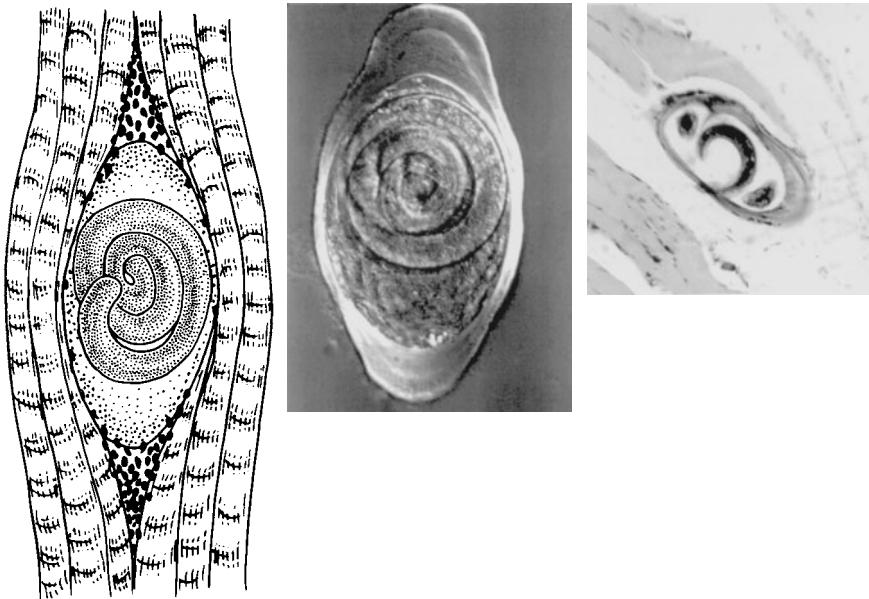
**General Comments**

Although recommendations have been made to use several species designations, some publications still use the single species designation *T. spiralis*. Genetic relationships among many *Trichinella* isolates are being assessed by dot blot hybridization, restriction endonuclease, and gel electrophoresis techniques. Taxonomic changes will continue to occur.

Human infection is initiated by ingestion of raw or poorly cooked pork, bear, walrus, or horse meat, or meat from other mammals (carnivores and omnivores) containing viable, infective larvae. After digestion, the encysted larvae invade the intestinal mucosa, develop through four larval stages, mature, and mate by day 2. By day 6 of infection, the female worms begin to deposit motile larvae, which are carried by the intestinal lymphatic system or mesenteric venules to the body tissues, primarily striated muscle. Deposition of larvae continues for 4 to 6 weeks, with each female producing up to 1,500 larvae in the nonimmune host. Newborn larvae can penetrate almost any tissue but can continue their development only in striated muscle cells. With the exception of *T. pseudospiralis*, *T. papuae*, and *T. zimbawensis*, invasion of striated muscle cells stimulates the development of nurse cells. As the larvae begin to coil, the nurse cell completes the formation of the cyst within ca. 2 to 3 weeks. In the human host, the cyst measures about 400 by 260 μm, and within the cyst, the coiled larva is 800 to 1,000 μm long. At this point, the larvae are fully infective.

The most active muscles, which have the greatest blood supply, including the diaphragm, muscles of the larynx, tongue, jaws, neck, and ribs, the biceps, gastrocnemius, and others, are invaded.

The cyst wall develops from the host's immune response to the presence of the larvae, and the encysted larvae may remain viable for many years, although calcification can occur within less than a year. Just five larvae per g of body muscle can cause death, although 1,000 larvae per g have been recovered from individuals who died from causes other than trichinosis.



Images from left to right: The drawing shows an encysted larva; both photographs show larvae, the one on the right being sectioned (histopathology).

## Additional Information

Preventive measures for pork containing temperate-zone strains include refrigeration at 5°F ( $-15^{\circ}\text{C}$ ) for not less than 20 days, at  $-10^{\circ}\text{F}$  for 10 days, or at  $-20^{\circ}\text{F}$  for 6 days or deep freezing ( $-37^{\circ}\text{C}$ ). Smoking, salting, and drying are not effective. In 1981, the USDA issued a news release suggesting that microwave cooking might not kill the larvae. The current recommendation states that "all parts of pork muscle tissue must be heated to a temperature not lower than  $137^{\circ}\text{F}$  ( $58.3^{\circ}\text{C}$ )."<sup>1</sup> An internal meat thermometer should be used when cooking pork. Reduction in the number of cases is due primarily to regulations requiring heat treatment of garbage and low-temperature storage of the meat. Occasional outbreaks are frequently due to problems with feeding, processing, and cooking of pigs raised for home use.

Recent information also confirms the need to review the intentional feeding of animal products and kitchen waste to horses, a high-risk practice requiring implementation of regulations to ensure that such feeds are safe for horses, as is currently required for feeding to swine.

**Note:** Up to 10 to 20% of patients with trichinosis have CNS involvement; the mortality rate may reach 50% if they are not treated.

Symptoms of trichinosis are generally separated into three phases. Phase 1 is related to the presence of the parasite in the host prior to muscle invasion. Phase 2 is related to the inflammatory and allergic reactions due to muscle invasion; there may also be an incubation period of up to 50 days. Phase 3 is the convalescent phase or chronic period.

Damage can be classified as (i) intestinal and (ii) muscle penetration and larvae encapsulation. Damage caused in any phase of the infection is usually based on the original number of ingested cysts. Early symptoms include diarrhea, nausea, abdominal cramps, and general malaise, all of which may suggest food poisoning, particularly if several people are involved. Diarrhea can be prolonged, lasting up to 14 weeks (average, 5.8 weeks) with little or no muscle symptoms. It is unknown whether this new clinical presentation is related to variant biological behavior of Arctic *Trichinella* organisms, to previous exposure to the parasite, or to other factors. During muscle invasion, there may be fever, facial (periorbital) edema, and muscle pain, swelling, and weakness. The extraocular muscles are usually the first to be involved, followed by the muscles of the jaw and neck, limb flexors and back. Problems in chewing, swallowing, or breathing can be seen. The most severe symptom is myocarditis (after week 3); death may occur between weeks 4 and 8. Other severe symptoms may involve the central nervous system. Although *Trichinella* encephalitis is rare, it is life-threatening. CT, angiogram, and electroencephalogram are of no diagnostic assistance.

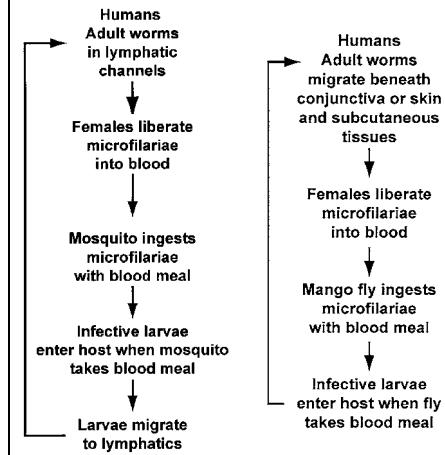
**Note:** Peripheral eosinophilia of at least 20%, often over 50%, and possibly up to 90% is present during the muscle invasion phase of the infection.

## NEMATODES • Blood and Tissue

### Filarial Worms

<b>Pathogenic</b>	Yes, some more than others
<b>Disease</b>	Filarisis
<b>Acquired</b>	Bite of various arthropods
<b>Body site</b>	Blood, lymph, tissues
<b>Symptoms</b>	Few to elephantiasis; depends on body site (lymphatics, tissues, etc.)
<b>Clinical specimen</b>	Blood, multiple draws (in EDTA); skin snips
<b>Epidemiology</b>	Sporadic distribution; arthropod-to-human transmission
<b>Control</b>	Vector control

### LIFE CYCLE



### Diagnosis

Presumptive diagnosis of filariasis must include lymphangitis and lymphedema. Confirmation of filarial infections is based on detecting microfilariae in blood or tissues. Microfilariae can be identified to the species level by the presence or absence of a sheath and the position of body nuclei in stained specimens. Definitive diagnosis is based on detecting microfilariae for *W. bancrofti*, *B. malayi*, *Loa loa*, *Mansonella ozzardi*, and *M. perstans* in the circulating blood. Microfilariae of *O. volvulus* and *M. streptocerca* are detected primarily in the skin, although they are occasionally detected in the blood. Microfilariae may also be found in hydrocele fluid and urine, particularly in patients who have high microfilaremias or have been treated recently with diethylcarbamazine.

The optimal time for drawing blood to detect periodic infections of *W. bancrofti*, *B. malayi*, and *B. timori* is between 10 p.m. and 4 a.m. Blood to detect subperiodic species of *W. bancrofti* and *B. malayi* may be drawn any time. Blood for *L. loa* should be drawn between 10 a.m. and 4 p.m., and blood to detect *Mansonella* infections can be drawn at any time. Finger-prick or earlobe blood may be taken for direct wet, thin, and thick blood smears. Blood films may be stained with Giemsa or Delafield's hematoxylin stain. Giemsa stain does not stain the microfilarial sheath adequately, although hematoxylin stains do. Examination of a blood film for microfilariae should include low-power review of the entire film. Sheathed microfilariae often lose their sheath when drying on thick films.

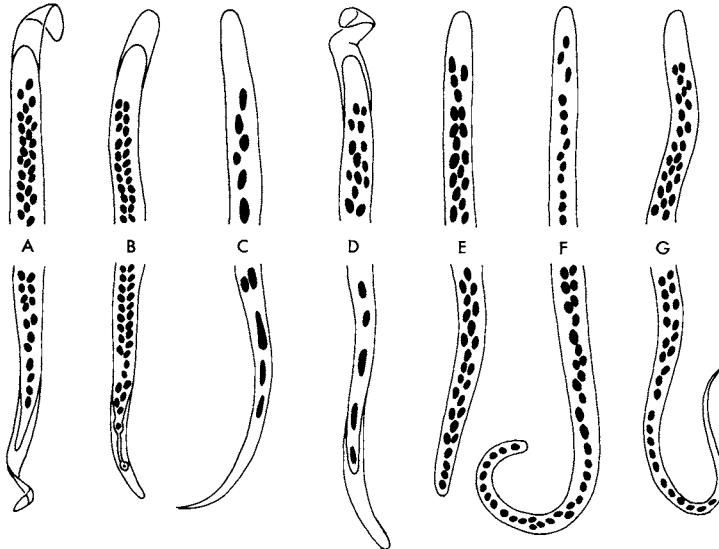
Serologic tests are more meaningful in patients who have not resided in the areas of endemicity for extended periods.

### General Comments

Infections are transmitted to humans by the bites of obligate blood-sucking arthropods that had become infected through ingesting larvae (microfilariae) in a blood meal from a mammalian host. Each parasite has a complex life cycle, and human infections are not readily established unless there is intense and prolonged exposure to infective larvae. After exposure, it may take years before significant pathologic changes in the human host are evident. Adult stages inhabit the lymphatic system, subcutaneous tissues, or deep connective tissues. Adult females produce microfilariae, i.e., prelarvae that may retain the egg membrane (sheathed microfilariae) or may lose it (unsheathed microfilariae). Once released by the female worm, the highly motile and threadlike microfilariae can be detected in the peripheral blood or cutaneous tissues, depending on the species. They may survive for 1 to 2 years, are not infective for other vertebrate hosts, and do not develop further in the host.

Adult *O. volvulus* worms lie within fibrous tissue capsules in the dermis and subcutaneous tissues, and the microfilariae are usually nearby to be ingested by a species of *Simulium* (blackfly or buffalo gnat). Humans are infected when bitten by the infected fly, and larvae are deposited into the bite site. Microfilariae are normally found in the dermis.

When *Dirofilaria* worms lodge in the pulmonary artery branches, they cause an infarct. These lesions are usually on the periphery of the lungs and are sharply defined (coin lesion). There is a central necrotic area surrounded by a granulomatous inflammation and a fibrous wall. Dead or dying worms may be found in the lesion. Reported cases highlight the morphologic variation seen in human pulmonary dirofilariasis and emphasize the need to consider this diagnosis in all cases of necrotizing granulomas of the lungs.



Images from left to right: Diagrams of human microfilariae—*W. bancrofti*, *B. malayi*, *O. volvulus*, *L. loa*, *M. perstans*, *M. streptocerca*, and *M. ozzardi*. (Illustration by Nobuko Kitamura.)

## Additional Information

Three species, *W. bancrofti*, *B. malayi*, and *O. volvulus*, account for most infections. There are 90 million people currently infected (two-thirds live in China, India, and Indonesia) with *W. bancrofti*, *B. malayi*, and *B. timori*.

*O. volvulus* has infected 45 million to 50 million people in Africa and Central and South America, of whom approximately 1 million are blind. Symptoms of heavy *O. volvulus* infections include dermatitis, onchocercomas (subcutaneous nodules containing adult worms), lymphadenitis, and blindness. Individuals with onchocerciasis may have clinically normal skin whereas others may have pruritus and disfiguring skin lesions. The pruritus may cause sleeplessness, fatigue, and weakness. The skin may be painful, hot, and edematous, eventually resulting in permanent thickening. Acute attacks of onchodermititis have resulted in a purplish skin discoloration known as mal morado in Central America. Chronically infected skin loses its elasticity and becomes thickened. In Central America, these skin changes are frequently seen as noticeable thickening of the earlobes and thickening of facial skin to mimic leonine facies (seen in lepromatous leprosy). In Africa, the same skin changes occurring in the hip region produce a condition known as hanging groin, which may predispose infected individuals to inguinal and femoral hernias. Lymphadenopathy in the inguinal and femoral areas is common among Africans.

**Note:** Patients who have been treated for lymphatic filariasis should have blood specimens reexamined for microfilariae 2 to 6 weeks posttherapy. Onchocerciasis patients should have skin snips examined 3 to 6 months posttherapy.

The clinical manifestations of filariasis vary and may depend on host factors and parasite strains. Some

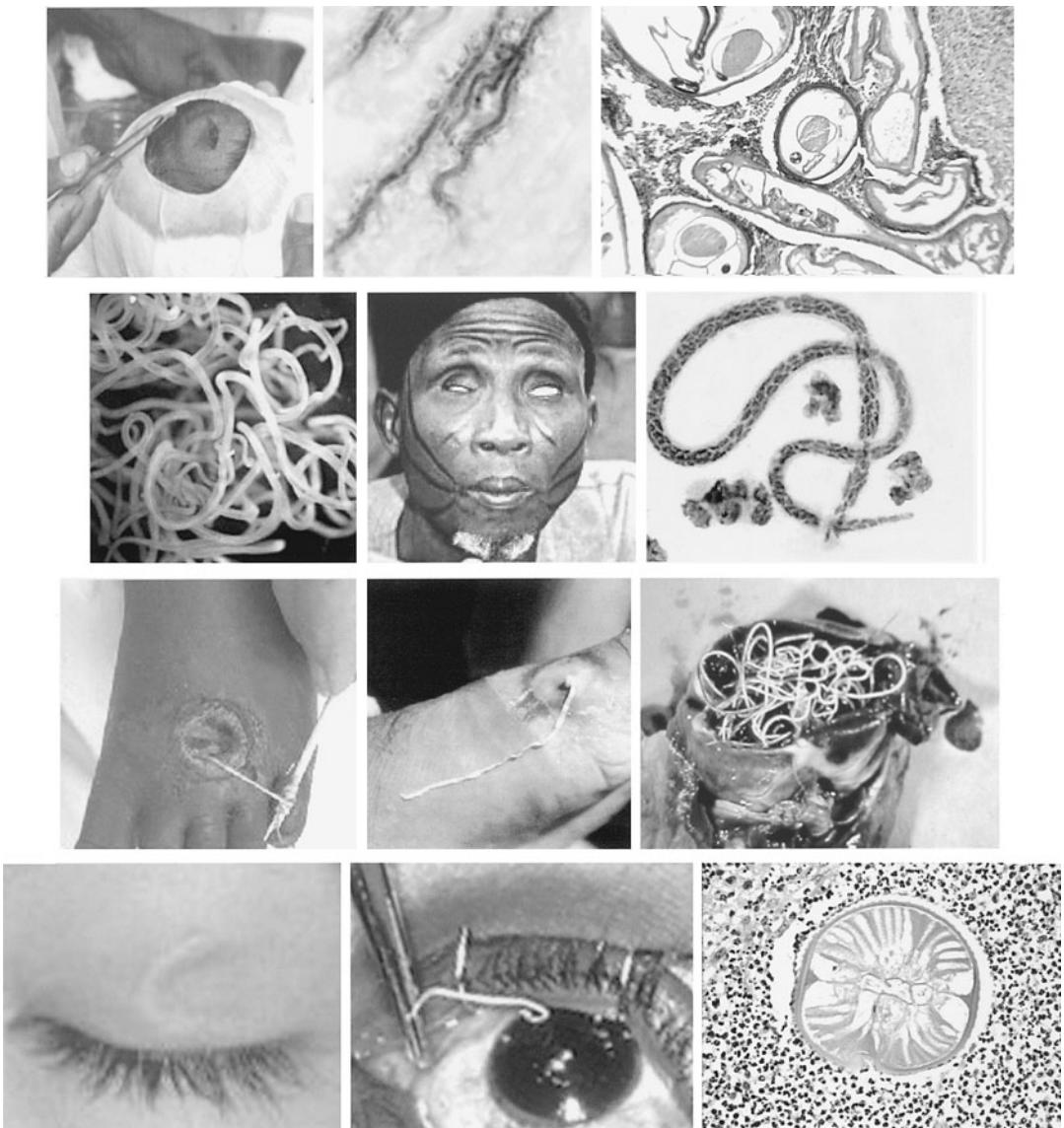
patients harbor adult worms without a peripheral microfilaremia, or the microfilaremia may be too low to be detected by the usual laboratory procedures. Other patients have a heavy microfilaremia but are clinically asymptomatic. Many patients who were thought to be microfilaricemic but clinically asymptomatic have symptoms of elephantiasis.

Early manifestations include high fevers (filarial or elephantoid fever), lymphangitis, and lymphadenitis. Filarial fever usually begins with a high fever and chills that last 1 to 5 days before spontaneously subsiding; patients often do not have a microfilaremia. The lymphangitis extends distally from the affected nodes where the filarial worms reside. Lymphadenitis and lymphangitis develop in the lower extremities more commonly than in the upper. There can also be genital (almost exclusively a feature of *W. bancrofti* infection) and breast involvement. The lymph nodes most often affected are the epitrochlears and femorals. They are firm, discrete, and tender and tend to remain enlarged. The lymph vessel is indurated and inflamed. The overlying skin is tense, erythematous, and hot, and the surrounding area is edematous. Occasionally abscesses form at the lymph node or along the lymphatic system and may take 2 to 3 months to heal.

In *Dirofilaria* infections, adult worms reside in the right heart of dogs and microfilariae are found in the blood, where they are ingested by mosquitoes or *Simulium* black flies. After biological development in the vector, dogs and humans may be infected with the infective larvae during a blood meal. Development to a mature adult takes approximately 180 days in the dog. In humans, the worms do not reach maturity and no microfilariae can be detected. The cuticle of nematodes contains chitin, which can be stained with nonspecific whiteners such as calcofluor white. *Dirofilaria* larvae stained with calcofluor can be easily recognized in tissue sections, whereas the parasite may be difficult to identify using routine histologic stains.



**Plate 7.13** Row 1 (left to right): *Brugia malayi* microfilaria (note the sheath and two terminal nuclei), *Brugia malayi* head (sheath not visible), and *Brugia malayi* tail (again, note the sheath and two terminal nuclei); row 2 (left to right): *Loa loa* microfilaria (note the sheath), *Loa loa* tail (note that nuclei run all the way to the end of the tail; the sheath is not visible), and *Loa loa* tail (note that nuclei go to the tip of the tail; the sheath is visible); row 3: *Loa loa* in eye (left and middle) and *Loa loa* calabar swelling (note the swollen left knee) (right) (all three images from *A Pictorial Presentation of Parasites*: a cooperative collection prepared and/or edited by H. Zaiman); row 4 (left to right): *Wuchereria bancrofti* head of microfilaria (note the nuclei and sheath), *Wuchereria bancrofti* tail (note that the nuclei end before the end of the tail; the sheath is visible), and elephantiasis (occurs with certain types of filariasis) (right image from *A Pictorial Presentation of Parasites*: a cooperative collection prepared and/or edited by H. Zaiman).



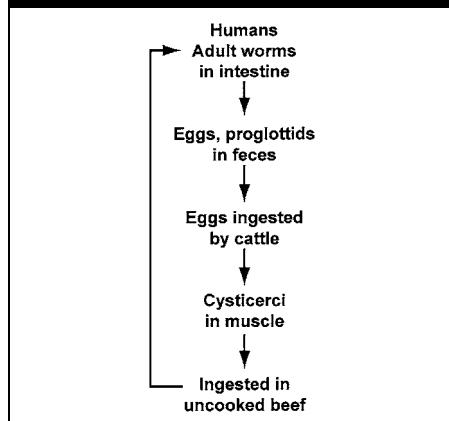
**Plate 7.14** Row 1 (left to right): *Onchocerca volvulus* (removal of nodule from scalp), *Onchocerca volvulus* (organisms seen in skin biopsy), and *Onchocerca volvulus* (cross section of worms within a nodule); row 2 (left to right): *Onchocerca volvulus* (worms removed from nodule), *Onchocerca volvulus* (example of "river blindness"), and *Mansonella ozzardi* (this microfilaria does not have a sheath); row 3: *Dracunculus medinensis* (worm removal) (left and middle) and *Dirofilaria* spp. in the heart of a dog (right) (three images courtesy of Armed Forces Institute of Pathology); row 4 (left to right): *Dirofilaria* spp. (worm in eyelid), *Dirofilaria* spp. (worm being removed from eye) (two images from *A Pictorial Presentation of Parasites: a cooperative collection prepared and/or edited by H. Zaiman*), and *Dirofilaria* spp. (worm in cross section), (courtesy of A. Linscott).

## CESTODES • Intestinal

### Taenia saginata

<b>Pathogenic</b>	Yes
<b>Disease</b>	Beef tapeworm disease, taeniasis
<b>Acquired</b>	Ingestion of infective cysts (immature tapeworm larvae, cysticerci) from infected (raw or poorly cooked) beef
<b>Body site</b>	Intestine
<b>Symptoms</b>	Vague to none, discomfort or embarrassment caused by proglottids crawling from the anus
<b>Clinical specimen</b>	Stool or individual proglottids
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Cattle should not be allowed to graze on ground contaminated by human sewage.

#### LIFE CYCLE



### Diagnosis

Few symptoms are associated with the presence of the adult worm in the intestine. Although rare symptoms (obstruction, diarrhea, hunger pains, weight loss, or appendicitis) have been reported, the most common complaint is the discomfort and embarrassment caused by the proglottids crawling from the anus. This may be the first clue that the patient has a tapeworm infection. Occasionally, the proglottids are also seen on the surface of the stool after it is passed.

The standard O&P exam is recommended for recovery and identification of *T. saginata* eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. The eggs are most easily seen on a direct wet smear or a wet preparation of the concentration sediment.

Since the eggs of *T. saginata* and *T. solium* look identical, identification to the species level is normally based on recovery and examination of gravid proglottids, in which the main lateral branches are counted (count on one side only; 15 to 20 for *T. saginata* and 7 to 13 for *T. solium*). Often the gravid proglottids of *T. saginata* are somewhat larger than those of *T. solium*, but this difference may be minimal or impossible to detect. The scolex has four suckers and no hooks.

Preliminary examination of the gravid proglottid may not allow identification without clearing or injection of the uterine branches with India ink.

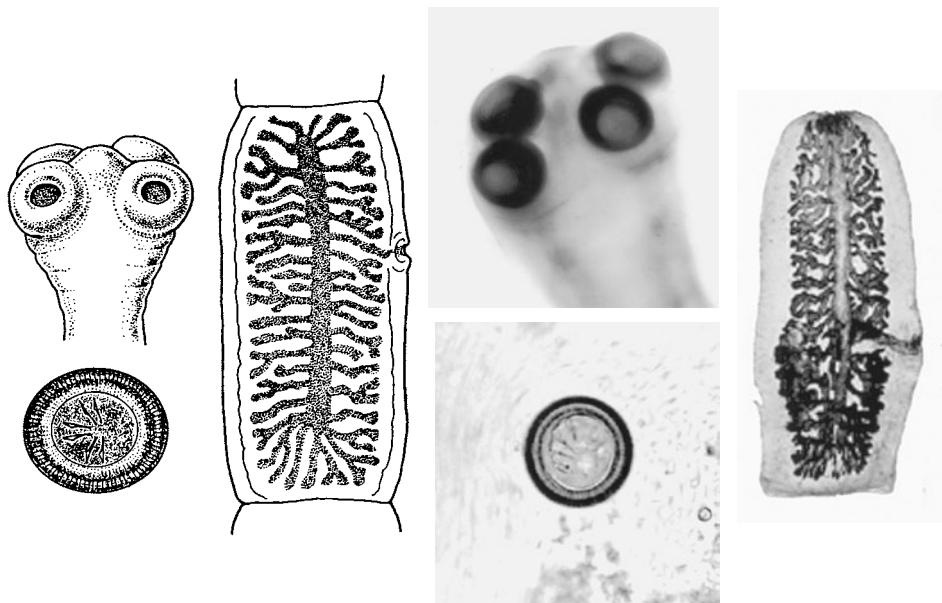
**Note:** Since it is possible that the proglottid is *T. solium*, with the inherent problem of egg ingestion and cysticercosis, all specimens should be handled with extreme caution.

### General Comments

*T. saginata* was apparently differentiated from *T. solium* in the late 1700s; however, cattle were not identified as the intermediate host until 1863. This infection occurs worldwide and is generally much more common than *T. solium* infection, particularly in the United States. The overall impact on human health is much lower than that of *T. solium*, since *T. saginata* cysticercosis is quite rare.

The life cycle is very similar to that of *T. solium*. Infection with the adult worm is initiated by ingestion of raw or poorly cooked beef containing encysted *T. saginata* larvae. The larva is digested out of the meat in the stomach, and the tapeworm evaginates in the upper small intestine and attaches to the intestinal mucosa, where the adult worm matures in 5 to 12 weeks. The adult worm grows up to 25 ft but often measures only about half this length. The scolex is "unarmed" and has four suckers with no hooks. The proglottids usually number 1,000 to 2,000, with the mature proglottids being broader than long and the gravid proglottids being narrower and longer. Although a single worm is usually found, multiple worms can be present (personal observation).

The eggs can remain viable in the soil for days to weeks. On ingestion by cattle, the oncospheres hatch in the duodenum, penetrate the intestinal wall, and are carried via the lymphatics or bloodstream, where they are filtered out in the striated muscle. They develop into the bladder worm or cysticercus within approximately 70 days. The mature cysticercus is 7.5 to 10 mm wide by 4 to 6 mm long and contains the immature scolex, which has no hooks (unarmed). Other animals that harbor cysticerci include buffalo, giraffe, llama, and possibly reindeer. Actual cases of human cysticercosis with *T. saginata* are rare, and some reported cases may have been inaccurately diagnosed.



**Images from left to right:** The drawings depict the scolex, egg, and gravid proglottid of *T. saginata*; the photographs show the same structures. Note the number of uterine branches (many more than in *T. solium*). Also, note that the scolex does not contain hooklets, like that of *T. solium* (see p. 353).

### Description (Eggs)

The eggs are usually spheroidal and yellow-brown. They are thick-shelled eggs, measuring 31 to 43  $\mu\text{m}$ ; they contain a six-hooked oncosphere (embryo). They are routinely found in the stool, even if gravid proglottids are not found in the specimen.

*T. saginata* eggs look like those of *T. solium* (pork tapeworm). Since *T. solium* eggs are infectious for humans, all clinical specimens containing proglottids and/or eggs must be handled very carefully to avoid accidental ingestion of infective *T. solium* eggs and resulting cysticercosis.

**Note:** In proficiency-testing specimens, the eggs may be harvested from gravid or mature proglottids. Some eggs have a gelatinous coating that may be confusing. This coating is generally found on eggs that have been recovered from the mature proglottids and not from the gravid proglottids at the end of the strobila. This extra coating should not cause confusion; the six-hooked embryo can still be seen within the eggshell. The true tapeworm egg has a radially striated shell that is relatively thick.

### Description (Adults)

The adult tapeworm consists of the attachment organ (scolex), to which is attached a chain of segments or proglottids called the strobila. Each proglottid contains a male and female reproductive system. The proglottids are classified as immature, mature, or gravid; gravid proglottids are found at the end of the strobila and contain the fully developed uterus full of eggs. The branched uterine structure in the gravid proglottids is often used as the main criterion for identification to the species level. The

scolex and eggs can also be used to identify a cestode to the species level. The adult worms can reach about 15 to 20 ft long and may survive for up to 25 years.

The scolex is quadrate shaped; it has four suckers and no rostellum or hooklets.

The gravid proglottids can be found in feces and are longer than wide (19 by 17 mm) with 15 to 20 lateral branches on each side of the central uterine stem. They usually appear singly and can actively crawl like an "inch-worm"; they may actually migrate under a fresh stool specimen.

### Additional Information

Recent epidemiologic studies of taeniasis in Southeast Asia indicate the presence of a form of human *Taenia* spp., which can be distinguished from *T. saginata* and *T. solium*. This newly recognized cestode was originally called the Taiwan *Taenia* sp. and was first found in Taiwanese aborigines. However, it is now referred to as the Asian *Taenia* sp. or *T. asiatica* since it has also been found in a number of other Asian countries.

Cysticerci of *T. asiatica* develop in the liver of pigs, cattle, and goats and are much smaller than those of *T. saginata* or *T. solium*. The adult worm matures in 10 to 12 weeks, measuring 4 to 8 m long, and contains 300 to 1,000 proglottids. In some indigenous populations in Taiwan, 10 to 20% of the people are infected, primarily from the ingestion of pork meat and viscera. Multiple worms are often present, and in one area of Taiwan during the late 1990s, the infection rate with *T. asiatica* was 11%. A history of eating raw pig liver is relevant for *T. asiatica*.

## CESTODES • Intestinal

### *Taenia solium*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Pork tapeworm disease, taeniasis, cysticercosis
<b>Acquired</b>	<p><b>Adult worm:</b> Ingestion of infective cysts (immature tapeworm larvae, cysticerci) from infected (raw or poorly cooked) pork</p> <p><b>Cysticercosis:</b> Ingestion of infective eggs from adult tapeworm; development of cysticerci in human tissues, similar to that seen in the pig</p>
<b>Body site</b>	<p><b>Adult worm:</b> Intestine</p> <p><b>Cysticerci:</b> Primarily CNS and muscle</p>
<b>Symptoms</b>	<p><b>Adult worm:</b> Vague to none, discomfort or embarrassment caused by proglottids crawling from the anus</p> <p><b>Cysticercosis:</b> Dependent on the body site</p>
<b>Clinical specimen</b>	Stool or individual proglottids
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Pigs should not be allowed to graze on ground contaminated by human sewage.

### Diagnosis

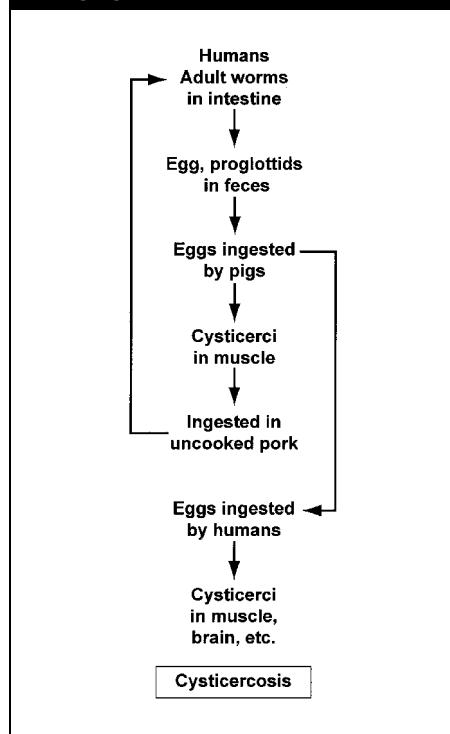
The standard O&P exam is recommended for recovery and identification of *T. solium* eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. The eggs are most easily seen on a direct wet smear or a wet preparation of the concentration sediment.

Since *T. solium* and *T. saginata* eggs look identical, identification to species level is normally based on the recovery and examination of gravid proglottids, in which the main lateral branches are counted (7 to 13 for *T. solium* and 15 to 20 for *T. saginata*). Often the gravid proglottids of *T. saginata* are larger than those of *T. solium*, but this difference may be minimal or impossible to detect. The scolex has four suckers and an armed rostellum (hooklets present).

Preliminary examination of the gravid proglottid may not allow identification without clearing or injection of the uterine branches with India ink.

**Note:** Since it is possible that the unidentified proglottid is *T. solium*, with the inherent problem of egg ingestion and cysticercosis, all specimens should be handled with extreme caution.

### LIFE CYCLE



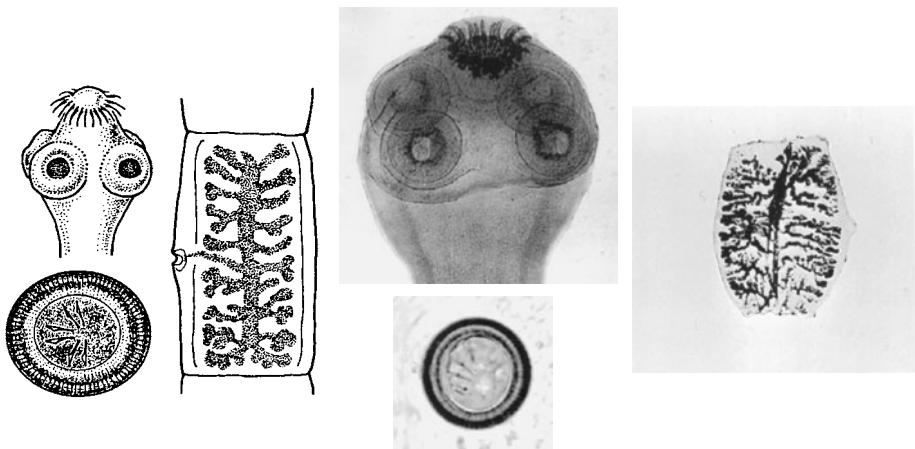
### General Comments

*T. solium* infection may have been recognized since biblical times, with the life cycle being delineated in the mid-1850s. The tapeworm is found in many parts of the world and is considered an important human parasite where raw or poorly cooked pork is eaten.

Cysticercosis infections with *T. solium* (larvae) are relatively common in certain parts of the world but rare in the United States. This extraintestinal infection is far more serious than the adult worm in the intestine.

Infection with the adult worm is initiated by ingestion of raw or poorly cooked pork containing encysted *T. solium* larvae. The larva is digested out of the meat in the stomach, and the tapeworm head evaginates in the upper small intestine, attaches to the intestinal mucosa, and grows to the adult worm within 5 to 12 weeks. Although usually a single worm is present, there may be multiple worms in the intestine. The adult worm reaches 2 to 7 m long and may survive 25 years or more.

On ingestion by hogs or humans, the eggs hatch in the duodenum or jejunum after exposure to gastric juice in the stomach. The released oncospheres penetrate the intestinal wall, are carried via the mesenteric venules throughout the body, and are filtered out in the subcutaneous and intramuscular tissues, the eye, the brain, and other body sites.



Images from left to right: The drawings depict the scolex, egg, and gravid proglottid of *T. solium*; the photographs show the same structures. Note the number of uterine branches (many fewer than that in *T. saginata*). Also note that the scolex has hooklets, unlike that of *T. saginata* (see p. 351).

### Description (Eggs and Larvae)

Eggs usually pass from the uterus through the ruptured wall, where the proglottids break off from the strobila. The eggs are round or slightly oval (31 to 43  $\mu\text{m}$ ), have a thick striated shell, and contain a six-hooked embryo or oncosphere. They may remain viable in the soil for weeks.

*T. solium* eggs look like those of *T. saginata* (beef tapeworm). Since *T. solium* eggs are infectious for humans, all clinical specimens containing proglottids and/or eggs must be handled very carefully to avoid accidental ingestion of infective *T. solium* eggs and resulting cysticercosis.

The cysticercus is an ovoid, milky white bladder with the head invaginated into the bladder. The host generally produces a fibrous capsule around the bladders, unless they are located in the brain, particularly the ventricles. The laboratory infrequently sees material from CNS system tissues.

**Note:** In proficiency testing specimens, the eggs may be harvested from gravid or mature proglottids. In some cases the eggs have a gelatinous coating that may be confusing. See the note under *T. saginata*.

### Description (Adult)

The adult tapeworm is composed of the attachment organ (scolex), to which is attached a chain of segments or proglottids called the strobila. Each proglottid contains a male and female reproductive system. The proglottids are classified as immature, mature, or gravid; gravid proglottids are found at the end of the strobila and contain the fully developed uterus full of eggs. The branched uterine structure in the gravid proglottids is often used as the main criterion for identification to the species level. The scolex and eggs can also be used to identify a cestode to the species level. The adult worms can reach about 15 to 20 ft long and may survive for up to 25 years.

The scolex of *T. solium* is quadrate shaped; it has four suckers and a rounded rostellum with a double set of hooklets.

The gravid proglottids are found in feces and are approximately square with 7 to 13 lateral branches on each side of the central uterine stem. They usually appear singly and can actively crawl like an "inchworm"; they may migrate under a fresh stool specimen, although this type of motility is more often seen in *T. saginata*. Occasionally two or three proglottids are passed together (attached).

### Adult-Onset Epilepsy

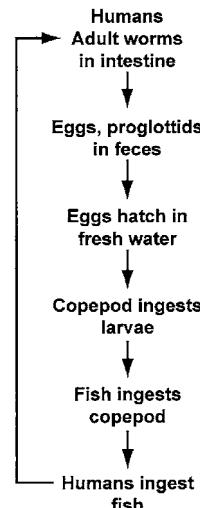
The presence of cysticerci in the brain is the most common parasitic infection of the human nervous system and the most common cause of adult-onset epilepsy throughout the world. In Latin America it is unusual for both brain and muscle cysticercosis to occur in the same patient; fewer than 6% of patients have cysticerci in both sites. However, elsewhere, subcutaneous involvement by *T. solium* cysticerci has been found in up to 78.5% of patients with cerebral cysticercosis. Possible reasons for such differences include (i) the immune status of the patient, (ii) the human leukocyte antigen type, (iii) the nutritional status of the patient, (iv) the burden of eggs infecting the patient, and (v) a difference in the strains of *T. solium*.

## CESTODES • Intestinal

### *Diphyllobothrium latum*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Broad fish tapeworm disease <i>Diphyllobothriasis</i>
<b>Acquired</b>	<i>Adult worm</i> : Ingestion of infective larvae from infected freshwater fish (raw or poorly cooked fish)
<b>Body site</b>	<i>Adult worm</i> : Intestine
<b>Symptoms</b>	<i>Adult worm</i> : Vague to none
<b>Clinical specimen</b>	Intestinal: Stool or proglottids
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Dogs may serve as carriers of the adult worms and infected dogs should be periodically treated.

#### LIFE CYCLE



#### Diagnosis

Diagnosis is usually based on recovery and identification of the characteristic eggs or proglottids. If the egg operculum is difficult to see, the coverslip of the wet preparation can be tapped and the pressure may cause the operculum to pop open, making it more visible. The eggs are unembryonated at the time they are passed in the stool.

These eggs can be confused with those of *Paragonimus westermani*; however, *P. westermani* eggs have opercular shoulders into which the operculum fits while eggs of *D. latum* have a very smooth opercular outline with no shoulders.

Proglottids are often passed in chains (a few inches to several feet), and this is a clue to *D. latum*. The overall proglottid morphology with the rosette uterine structure also facilitates identification.

#### General Comments

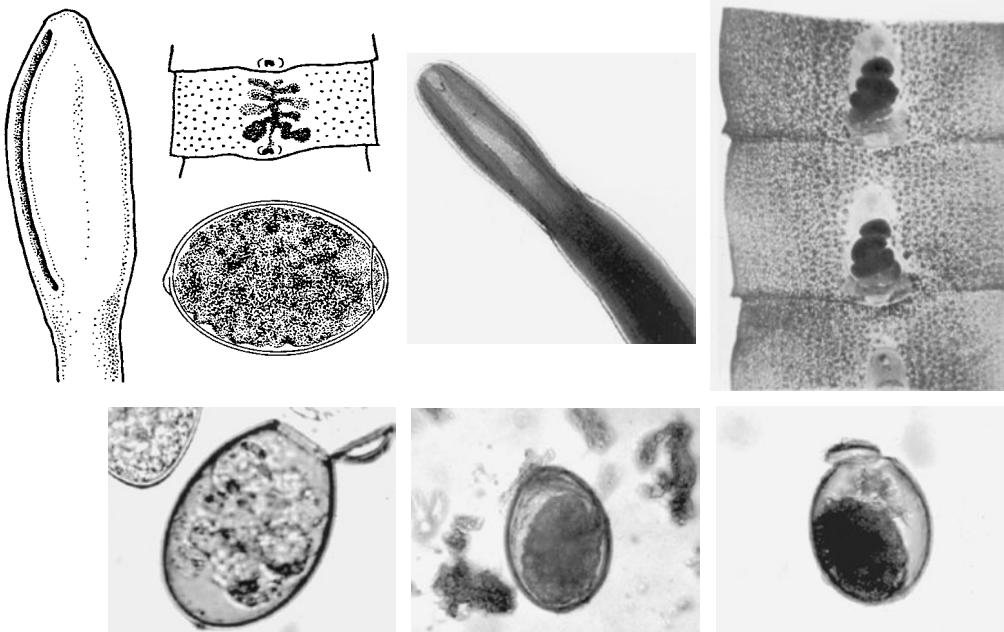
Symptoms depend on the number of worms present, the amounts and types of by-products produced by the worm, the patient's reaction to these by-products, and the absorption of various metabolites by the worms. There may be occasional intestinal obstruction, diarrhea, abdominal pain, or anemia. If the worm is attached at the jejunal level, there may very rarely be vitamin B<sub>12</sub> deficiency resembling pernicious anemia. Heavy or long-term infections may cause megaloblastic anemia due to parasite-

mediated dissociation of the vitamin B<sub>12</sub>-intrinsic factor complex within the gut lumen, making B<sub>12</sub> unavailable to the host. This is more common in Finland, where some individuals have a genetic predisposition to pernicious anemia. In patients without this genetic predisposition, symptoms of *D. latum* infection may be absent or minimal, consisting of a slight leukocytosis with eosinophilia.

*D. latum* belongs to the pseudophyllidean tapeworm group, which is characterized by having a scolex with two bothria (sucking organs) rather than the typical four suckers seen in the *Taenia* tapeworms. Its distribution is worldwide, with increased outbreaks reported from time to time. The infection has been associated with a condition similar to pernicious anemia.

Infection with the adult worm is acquired by the ingestion of raw, poorly cooked, or pickled freshwater fish (pike, perch, lawyer, salmon, trout, white fish, grayling, ruff, turbot, etc.) containing the encysted plerocercoid larvae. After ingestion, the worm matures, with egg production beginning in week 5 or 6. The adult worm is ca. 10 m long and contains up to 3,000 proglottids.

After developing for 2 weeks in freshwater, the eggs hatch and the ciliated, coracidium larvae are ingested by the first intermediate host, the copepod. The copepods, containing the second larval stage (procercoid), are then ingested by fish, which may be ingested by larger fish. The final fish intermediate host may contain many plerocercoid larvae, which initiate the infection with the adult worm when ingested by humans.



**Images from left to right:** The drawings depict the scolex, egg, and gravid proglottid of *D. latum*; the photographs show the same structures. The gravid proglottids are often passed as a chain (which can be several feet long). Some of the eggs are seen with "popped" open opercula (trap-doors).

### Description (Eggs)

The eggs are broadly oval and operculated. They are yellow-brown and measure 58 to 75  $\mu\text{m}$  by 40 to 50  $\mu\text{m}$  in feces. They usually have a small knob at the abopercular end.

The eggs are unembryonated when passed in the feces. They are sometimes confused with eggs of *Paragonimus westermani*; however, *D. latum* eggs have no opercular shoulders into which the operculum fits.

### Additional Information

In areas where human infection is rare, *D. latum* infections have been found in other mammals. However, the natural transmission cycle from mammals other than humans does not seem to be sustained. Infection can result from ingestion of infected raw freshwater fish that has been shipped under refrigeration to areas where the infection is not endemic. Preventive measures include thorough cooking of all freshwater fish or freezing for 24 to 48 h at  $-18^\circ\text{C}$ . This infection has been called the Jewish housewives' disease, since the individual preparing the food may sample the dish (e.g., gefilte fish) prior to cooking and acquire the infection. Other groups who tend to eat raw or insufficiently cooked fish include the Russians, Finns, and Scandinavians. Raw fish marinated in lime juice (ceviche) is also a source of infection (*D. pacificum*) in Latin America. Since domestic dogs can serve as reservoir hosts, infected dogs should be periodically treated. Other factors include the continued dumping of wastewater into lakes and the possibility of animal reservoirs.

### Description (Adults)

The scolex of *D. latum* is elongate and spoon shaped and has two long sucking grooves, one on the dorsal surface and one on the ventral surface. The mature and gravid proglottids are wider than long, with the main reproductive structures (mainly the uterus) in the center of the gravid proglottid. This configuration of the uterine structure is called a rosette. Identification to species level is usually based on this typical morphology of the gravid proglottids. Both eggs and proglottids may be found in the stool. Often a partial chain of proglottids may be passed (a few inches to several feet).

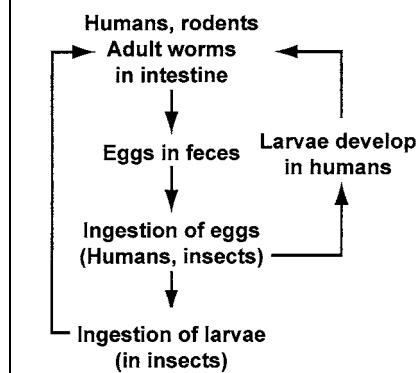
Proglottids are wider than long (3 by 11 mm) and have a rosette-shaped central uterine structure.

## CESTODES • Intestinal

### *Hymenolepis nana*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Dwarf tapeworm disease
<b>Acquired</b>	Ingestion of infective eggs or (less commonly) cysts (immature tapeworm larvae, cysticercoid) from infected grain beetles
<b>Body site</b>	Intestine
<b>Symptoms</b>	Vague to none; some patients have low-grade eosinophilia (5% or more).
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide, primarily human-to-human transmission
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The adult worm or proglottids are rarely seen in the stool; therefore, diagnosis is based on recovery and identification of the characteristic eggs. They are most easily identified in fresh specimens or those preserved in formalin-based fixatives. Specimens preserved in PVA contain eggs whose morphologic characteristics are not as well delineated as those in formalin-fixed specimens. These thin-shelled eggs also tend to collapse on the permanent stained smear and may be difficult to identify; the direct wet film or concentration wet mount is recommended.

The standard O&P exam is recommended for recovery and identification of *H. nana* eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. The eggs are most easily seen on a direct wet smear or a wet preparation of the concentration sediment.

*H. nana* is the only human tapeworm in which the intermediate host is not necessary and transmission is from person to person via the eggs. Children are usually infected more often than adults. Good personal hygiene is an important preventive measure. Infection from rats and mice is always a possibility, as is the accidental ingestion of infective insect intermediate hosts.

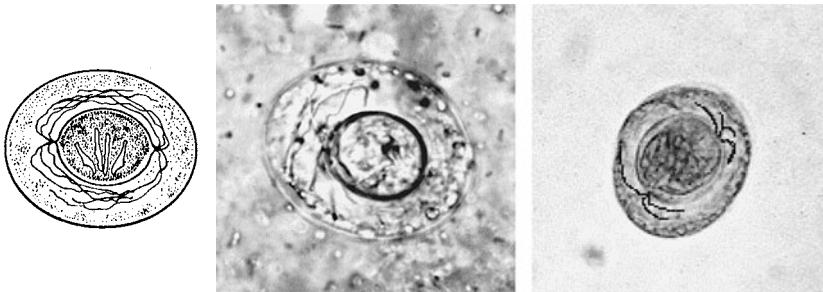
### General Comments

*H. nana* has been called the dwarf tapeworm; it occurs worldwide. The lack of an intermediate host was determined in the late 1800s. For this reason, *H. nana* has been considered the most common tapeworm worldwide. The infection is most common in children, although adults are also infected. Worldwide, 50 million to 75 million people are infected. There has been some discussion regarding placing *H. nana* in the genus *Rodentolepis*.

Infection is usually acquired by ingestion of *H. nana* eggs, primarily from human stool. The eggs hatch in the stomach or small intestine, and the liberated larvae, or oncospheres, penetrate the villi in the upper small intestine. The larvae develop into the cysticercoid stage in the tissue and migrate back into the lumen of the small intestine, where they attach to the mucosa. The adult worms mature within several weeks.

Although accidental ingestion of the insect intermediate host can result in development of the adult worms, this mode of infection is probably not common.

Heavy human infection can be attributed to internal autoinfection in which the eggs hatch in the intestine and follow the normal life cycle to the adult worm. This autoinfection feature of the life cycle can lead to complications in the compromised patient.



**Images from left to right:** The drawing depicts the *H. nana* egg; the photographs show the same structures. Note the six-hooked embryo (oncosphere) and the polar filaments that lie between the oncosphere and the thin eggshell. The key difference between this egg and that of the rat tapeworm (*H. diminuta*) is the lack of polar filaments in the latter.

### Description (Eggs)

Since *H. nana* and *H. diminuta* eggs look very much alike, identification depends on seeing the polar filaments in *H. nana* eggs. The eggs are round to oval with a thin shell and are 30 to 47  $\mu\text{m}$  in diameter. The oncosphere has two polar thickenings from which arise polar filaments that lie between the oncosphere and the shell.

**Note:** In proficiency-testing specimens, the eggs may not always have hooklets visible within the oncosphere. You may have to look very carefully for the polar filaments. These filaments and hooklets are more easily seen in stool specimens that have not been stored for a long time in formalin.

### Description (Adults)

The scolex has four suckers and a short rostellum with hooks. The adult worm is rarely seen in the stool. The eggs are released by disintegration of the gravid proglottids, pass out in the stool, and are immediately infectious.

The worms are very small compared with *Taenia* worms and measure up to 40 mm long. The more worms present, the shorter the total length of each worm.

### Additional Information

An infection with *H. nana* may cause no symptoms even with a heavy worm burden. Some patients complain of headache, dizziness, anorexia, abdominal pain, diarrhea, or possibly irritability. Some patients have low-grade eosinophilia (5% or more).

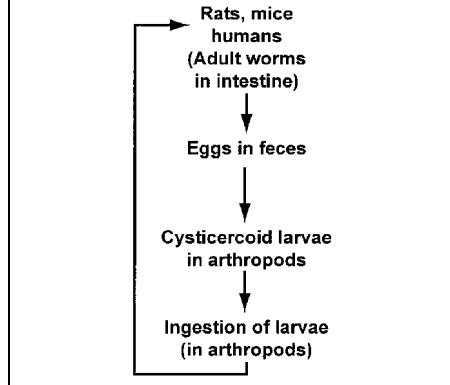
Heavy human infection can be attributed to internal autoinfection in which the eggs hatch in the intestine and follow the normal life cycle to the adult worm. This autoinfection feature of the life cycle can lead to complications in the compromised patient.

## CESTODES • Intestinal

### *Hymenolepis diminuta*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Rat tapeworm disease
<b>Acquired</b>	Ingestion of immature tapeworm larvae (cysticercoid) from infected arthropods
<b>Body site</b>	Intestine
<b>Symptoms</b>	Vague to none
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

The adult worm or proglottids usually disintegrate in the gut; therefore, diagnosis is based on recovery and identification of the characteristic eggs. They are most easily identified in fresh specimens or those preserved in formalin-based fixatives. Specimens preserved in PVA contain eggs whose morphologic characteristics are not as well delineated as those in formalin-fixed specimens. These thin-shelled eggs also tend to collapse on the permanent stained smear and may be difficult to identify; the direct wet film or concentration wet mount is recommended.

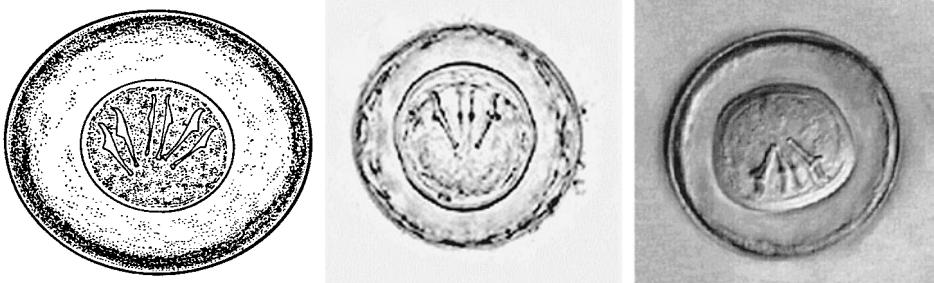
The standard O&P exam is recommended for recovery and identification of *H. diminuta* eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. The eggs are most easily seen on a direct wet smear or a wet preparation of the concentration sediment.

### General Comments

Although *H. diminuta* is commonly found in rats and mice, it is found infrequently in humans. It occurs worldwide in normal hosts, and approximately 200 to 300 human cases have occurred, primarily in India, the former Soviet Union, Japan, Italy, and certain areas of the southern United States (Tennessee, Georgia, and Texas).

The life cycle is very similar to that of *H. nana*; however, the arthropod intermediate host is obligatory. A number of different arthropods (lepidopterans, earwigs, myriapods, larval fleas, and beetles) can serve as intermediate hosts. After egg ingestion by the arthropods, the cysticercoid stage forms (similar in morphology to the cysticercoid stage of *H. nana*). Infection by accidental ingestion of the infected arthropod containing cysticercoids results in development of the adult worm in the intestine. Unlike *H. nana* eggs, *H. diminuta* eggs are not infectious from person to person. As with most tapeworms, the infection is usually tolerated very well by the host, with few if any symptoms.

This infection is a true zoonosis, since infected rats infect insects, which in turn are consumed by humans.



**Images from left to right:** The drawing depicts the *H. diminuta* egg; the photographs show the same structures. Note the six-hooked embryo (oncosphere) and the lack of polar filaments. The key difference between this egg and that of *H. nana* is the presence of polar filaments between the oncosphere and the thin eggshell in *H. nana* eggs.

### Description (Eggs)

Since *H. nana* and *H. diminuta* eggs look very much alike, identification depends on seeing the polar filaments in *H. nana* eggs. *H. diminuta* eggs are round to oval with a thin shell and are 60 to 79  $\mu\text{m}$  in diameter. The oncosphere has no polar thickenings and no polar filaments.

**Note:** In proficiency-testing specimens, the eggs may not always have hooklets visible within the oncosphere. The hooklets are more easily seen in stool specimens that have not been stored for a long time in formalin.

### Additional Information

As with most tapeworms, the infection is usually tolerated very well by the host, with few if any symptoms. Most infections occur in children younger than 3 years of age; however, infected adults have also been reported. Symptoms include diarrhea, anorexia, nausea, headache, and dizziness; they are most common in children with a heavy infection.

Since the infection is acquired from the accidental ingestion of infected intermediate arthropod hosts, avoidance of this type of exposure is recommended. Possible situations include swallowing ectoparasites from the rodent host or accidentally ingesting beetles in precooked cereals. Rat control programs might also decrease the possibility of human exposure.

### Description (Adults)

The scolex has four suckers and no hooks. The adult worm is rarely seen in the stool. *H. diminuta* is larger than *H. nana*, reaching at least 1 m in single infections in rats and perhaps even more in humans. The eggs are released by disintegration of the gravid proglottids and pass out in the stool.

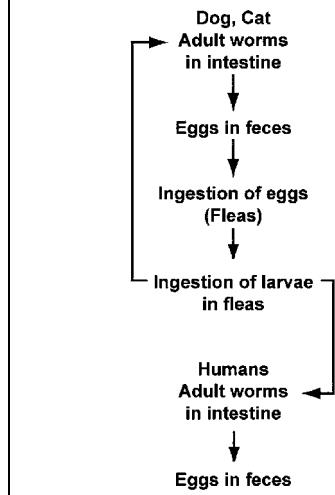
The worms are very small compared with *Taenia* worms and measure up to 1 m long. The more worms present, the shorter the total length of each worm.

## CESTODES • Intestinal

### *Dipylidium caninum*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Dog and cat tapeworm disease
<b>Acquired</b>	Ingestion of immature tapeworm larvae (cysticercoid) from infected arthropods
<b>Body site</b>	Intestine
<b>Symptoms</b>	Vague to none
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Worldwide
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated fruits and vegetables

#### LIFE CYCLE



### Diagnosis

Diagnosis depends on the recovery and identification of the characteristic egg packets or the proglottids. They are most easily identified in fresh specimens or those preserved in formalin-based fixatives. These egg packets also tend to collapse on the permanent stained smear and may be difficult to identify; the direct wet film or concentration wet mount is recommended. The proglottids look like white cucumber seeds when fresh and rice grains when dry.

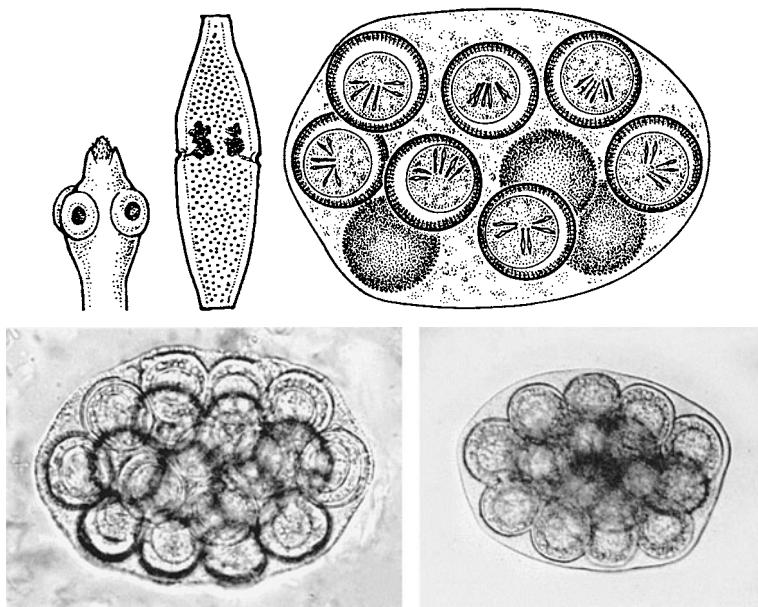
The standard O&P exam is recommended for recovery and identification of *D. caninum* eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. The eggs are most easily seen on a direct wet smear or a wet preparation of the concentration sediment.

### General Comments

*D. caninum* is common in dogs and cats, both domestic and wild, worldwide. Human infections have also been found in many areas of the world, including the United States. Most reported infections have been in children.

The life cycle is very similar to that of *H. diminuta*, in which the arthropod is an obligatory intermediate host. The adult worms are found in the dog or cat intestine, and gravid proglottids separate from the strobila and may migrate singly or in short chains out of the anus. The eggs are ingested by the larval stages of the dog, cat, or human flea, where they develop into cysticercoid larvae. When these fleas are ingested by the definitive host (dogs, cats, or humans), the adult worm develops within 3 to 4 weeks.

This infection is a true zoonosis, since infected dogs and cats infect fleas, which in turn are accidentally consumed by humans. Children may be more likely to accidentally swallow the infected fleas or may be more susceptible to infection. Periodic administration of antihelminthic agents to dogs and cats and use of flea powders will help reduce the risk of infection.



Images from left to right: (Top) The drawings depict the scolex, gravid proglottid, and egg packet of *D. caninum*. (Bottom) The photographs demonstrate the typical egg packets. Note the six-hooked embryo (oncosphere) and striated eggshells of the individual eggs within the egg packet. Individually, the eggs normally resemble *Taenia* eggs.

### Description (Eggs)

Groups of eggs (egg packets) may be found in the stool. Each egg measures 25 to 40  $\mu\text{m}$  and contains the six-hooked oncosphere. The individual eggs may closely resemble those of *Taenia* spp., particularly if they are released from the egg packet.

### Description (Adults)

The adult worms measure 10 to 70 cm long and have a scolex with four suckers and an armed rostellum. The single proglottids have been described as looking like cucumber seeds when moist and like rice grains when dry.

### Additional Information

The symptoms are related to the worm burden; however, in most patients (usually children) they consist of indigestion and appetite loss. Awareness of the infection may be due to the migration of proglottids from the anus.

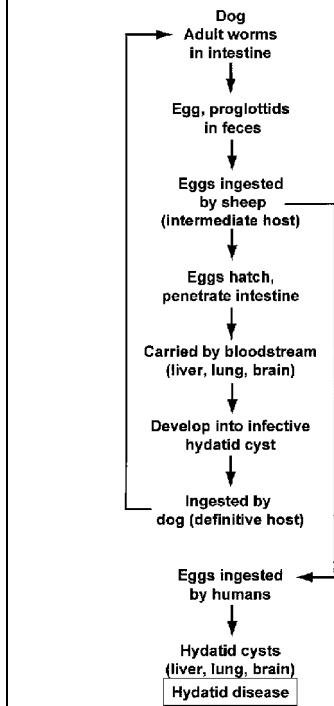
Most human cases have been in children, indicating that children may be more likely to accidentally swallow the infected fleas or may be more susceptible to infection. Periodic administration of anthelmintic agents to dogs and cats and use of flea powders will help reduce the risk of infection.

## CESTODES • Tissue

### *Echinococcus granulosus*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Hydatid disease; "sheep-sheepdog disease"
<b>Acquired</b>	Ingestion of eggs from the tape-worm in the dog's intestine
<b>Body site</b>	Hydatid cysts in liver, lungs, and other tissues
<b>Symptoms</b>	Vague to none
<b>Clinical specimen</b>	Aspirated fluid from hydatid cyst; routine histology
<b>Epidemiology</b>	Worldwide
<b>Control</b>	Improved adult dog tapeworm prophylaxis, no feeding of sheep entrails to dogs.

### LIFE CYCLE



### Diagnosis

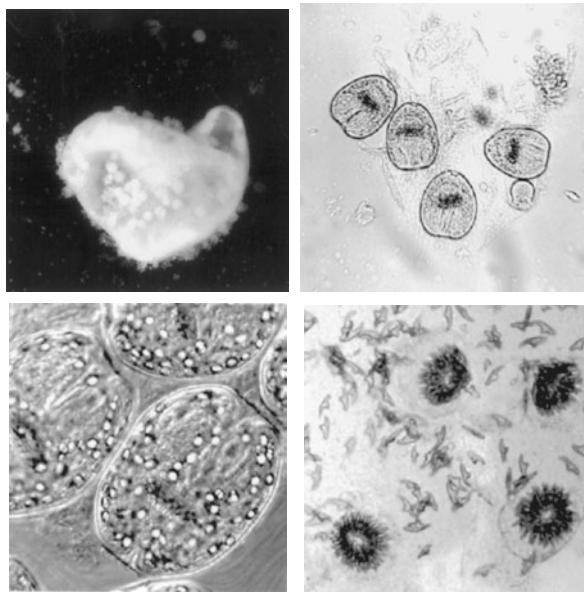
Hydatid cysts should be considered in patients with abdominal masses with no clearly defined diagnosis. Eosinophilia is present in 20 to 25% of patients but is merely suggestive. Many asymptomatic cysts are first discovered after radiologic studies. The cyst usually has a well-defined margin with occasional fluid level markings. These studies can also be helpful in diagnosing osseous involvement. Scans may also demonstrate a space-occupying lesion, particularly in the liver.

Serologic tests are available, including an enzyme-linked immunoelectrotransfer blot (EITB) test which apparently offers greater sensitivity and specificity than do the EIA and arc-5 double-diffusion assay (DD5); when the tests were run simultaneously, the greatest number of cases was detected by using a combination of the EITB and DD5 tests. Newer EIA procedures appear to provide greater than 90% sensitivity and specificity compared to the EITB.

### General Comments

Four species are recognized: *E. granulosus* (which causes cystic disease), *E. multilocularis* (which causes alveolar disease), *E. vogeli* (which causes polycystic disease), and *E. oligarthus* (which causes polycystic disease).

*E. granulosus* adult worms are very small (3 to 6 mm long) and consist of a scolex, neck, and only a single proglottid at each stage of development (immature, mature, and gravid). There may be several hundred worms in the intestine of the canine host (usually the dog). The worms may survive in the host for up to 20 months, and each gravid proglottid contains few eggs compared with some of the other, larger tapeworms. After the gravid proglottids and eggs are passed in the feces, they may be swallowed by an intermediate host, including humans, where they hatch in the duodenum. The released oncospheres penetrate the intestine and are carried via the bloodstream, where they are filtered out in the various organs. The most common site in humans is the liver (60 to 70% of cases). Usually by the fifth month, the wall of the hydatid cyst has become differentiated into an outer friable, laminated, nonnucleated layer and an inner nucleated germinal layer. Various daughter cysts (brood capsules) bud off from the inner germinal layer and may remain attached or float free in the interior of the fluid-filled cyst. The individual scolices bud off from the inner wall of the daughter cysts; these scolices and free daughter cysts are called hydatid sand. Each scolex normally invaginates to protect the hooklets. Although not every cyst produces daughter cysts and/or scolices, this general tissue organization is called a unilocular cyst, in which the cyst contents are held within a single limiting cyst wall.



Images from left to right: (Top) The photographs depict the hydatid cyst showing daughter scolices and the immature scolices (hydatid sand). (Bottom) Higher magnification of the immature scolices and the hooklets that remain after the scolices have disintegrated.

## Additional Information

Once the cyst is discovered and surgical removal is selected, some of the cyst fluid can be aspirated and submitted for microscopic examination to detect the presence of hydatid sand, confirming the diagnosis. This procedure is risky because of possible fluid and/or tissue leakage or dissemination. Cyst aspiration is usually performed at the time of surgery. Hydatid sand is not always present. Also, if the cyst is old, the daughter cysts and/or scolices may have disintegrated, so only the hooklets are left. These may be difficult to find and identify if the cyst contains debris.

If a drop of centrifuged fluid is placed on a slide, another slide is placed on top, and the two slides are rubbed back and forth over the fluid, the grating of the hooks on the glass may be felt and heard (hydatid sand sounds like glass grating on sand grains). If the individual scolices are intact, routine microscopic examination of the centrifuged fluid as a wet mount will confirm the diagnosis. If the cyst is sterile (no daughter cysts or scolices), the diagnosis could be confirmed histologically from the cyst wall.

Hydatid disease in humans is potentially dangerous; however, size and organ location greatly influence the outcome. Most hydatid cysts reside in the liver, causing symptoms including chronic abdominal discomfort, occasionally with a palpable or visible abdominal mass. If cysts are in a vital area or bone (osseous), even relatively small cysts can cause severe damage. Some unilocular cysts remain undetected for many years until they become large enough to crowd other organs. Cysts in the lungs

are usually asymptomatic until there is cough, shortness of breath, or chest pain.

During the life of the cyst, there may be small fluid leaks into the systemic circulation that sensitize the patient. Later, if the cyst bursts or there is a large fluid leak, serious allergic sequelae, including anaphylactic reactions, may occur. Release of cyst tissue may lead to abscess formation, emboli, and/or the development of additional young cysts at secondary sites.

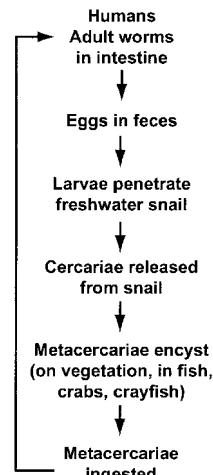
**Note:** The percentage of infected hosts varies throughout the world, but human infection is still much less common than infection of any of the reservoir hosts. The risk of infection depends on the association between humans and dogs. Those at high risk include populations where dogs are used to herd sheep and are also intimate members of the family, often having unrestricted access to the house and family members. Cystic echinococcosis has been recorded in 21 of China's 31 provinces, autonomous regions, and municipalities (approximately 87% of the territory). Hydatid disease caused by *E. granulosus* is a zoonosis of major public health concern throughout Latin America, particularly in the Andean and South Cone regions. It is also widely found throughout Arab North Africa and the Middle East. In areas of endemic infection around the world, the practice of giving raw viscera of slaughtered livestock to the dogs enhances transmission; however, in areas where this practice has been curtailed, prevalence figures have decreased.

## TREMATODES • Intestinal

### *Fasciolopsis buski*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Fasciolopsiasis, giant intestinal-fluke disease
<b>Acquired</b>	Ingestion of infective metacercariae encysted on plant material
<b>Body site</b>	Intestine
<b>Symptoms</b>	None to abdominal pain and diarrhea
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Bangladesh, Cambodia, central and southern China, India, Indonesia, Laos, Malaysia, Pakistan, Taiwan, Thailand, and Vietnam; human-to-human and animal-to-human transmission (dogs, pigs, rabbits)
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated plant material

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *Fasciolopsis* eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. The eggs are detected in feces by direct microscopy or by concentration techniques. Infrequently, adult worms are detected in the stool in very heavy infections or during therapy.

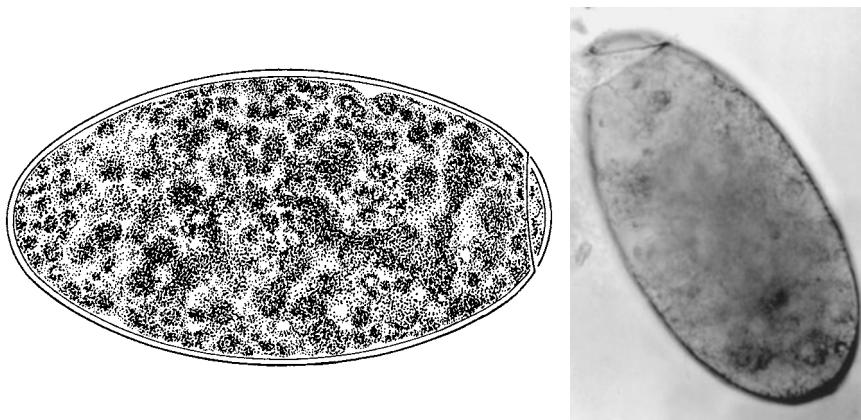
The zinc sulfate flotation method is not recommended because of the operculum and the fragility of the eggshell. In addition to the surface material, one would have to examine the sediment because the eggs sink to the bottom. The formalin-ether sedimentation technique of Ritchie is recommended instead. The sedimented material can be examined with or without iodine. The eggs are brownish yellow and may contain fully formed miracidia, depending on the species.

The eggs are ellipsoidal, operculate, and yellow-brown. They measure 130 to 140 µm by 80 to 85 µm, with the operculum found at the more pointed end of the transparent eggshell. Humans become infected by ingesting the raw or undercooked plants containing the metacercariae. The metacercariae excyst, attach to the duodenal or jejunal mucosa, and develop into an adult worm within 3 months. The adult life span seldom exceeds 6 months to 1 year.

The eggs of *Echinostoma ilocanum*, *Fasciola hepatica*, *F. buski*, and *Gastroplocooides hominis* are similar in size and shape; therefore, exact diagnosis cannot be made from examining the eggs. It is possible to detect adult worms in the stool in heavy infections when they lose their ability to remain attached to the intestinal mucosa.

### General Comments

Eggs deposited by the adult worms are passed in the feces. They contain immature miracidia when passed in the stool and require a period for embryonation in the outside environment. All eggs of intestinal trematodes have an operculum, or cap, from which the miracidium can escape. *Fasciolopsis* eggs hatch in freshwater to release a free-swimming miracidium larva that must find the snail host to which it is adapted or die. Once the miracidium has penetrated the snail's soft tissues, it begins to develop into a first-generation sporocyst (an elongated sac without distinct internal structures in which germ balls proliferate). These germ balls develop into rediae that contain a mouth, pharynx, blind cecum, and birth pore. Within the rediae, the germ balls again proliferate, developing into cercariae. On reaching maturity, the cercariae escape from the snail host into the water. *Fasciolopsis* rediae develop a second generation of rediae before forming cercariae. The cercariae are free-living in the water, and they must find their next intermediate host or die. When the appropriate host or vegetation is found, the cercariae lose their tails and encyst (becoming metacercariae). *Fasciolopsis* cercariae encyst on aquatic vegetation. On ingestion of uncooked vegetation or of raw or inadequately cooked mollusks or fish, the metacercariae excyst in the small intestine and develop into mature hermaphroditic adults in the intestinal tract.



**Images from left to right:** Drawing and photograph of *F. buski* egg. The operculum blends into the shell; there are no opercular shoulders into which the operculum fits. In the photograph, the operculum has popped open. This egg and that of several other trematodes, including *F. hepatica*, look almost identical and cannot be differentiated visually.

## Description

The eggs of *E. ilocanum*, *F. hepatica*, *F. buski*, and *G. hominis* are similar in size and shape, so exact identification cannot be made from examining the eggs. Adult worms can be found in the stool in heavy infections, when they can no longer remain attached to the intestinal mucosa.

Since the eggs of *Fasciola* and *Fasciolopsis* are so similar in size and shape, if either is found the report can indicate that the two trematodes cannot be separated into appropriate genera on the basis of egg morphology.

## Additional Information

*F. buski* reservoir hosts include dogs, pigs, and rabbits. The infection is common in Bangladesh, Cambodia, central and southern China, India, Indonesia, Laos, Malaysia, Pakistan, Taiwan, Thailand, and Vietnam; it has also been found in Japan. Drainage of farm waste, use of manure for cultivation, and defecation in or near ponds or lakes that contain snails from the family Planorbidae, with water plants acting as vectors, support the life cycle. Metacercariae encyst on freshwater vegetation such as water chestnuts, bamboo shoots, or water caltrops, and the infection is acquired when these infested plants are consumed raw or the outer coat is peeled off the nut with the teeth, resulting in accidental ingestion. To prevent infection, plants should be cooked or immersed in boiling water for a few seconds before being eaten or peeled. In areas of endemicity, the use of unsterilized night soil for fertilizer should be prohibited.

The disease occurs focally and is most prevalent in school-age children. In areas of endemic infection, the prevalence in children ranges from 57% in mainland China to 25% in Taiwan and from 50% in Bangladesh and 60% in India to 10% in Thailand. Control programs are not fully successful because of long-standing traditions of eating raw aquatic plants and using untreated water.

In light infections, the adults inhabit the duodenum and jejunum; in heavy infections, they reside in the stomach and most of the intestinal tract. The attachment of worms to the mucosal wall produces local inflammation with hypersecretion of mucus, hemorrhage, ulceration, and possible abscess formation. In heavy infections, the worms may cause bowel obstruction, acute ileus, and absorption of toxic or allergic worm metabolites, producing general edema and ascites. A marked eosinophilia and leukocytosis are common. Few symptoms are associated with light infections, but in heavier infections the patient may experience abdominal pain and diarrhea. In heavy infections, the stools are profuse and yellow-green and contain increased amounts of undigested food, suggesting a malabsorption process. The disease can be fatal, depending on the worm burden.

## TREMATODES • Liver and Lungs

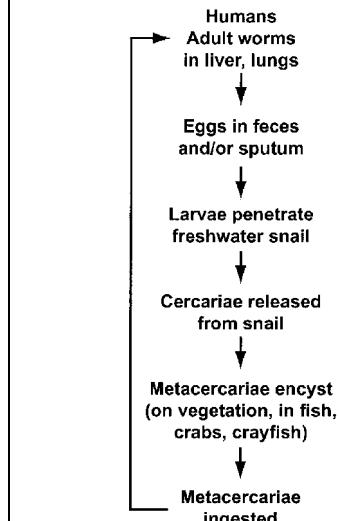
### *Paragonimus westermani*

### *Paragonimus mexicanus*

### *Paragonimus kellicotti*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Paragonimiasis
<b>Acquired</b>	Ingestion of encysted metacercariae in fish, crabs, and crayfish
<b>Body site</b>	Lungs, extrapulmonary sites
<b>Symptoms</b>	None to chronic cough, vague chest pains, hemoptysis
<b>Clinical specimen</b>	<i>Intestinal:</i> Stool <i>Lung:</i> Sputum
<b>Epidemiology</b>	Far East; North, Central, and South America; human-to-human and animal-to-human transmission (dogs, cats)
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate cooking of fish, crabs, crayfish

#### LIFE CYCLE



### Diagnosis

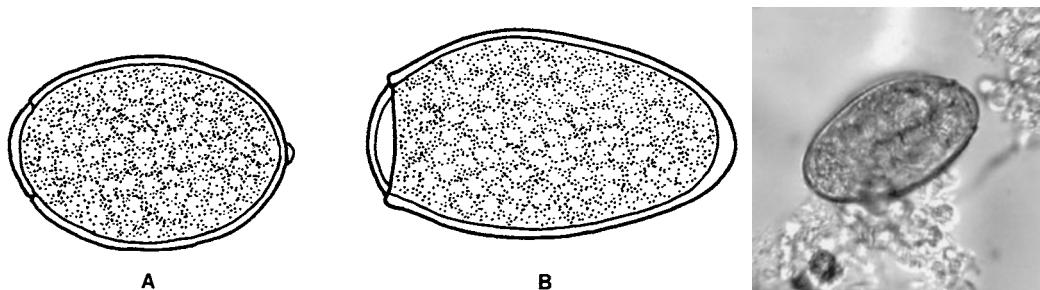
Individuals with symptoms of chronic cough, vague chest pains, and hemoptysis who have resided in an area where infections are endemic and have a history of eating raw crayfish or crabs should be suspected of having paragonimiasis. *Paragonimus* eggs can be detected in sputum and in stool; both specimens should be tested to improve the overall detection rate. Small numbers of eggs are present intermittently in the sputum and feces. For light infections, up to seven sputum examinations have been recommended. Frequently, pulmonary paragonimiasis is misdiagnosed as pulmonary tuberculosis. The Ziehl-Neelsen method for detecting mycobacteria destroys the eggs of *Paragonimus*. The typical findings of cough, hemoptysis, and eggs in the feces or sputum may be absent in patients with ectopic or pleural infection.

Skull films generally reveal round or oval cystic calcifications, described as looking like bubbles. CT scans reveal multilocular cysts with edema, migration tracks, peripheral density, bronchial wall thickening, and centrilobular nodules; ring enhancement may be visible after the use of contrast. MRI demonstrates areas of granulomatous inflammation surrounding the lesions; MRI of the brain may reveal multiple conglomerated iso- or low-signal intensity round nodules with peripheral rim enhancement. CT and MRI of the liver may reveal a cluster of small cysts with rim enhancement in the subcapsular area. Solitary nodular lesions often mimic lung cancer, tuberculosis, or fungal diseases. When a pulmonary mass lesion or empyema is detected in patients who live in areas of endemic infection, paragonimiasis should always be included in the differential diagnosis.

Immunodiagnostic tests have been used to diagnose pulmonary and extrapulmonary infections. These serologic assays are available in areas of endemicity or in specialized diagnostic centers.

### General Comments

The adult worm is a plump, ovoid, reddish brown fluke found encapsulated in the lung. Eggs deposited by the worms are ovoid, brownish yellow, unembryonated, and thick shelled, with an operculum at one end and opercular shoulders. Eggs measure 80 to 120  $\mu\text{m}$  by 45 to 65  $\mu\text{m}$ . *P. westermani* eggs are often confused with *Diphyllobilium* eggs because both are operculated, unembryonated, and somewhat similar in size. *P. westermani* eggs have opercular shoulders and a thickened shell at the abopercular end. Eggs escape from the encapsulated tissue through the bronchioles, are coughed up and voided in the sputum, or are swallowed and passed out in the feces. They hatch in water in 2 to 3 weeks, releasing a miracidium to infect a susceptible snail host. Cercariae are released after sporocyst and rediae generations. Crabs and crayfish are infected by cercariae via the gill chamber or on ingestion of an infected snail. Cercariae encyst in the gill vessels and muscles. Humans are infected by ingesting uncooked crabs or crayfish containing metacercariae. The metacercariae excyst in the duodenum and migrate through the intestinal wall into the abdominal cavity. The larvae migrate around or through the diaphragm into the pleural cavity and the lungs. The larvae mature to adults in the vicinity of the bronchioles, where they discharge their eggs into the bronchial secretions.



Images from left to right: The drawings on the left show *D. latum* (A) and *P. westermani* (B). Although the eggs are different sizes, there is some similarity between the two. The photograph shows *P. westermani*. Note the opercular shoulders visible with *P. westermani*. It is critical that these eggs be measured carefully, including specimens used for proficiency testing.

## Description

*Paragonimus* and *D. latum* eggs are similar in size and shape. Remember to look for the opercular shoulders and thickening at the abopercular end of the *Paragonimus* eggs. *D. latum* eggs do have an operculum but no opercular shoulders. Also, there is a "knob" at the abopercular end in *D. latum* eggs, although it is often difficult to see.

Since the eggs of these two trematodes mentioned above are so similar in size and shape, it is important to measure the eggs and review several in order to assign the eggs to the correct genus.

**Note:** The most serious consequence of paragonimiasis is cerebral complications, which are commonly found in younger age groups. Most patients with extrapulmonary lesions have an associated lung lesion or a history of lung disease. Symptoms include fever, headache, nausea, vomiting, visual disturbances, motor weakness, localized or generalized paralysis and possibly death.

Pulmonary paragonimiasis is rarely fatal; however, cerebral disease is characterized by chronic morbidity and symptoms including epilepsy, dementia, and other neurologic sequelae. About 5% of patients with cerebral disease die due to hemorrhage in the first 2 years of the disease.

Other body sites that have been infected include the breast, lymph nodes, heart, pericardium, mediastinum, kidney, adrenal gland, omentum, bone marrow, stomach wall, bladder, spleen, pancreas, and reproductive organs. While ectopic lesions are usually thought to be caused by worm migration, dissemination of eggs to other body sites may also be responsible.

## Additional Information

Migration of larval forms through the intestinal wall into the abdominal cavity is generally not associated with symptoms. Once the larvae have reached the peritoneal cavity, they migrate through organs and tissues, producing localized hemorrhage and leukocytic infiltrates. When they reach the lungs and mature, a pronounced tissue reaction occurs with infiltration of eosinophils and neutrophils. A fibrotic capsule forms around the worm. The cysts contain purulent fluid with flecks or "iron filings" composed of brownish yellow eggs. Many cysts perforate into the bronchioles, releasing their contents of eggs, necrotic debris, metabolic by-products, and blood into the respiratory tract. The eggs may also enter the pulmonary tissue or be carried by the circulatory system to other body sites, where they cause a granulomatous reaction. Larval forms may end up in many ectopic sites other than the lungs. Cysts have been detected in the liver, intestinal wall, muscles, brain, and peritoneum.

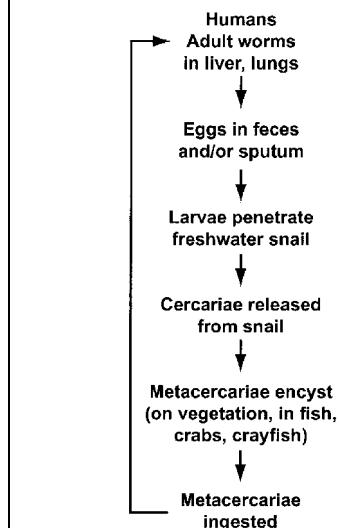
Symptoms of paragonimiasis depend largely on the worm burden of the host and are usually insidious in onset and mild in chronic cases. Light infections may be asymptomatic, although peripheral blood eosinophilia and lung lesions may be noted on X-ray. As the cyst ruptures, a cough develops with increased production of viscous blood-tinged sputum (rusty sputum which may have a foul fish odor) and increasing chest pain. The patient may experience increasing dyspnea with chronic bronchitis and be misdiagnosed as having tuberculosis. There will generally be a moderately high peripheral blood eosinophilia and leukocytosis with elevated serum IgG and IgE.

## TREMATODES • Liver and Lungs

### *Fasciola hepatica*

<b>Pathogenic</b>	Yes
<b>Disease</b>	Fascioliasis
<b>Acquired</b>	Ingestion of infective metacercariae encysted on plant material
<b>Body site</b>	Bile ducts
<b>Symptoms</b>	None to abdominal pain and diarrhea
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	Bolivia, Ecuador, Egypt, France, Iran, Peru, and Portugal; human-to-human and animal-to-human transmission (cattle, goats, sheep)
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate washing of contaminated plant material

#### LIFE CYCLE



### Diagnosis

The standard O&P exam is recommended for recovery and identification of *F. hepatica* eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. The eggs are detected in feces by direct microscopy or by concentration techniques. Infrequently, adult worms are detected in the stool in very heavy infections or when the patient is undergoing therapy.

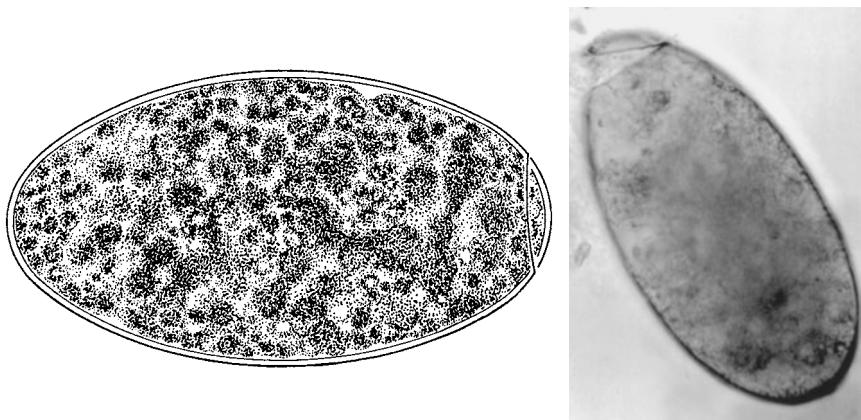
The zinc sulfate flotation method is not recommended because of the operculum and the fragility of the eggshell. In addition to the surface material, one would have to examine the sediment because the eggs sink to the bottom. The formalin-ether sedimentation technique of Ritchie is recommended instead. The sedimented material can be examined with or without iodine. The eggs appear brownish yellow and may contain fully formed miracidia, depending on the species.

The eggs are unembryonated, operculated, large, ovoid, and brownish yellow and measure 130 to 150  $\mu\text{m}$  by 63 to 90  $\mu\text{m}$ . Humans become infected by ingesting raw or undercooked plants containing the metacercariae. The metacercariae excyst, attach to the duodenal or jejunal mucosa, and develop into adult worms within 3 months. The adult worms can grow to 30 mm long and 13 mm wide and can live for more than 10 years.

### General Comments

Adult worms, which may live for 9 years in the bile ducts, produce eggs that are carried by the bile fluid into the intestinal lumen and passed into the environment with the feces. The miracidium develops within 1 to 2 weeks and escapes from the egg to infect the snail intermediate host, *Lymnaea* sp. Cercariae are liberated from the snail after a sporocyst generation and two or three rediae generations. Cercariae encyst on water vegetation, e.g., watercress. Humans are infected by ingesting this vegetation raw. Metacercariae excyst in the duodenum and migrate through the intestinal wall into the peritoneal cavity. Larvae enter the liver by penetrating the capsule (Glisson's capsule) and inhabit the liver parenchyma for up to 9 weeks; they finally enter the bile ducts, where they mature and produce eggs, which are passed out in feces.

The incubation period ranges from a few days to a few months. Symptoms reflect the phase of the infection, as well as the number of parasites in the host. In the acute phase, symptoms may occur for weeks to months. In the more chronic phases of the disease, the patient generally has few to no symptoms once the flukes have lodged in the biliary passages. Other body sites include the intestinal wall, lungs, heart, brain, and skin. Symptoms mimic those seen with visceral larva migrans and include vague abdominal pain.



**Images from left to right:** Drawing and photograph of *F. hepatica* egg. The operculum blends into the shell; there are no opercular shoulders into which the operculum fits. In the photograph, the operculum has popped open. This egg and that of *F. buski* (and several other trematodes) look almost identical and cannot be differentiated visually.

## Description

*E. ilocanum*, *F. hepatica*, *F. buski*, and *G. hominis* eggs are similar in size and shape, so exact identification cannot be made from examining the eggs. Adult worms can be found in the stool in heavy infections, when they can no longer remain attached to the intestinal mucosa.

Since *Fasciola* and *Fasciolopsis* eggs are so similar, if either is found the report can indicate that the two trematodes cannot be separated into appropriate genera on the basis of egg morphology. Multiple stool examinations may be needed to detect light infections.

Fascioliasis is primarily a zoonotic disease involving liver infections with adult flukes. In areas of endemicity where uncooked goat and sheep livers may be eaten, such as Lebanon, adult worms may attach to the pharyngeal mucosa, causing suffocation (halzoun syndrome). This condition is temporary, although distressing. The adult worms may lodge on the pharyngeal mucosa, causing edema and congestion of the soft palate, pharynx, larynx, nasal fossae, and eustachian tube. Symptoms include dyspnea, dysphagia, deafness, and occasionally suffocation. Some cases may be caused by infection with larval linguatulids, rather than adult worms of *F. hepatica*.

Eggs may be detected in the stool of individuals who have eaten *F. hepatica*-infected liver, yielding an erroneous laboratory result (a "spurious" infection). True and spurious infection can be distinguished by giving the patient a liver-free diet for at least 3 days. If the patient continues to pass eggs in the stool, the infection is probably genuine.

## Additional Information

The degree of damage depends on the worm burden. Linear lesions of 1 cm or greater can be found. Hyperplasia of the bile ducts occurs, possibly as a result of toxic products produced by the larvae. Symptoms associated with this migratory phase include fever, epigastric and right upper quadrant pain, and urticaria, while some patients remain asymptomatic. Leukocytosis, eosinophilia, and mild to moderate anemia occur in many patients. Levels of IgG, IgM, and IgE in serum are usually elevated. In sheep, the migratory phase produces such extensive liver parenchyma damage that the disease is known as liver rot.

Larvae may be found in ectopic foci after penetrating the peritoneal cavity. Worms in human infections have been discovered in many areas of the body other than the liver.

Once the worms are established in the bile ducts and have matured, they produce considerable damage from mechanical irritation and metabolic by-products as well as obstruction. The degree of pathology depends on the number of flukes penetrating the liver.

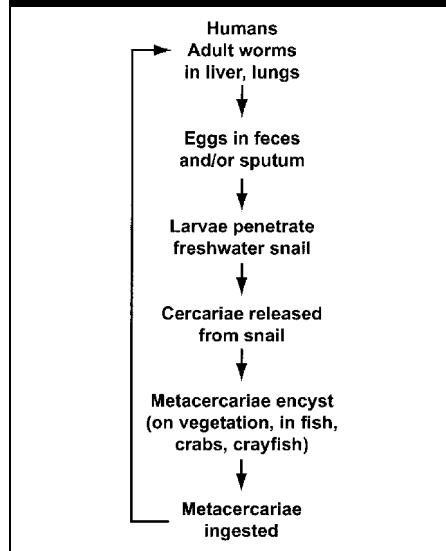
The infection produces hyperplasia of the biliary epithelium and duct fibrosis with portal or total biliary obstruction. The gallbladder undergoes similar damage and may even harbor adult worms. Adult worms may reinvade the liver parenchyma, producing abscesses.

## TREMATODES • Liver and Lungs

### *Clonorchis sinensis* (*Opisthorchis sinensis*)

<b>Pathogenic</b>	Yes
<b>Disease</b>	Clonorchiasis
<b>Acquired</b>	Ingestion of infective metacercariae encysted in raw or poorly cooked freshwater fish
<b>Body site</b>	Bile duct and liver
<b>Symptoms</b>	None to acute pancreatitis, cholecystitis, and choledolithiasis (may be the result of worm invasion); biliary tract obstruction
<b>Clinical specimen</b>	Stool
<b>Epidemiology</b>	China, Japan, Korea, Malaysia, Singapore, Taiwan, and Vietnam; human-to-human and animal-to-human transmission (dogs, cats, fish-eating mammals)
<b>Control</b>	Improved hygiene, adequate disposal of fecal waste, adequate cooking of freshwater fish

#### LIFE CYCLE



#### Diagnosis

The standard O&P exam is recommended for recovery and identification of *C. sinensis* eggs in stool specimens, primarily from the wet preparation examination of the concentration sediment. The eggs are detected in feces by direct microscopy or concentration techniques. Infrequently, adult worms are detected in the stool in very heavy infections or during therapy.

The zinc sulfate flotation method is not recommended because of the operculum and the fragility of the eggshell. In addition to the surface material, one would have to examine the sediment because the eggs sink to the bottom. The formalin-ether sedimentation technique of Ritchie is recommended instead. The sedimented material can be examined with or without iodine. The eggs appear brownish yellow and may contain fully formed miracidia, depending on the species.

*C. sinensis* and *O. viverrini* eggs are fully embryonated when laid and measure 28 to 35  $\mu\text{m}$  by 12 to 19  $\mu\text{m}$ . They are ovoid, with a thick, light brownish yellow shell and an operculum. There are distinct opercular shoulders surrounding the operculum. The eggs are similar in size and shape to those of *H. heterophyes* and *M. yokogawai* and cannot be readily differentiated. If a patient has not resided in or recently visited areas where infections are endemic, the infection is probably due to *C. sinensis* or *O. viverrini*. The infection may be confirmed by detecting eggs in the bile fluid (duodenal aspirate), by recovering adult worms, or from the clinical history. Some strains produce eggs with a comma-shaped appendage at the abopercular end. Multiple egg measurements are usually required to determine size differences, but absolute identification of the small trematode eggs can be very difficult.

In patients with biliary obstruction, eggs are not found in the stool specimens; needle aspiration, surgery, or au-

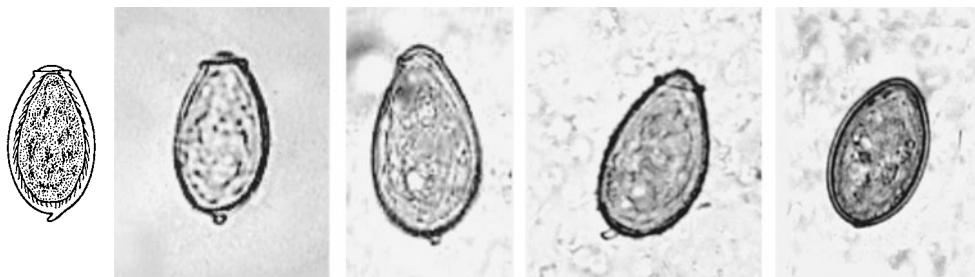
topsy specimens may be required to confirm their presence. In these patients, biliary obstruction must be differentiated from enlarged gallbladder, cholangitis with jaundice, liver carcinoma, and cholangiocarcinoma. Cholangiography, ultrasonography, and liver scans may reveal lesions consistent with infection.

#### General Comments

The infections caused by the liver and lung trematodes are food borne and have considerable economic and public health impact. More than 50 million people have acquired food-borne trematode infections. Approximately 601, 293, 91, and 80 million people are at risk of infection with *C. sinensis*, *Paragonimus* spp., *Fasciola* spp., and *Opisthorchis* spp., respectively. Of great public health concern is cholangiocarcinoma associated with *Clonorchis* and *Opisthorchis* infections, severe liver disease associated with *Fasciola* infections, and the misdiagnosis of tuberculosis in those infected with *Paragonimus* spp.

Adult *Clonorchis* worms deposit eggs in the bile ducts, and the eggs are discharged with the bile fluid into the feces and passed out into the environment. Eggs are ingested by the snail host, and the miracidium hatches to infect the snail. Sporocyst and rediae generations are produced before cercariae are released to encyst in the skin or flesh of freshwater fish. Humans are infected by ingesting the metacercariae in uncooked fish.

Metacercariae excyst in the duodenum, enter the common bile duct, and travel to the distal bile capillaries, where the worms mature. The life cycle takes approximately 3 months in humans.



**Images from left to right:** The drawing and first three eggs are those of *C. sinensis*, while the photograph of the egg on the right is another similar egg, either *H. heterophyes* or *M. yokogawai*. Differentiation of these three trematodes can be difficult.

## Description

The eggs of *C. sinensis*, *H. heterophyes*, and *M. yokogawai* are similar in size and shape, so exact identification may not be possible from examining the eggs. Adult worms can be found in the stool in heavy infections when they can no longer remain attached to the intestinal mucosa.

Since the eggs of the three trematodes mentioned above are so similar, if any of the three are found, the report can indicate that they cannot be separated into appropriate genera on the basis of egg morphology.

**Note:** Light infections generally cause no symptoms. In heavier infections, the patient may experience dull pain and abdominal discomfort that may last 1 to 2 h, often in the afternoon. As the disease progresses, the pain persists and may become so severe that the patient is unable to work. Patients who have had the disease for a long time have liver enlargement with some degree of functional impairment secondary to biliary obstruction. Acute infections caused by ingestion of large numbers of metacercariae will, within a month, cause fever, chills, diarrhea, epigastric pain, enlarged tender liver, and possibly jaundice. The acute symptoms last for about 1 month and subside at about the time eggs are detected in the stool.

Infection can be prevented by thorough cooking of all freshwater fish. Night soil used without disinfection for fertilizer should not be applied in lakes or ponds containing susceptible snails; night soil should be stored prior to use since *C. sinensis* eggs die within 2 days when stored at 26°C.

## Additional Information

As the worms mature, an inflammatory response is seen in the biliary epithelium, related to the intensity and duration of infection. Lesions are due to mechanical irritation and toxic products. In light infections, there is little or no change in liver parenchyma, while in heavy infections there is thickening and localized dilations of the bile ducts with hyperplasia of the mucinous glands. Biliary tract obstruction causes bile retention, infiltration of lymphocytes and eosinophils, and fibrosis. Many patients have recurrent pyogenic cholangitis. Acute pancreatitis, cholecystitis, and cholelithiasis may be due to worm invasion. Cirrhosis is probably related to malnutrition. Serum IgE and *C. sinensis*-specific IgE levels are elevated in infected individuals. In acute infections there is an increase in the levels of IgM followed by IgA and IgG. In chronic infections, the IgA level returns to normal while the IgG and IgM levels remain elevated.

*C. sinensis* has been linked to neoplasms of the bile duct or cholangiocarcinoma, which are usually seen in areas where clonorchiasis is endemic. There was no direct link between infection and carcinoma, but one of the first steps in malignant transformation may be induced by the biliary tract hyperplasia caused by the worms.

Identification of patients with deteriorating liver function before cholangiocarcinoma develops remains an important goal in the management of primary sclerosing cholangitis, particularly in areas where *C. sinensis* infection is endemic. Cofactors may play a role; liver flukes are promoters and not initiators of cholangiocarcinoma.

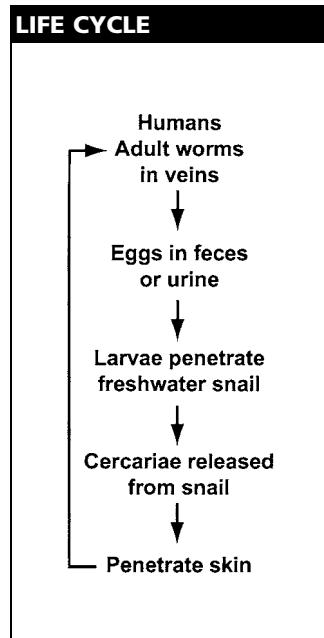
## Aquaculture

There has been a dramatic increase in aquaculture of fish from 5.3% in 1970 to 32.2% in 2000. Freshwater fish production now accounts for 45.1% of the total aquaculture production. The increased production of grass carp, an important species that serves as an intermediate host for food-borne trematodes, has increased from >10,000 tons in 1950 to >3 million tons in 2002. Growth of this industry must be monitored for potential problems related to increased disease in which infection is transmitted through the ingestion of raw or poorly cooked fish.

## TREMATODES • Blood

### *Schistosoma* spp. (*S. mansoni*, *S. haematobium*, *S. japonicum*, *S. mekongi*, *S. intercalatum*)

<b>Pathogenic</b>	Yes
<b>Disease</b>	Schistosomiasis
<b>Acquired</b>	Skin penetration by cercariae released from freshwater snails
<b>Body site</b>	Veins overlying intestine/bladder
<b>Symptoms</b>	Cercarial dermatitis, high fever, hepatosplenomegaly, lymphadenopathy, eosinophilia, and dysentery
<b>Clinical specimen</b>	Intestinal: Stool Bladder: Urine
<b>Epidemiology</b>	<i>S. mansoni</i> : western and central Africa, Egypt, Malagasy, Arabian peninsula, Brazil, Suriname, Venezuela, West Indies <i>S. japonicum</i> : Far East, China, Indonesia, Japan, Philippines <i>S. haematobium</i> : Africa, Asia Minor, Cyprus, islands off Africa's east coast, southern Portugal; focus in India. <i>S. mekongi</i> : Mekong River basin in Kampuchea, Laos, and Thailand
<b>Control</b>	Improved hygiene, adequate disposal of fecal and urine waste, snail control



### Diagnosis

The standard O&P exam is recommended for recovery and identification of schistosome eggs in stool and urine specimens, primarily from the wet preparation examination of the concentration sediment. Spot urines and 24-h urines should be examined, as well as several stools.

*S. mansoni* eggs are yellow-brown (114 to 180 µm long by 45 to 73 µm wide), elongate and ovoid, and have a large lateral spine projecting from the egg near one end. *S. haematobium* eggs are yellow-brown and have a distinct terminal spine (112 to 170 µm by 40 to 70 µm). *S. japonicum* eggs are more spherical (55 to 85 µm by 40 to 60 µm). A minute lateral spine occurs at one end in some strains. *S. mekongi* eggs are similar in shape to *S. japonicum* eggs but smaller. They are subspherical (30 to 55 µm by 50 to 65 µm) and have a small lateral spine near one end.

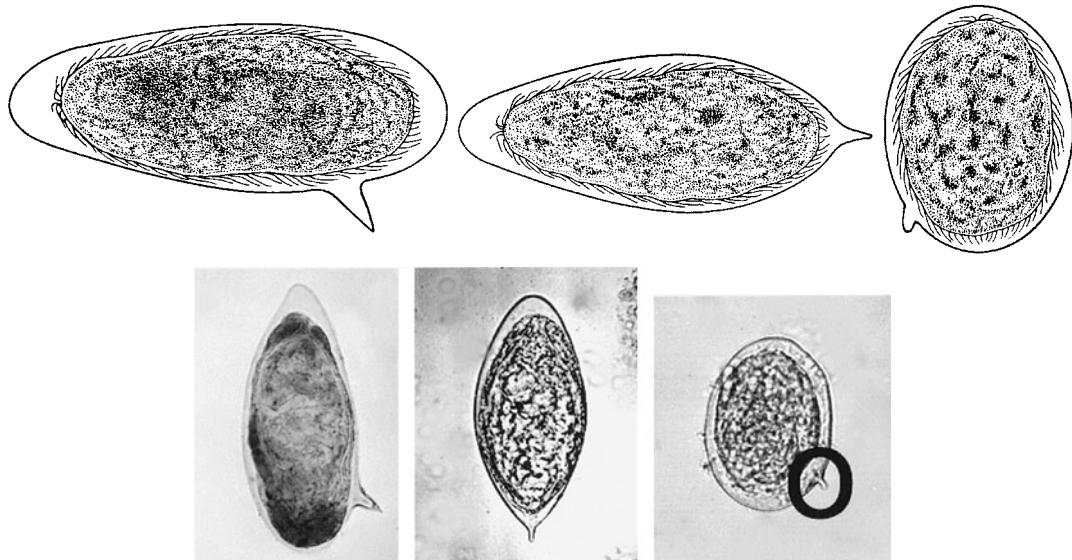
**Note:** All specimens must be collected without preservatives. It is important to be able to distinguish living from dead eggs. Do not use the zinc sulfate flotation concentration; eggs do not float.

### General Comments

Humans are the definitive host for *S. mansoni*, *S. japonicum*, *S. haematobium*, *S. mekongi*, *S. malayi*, and *S. intercalatum*. *S. mattheei*, which causes infections in sheep, cattle, and horses, also infects humans and can cause disease.

Humans are infected by penetration of cercariae through intact skin. Cercariae contain glands whose material is used to penetrate skin and a bifurcated tail that is lost when the cercariae penetrate the skin. After entry, the organism is termed a schistosomulum; it migrates through the tissues and finally invades a blood vessel, where it is carried to the lungs and then the liver. Once within the liver sinusoids, the worms mature into adults. *S. haematobium* adults are found primarily in the blood vessels of the vesical, prostate, and uterine plexuses. *S. mansoni* and *S. japonicum* adults are found in the inferior and superior mesenteric veins.

Egg deposition occurs in the small venules of the intestine and rectum (*S. japonicum* and *S. mansoni*) or the venules of the bladder (*S. haematobium*). A mature female worm produces 300 to 3,000 eggs per day depending on species. The eggs are immature when first laid and take ca. 10 days to develop a mature miracidium. Egg deposition takes place intravascularly, and the eggs work their way through the tissues either into the lumen of the bladder and urethra (*S. haematobium*) or the intestine (*S. mansoni* and *S. japonicum*) to be released from the body in the urine or feces.



Images from left to right: The eggs on the left with the large lateral spines are *S. mansoni*, those in the middle with the terminal spines are *S. haematobium*, and those on the right with the very small lateral spines are *S. japonicum*.

### Additional Information

Eggs become trapped in the fine venules and are able to pass through the tissues, escaping into the intestine or bladder. They liberate soluble antigens, evoking minute abscesses which facilitate their passage into the lumen. When they pass through the wall of the intestine or bladder, symptoms include fever, abdominal pain, liver tenderness, urticaria, and general malaise. In *S. haematobium* infections, there may be hematuria at the end of micturition and possibly dysuria; in *S. japonicum* and *S. mansoni* infections, blood and mucus occur in the stools, and the patient may have diarrhea or dysentery.

Although many eggs remain where deposited, others are swept into the circulation and filtered out in the liver, leading to hepatosplenic schistosomiasis. Hepatosplenomegaly is common in chronic *S. japonicum* and *S. mansoni* infections but less pronounced in *S. haematobium* infections. In some areas, *S. mansoni*, *S. japonicum*, and viral hepatitis are the most common cause of chronic liver disease.

**Note:** Egg viability can be determined by using the hatching test and/or seeing the cilia moving on the flame cells within the miracidium larva in the eggshell. Multiple stool or urine examinations should be performed for any individual suspected of having schistosomiasis. Occasionally, *S. mansoni* eggs are detected in the urine; adult worms may be found in vessels that are not their normal habitat, and this finding is known as crossover.

In active infections, eggs should contain live or mature miracidia. Examination to confirm flame cell activity must be done on fresh specimens using the microscopic wet mount or the hatching test; no preservatives can be used prior to the wet mount test or the hatching test.

Schistosomiasis affects 200 million to 300 million people in 77 countries and is a significant cause of disease in endemic areas. In Egypt, approximately 20% of the population is infected; prevalence rates in some villages are ca. 85%. In China, there are 1.52 million infected individuals. About 10% of infected people have serious disease; this represents 20 million to 30 million individuals worldwide. About half of the 180 million to 270 million infected individuals have symptoms.

Schistosomiasis symptoms are related to the stage of infection, previous host exposure, worm burden, and host response. Syndromes include cercarial dermatitis, acute schistosomiasis (Katayama fever), and tissue changes resulting from egg deposition.

Cercarial dermatitis follows skin penetration by cercariae and may partly be due to previous host sensitization. Few symptoms are associated with primary exposure, but both humoral and cellular immune responses are elicited later. After cercarial skin penetration, petechial hemorrhages with edema and pruritus occur. The subsequent maculopapular rash, which may become vesicular, may last 36 h or more. Cercarial dermatitis is more common with *S. haematobium* and *S. mansoni* infections. Dermatitis is a constant feature of human infection with avian schistosomes, with cercarial death occurring in the subcutaneous tissues and immediate hypersensitivity reactions at the invasion sites. Previous contact with cercariae leads to a more immediate intense immune response.

Clinical features of acute infection are high fever, hepatosplenomegaly, lymphadenopathy, eosinophilia, and dysentery. In chronic disease, symptoms include fever, abdominal pain, liver tenderness, urticaria, and general malaise.

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**S E C T I O N    8**

**Identification Aids**

**Table 8.1** Diagnostic characteristics for organisms in wet mounts

Specimen	Protozoa	Helminths
Stool, other specimens from gastrointestinal tract or urogenital system	Size, shape, stage (trophozoite, precyst, cyst, oocyst); motility (fresh specimens only), refractivity, cytoplasm inclusions (chromatoidal bars, glycogen vacuoles, axonemes, axostyles, median bodies, sporozoites)	Egg, larva, or adult: size, internal structure: Egg: shell characteristics (striations, bumpy or smooth, thick or thin), embryonated, opercular shoulders, abopercular thickenings or projections, hooklets, polar filaments, polar plugs, spines Larva: head and tail morphology, digestive tract Adult: nematode, cestode, or trematode

**Table 8.2** Diagnostic characteristics for organisms in permanent stained smears

Specimen	Protozoa	Helminths
Stool, other specimens from gastrointestinal tract and urogenital system	Size, shape, stage (trophozoite, precyst, cyst, oocyst, spore) Nuclear arrangement, cytoplasm inclusions (chromatoidal bars, vacuoles, axonemes, axostyles, median bodies, sporozoites, polar tubules)	Eggs, larvae, and/or adults may not be identified because of excess stain retention or distortion

**Table 8.3** Intestinal protozoa: trophozoites of common amebae

Characteristic	<i>Entamoeba histolytica</i> , <i>Entamoeba dispar</i>	<i>Entamoeba hartmanni</i>	<i>Entamoeba coli</i>	<i>Endolimax nana</i>	<i>Iodamoeba bütschlii</i>
Size <sup>a</sup> (diameter or length)	12–60 $\mu\text{m}$ ; usual range, 15–20 $\mu\text{m}$ ; invasive forms ( <i>E. histolytica</i> ) may be over 20 $\mu\text{m}$	5–12 $\mu\text{m}$ ; usual range, 8–10 $\mu\text{m}$	15–50 $\mu\text{m}$ ; usual range, 20–25 $\mu\text{m}$	6–12 $\mu\text{m}$ ; usual range, 8–10 $\mu\text{m}$	8–20 $\mu\text{m}$ ; usual range, 12–15 $\mu\text{m}$
Motility	Progressive with hyaline, fingerlike pseudopodia; motility may be rapid	Usually nonprogressive	Sluggish, nondirectional, with blunt, granular pseudopodia	Sluggish, usually nonprogressive	Sluggish, usually nonprogressive
Nucleus (no. and visibility)	Difficult to see in unstained preparations; 1 nucleus	Usually not seen in unstained preparations; 1 nucleus	Often visible in unstained preparation; 1 nucleus	Occasionally visible in unstained preparations; 1 nucleus	Usually not visible in unstained preparations; 1 nucleus
Peripheral nuclear chromatin (stained)	Fine granules, uniform in size and usually evenly distributed; may have beaded appearance	Nucleus may stain more darkly than <i>E. histolytica</i> although morphology is similar; chromatin may appear as solid ring rather than beaded (trichrome)	May be clumped and unevenly arranged on the membrane; may also appear as solid, dark ring with no beads or clumps	Usually no peripheral chromatin; nuclear chromatin may be quite variable	Usually no peripheral chromatin
Karyosome (stained)	Small, usually compact; centrally located but may be eccentric	Usually small and compact; may be centrally located or eccentric; often described as looking like a "bulls eye"	Large, not compact; may or may not be eccentric; may be diffuse and darkly stained	Large, irregularly shaped; may appear "blot-like"; many nuclear variations are common; may mimic <i>E. hartmanni</i> or <i>Dientamoeba fragilis</i>	Large, may be surrounded by refractile granules that are difficult to see ("basket nucleus")

(continued on next page)

**Table 8.3** Intestinal protozoa: trophozoites of common amebae (*continued*)

Characteristic	<i>Entamoeba histolytica</i> , <i>Entamoeba dispar</i>	<i>Entamoeba hartmanni</i>	<i>Entamoeba coli</i>	<i>Endolimax nana</i>	<i>Iodamoeba bütschlii</i>
Cytoplasm (stained)	Finely granular, "ground-glass" appearance; clear differentiation of ectoplasm and endoplasm; if present, vacuoles are usually small	Finely granular	Granular, with little differentiation into ectoplasm and endoplasm; usually vacuolated	Granular, vacuolated	Granular, may be heavily vacuolated
Inclusions (stained)	Both organisms may contain bacteria; presence of RBCs diagnostic for the true pathogen, <i>E. histolytica</i> ( <i>E. dispar</i> does not contain RBCs <sup>b</sup> )	May contain bacteria; no RBCs	Bacteria, yeast, other debris	Bacteria	Bacteria

<sup>a</sup> Sizes refer to wet preparation measurements. Organisms on a permanent stained smear may be 1 to 1.5  $\mu\text{m}$  smaller due to artificial shrinkage.

<sup>b</sup> *E. histolytica* and *E. dispar* appear identical; unless the trophozoite contains ingested RBCs, the organism identification should read "*Entamoeba histolytica/E. dispar group*."

**Table 8.4** Intestinal protozoa: cysts of common amebae

Characteristic	<i>Entamoeba histolytica</i> , <i>Entamoeba dispar</i>	<i>Entamoeba hartmanni</i>	<i>Entamoeba coli</i>	<i>Endolimax nana</i>	<i>Iodamoeba bütschlii</i>
Size <sup>a</sup> (diameter or length)	10–20 µm; usual range, 12–15 µm	5–10 µm; usual range, 6–8 µm	10–35 µm; usual range, 15–25 µm	5–10 µm; usual range, 6–8 µm	5–20 µm; usual range, 10–12 µm
Shape	Usually spherical	Usually spherical	Usually spherical; may be oval, triangular, or other shapes; may be distorted on permanent stained slide due to inadequate fixative penetration	Usually oval; may be round	May vary from oval to round; cyst may collapse due to large glycogen vacuole space
Nucleus (no. and visibility)	Mature cyst, 4; immature, 1 or 2; nuclear characteristics difficult to see on wet preparation	Mature cyst, 4; immature, 1 or 2; 2 nucleated cysts very common	Mature cyst, 8; occasionally 16 or more are seen; immature, 2 or more (occasionally seen)	Mature cyst, 4; immature, 2; very rarely seen and may resemble cysts of <i>Enteromonas hominis</i>	Mature cyst, 1
Peripheral chromatin (stained)	Peripheral chromatin present; fine, uniform granules, evenly distributed; nuclear characteristics may not be as clearly visible as in trophozoite	Fine granules evenly distributed on the membrane; nuclear characteristics may be difficult to see	Coarsely granular; may be clumped and unevenly arranged on membrane; nuclear characteristics not as clearly defined as in trophozoite; may resemble <i>E. histolytica</i>	No peripheral chromatin	No peripheral chromatin

(continued on next page)

**Table 8.4** Intestinal protozoa: cysts of common amebae (*continued*)

Characteristic	<i>Entamoeba histolytica</i> , <i>Entamoeba dispar</i>	<i>Entamoeba hartmanni</i>	<i>Entamoeba coli</i>	<i>Endolimax nana</i>	<i>Iodamoeba bütschlii</i>
Karyosome (stained)	Small, compact, usually centrally located but occasionally eccentric	Small, compact, usually centrally located	Large, may or may not be compact and/or eccentric; occasionally centrally located	Smaller than karyosome seen in trophozoites, but generally larger than those of <i>Entamoeba</i> spp.	Larger, usually eccentric refractile granules may be on one side of karyosome ("basket nucleus")
Cytoplasm, chromatoidal bodies (stained)	May be present; bodies usually elongate with blunt, rounded, smooth edges; may be round or oval	Usually present; bodies usually elongate with blunt, rounded, smooth edges; may be round or oval	May be present (less frequently than <i>E. histolytica</i> ); splinter shaped with rough, pointed ends	Rare chromatoidal bodies present; occasionally small granules or inclusions seen; fine linear chromatoidal bodies may be faintly visible on well-stained smears	No chromatoidal bodies present; small granules occasionally present
Glycogen (stained with iodine)	May be diffuse or absent in mature cyst; clumped chromatin mass may be present in immature cyst (stains reddish brown with iodine)	May or may not be present as in <i>E. histolytica</i>	May be diffuse or absent in mature cysts; clumped mass occasionally seen in mature cyst (stains reddish brown with iodine)	Usually diffuse if present (stains reddish brown with iodine)	Large, compact, well-defined mass (stains reddish brown with iodine)

<sup>a</sup> Sizes refer to wet preparation measurements. Organisms on a permanent stained smear usually measure 1 to 2  $\mu\text{m}$  less due to artificial shrinkage.

**Table 8.5** Intestinal protozoa: trophozoites of less common amebae

Characteristic	<i>Entamoeba polecki</i>	<i>Entamoeba gingivalis</i>
Source	Intestinal tract, stool	Mouth, poor oral hygiene
Size (diameter or length)	10–12 $\mu\text{m}$	5–20 $\mu\text{m}$ (average, 10–15 $\mu\text{m}$ )
Motility	Usually nonprogressive, sluggish (like <i>E. coli</i> )	Multiple pseudopodia, vary from long and lobose to short and blunt
Nucleus (no. and visibility)	Occasionally seen on a wet preparation; intermediate between <i>E. histolytica</i> and <i>E. coli</i> ; 1 nucleus	Similar to <i>E. histolytica</i> , 1 nucleus
Peripheral chromatin (stained)	Fine granules (may be interspersed with large granules) evenly arranged on membrane; chromatin may also be clumped at one or both edges of membrane	Fine granules, closely packed
Karyosome (stained)	Small, usually centrally located	Small, well defined, usually centrally located
Cytoplasm (stained)	Finely granular	Finely granular
Inclusions (stained)	May contain ingested bacteria	Ingested epithelial cells and host leukocytes

**Table 8.6** Intestinal protozoa: cysts of less common amebae

Characteristic	<i>Entamoeba polecki</i>	<i>Entamoeba gingivalis</i>
Size (diameter or length)	5–11 $\mu\text{m}$	No known cyst stage
Shape	Usually spherical	
Nucleus (no. and visibility)	Mature cyst, 1; may be visible in wet preparations (rarely 2 or 4 nuclei)	
Peripheral chromatin (stained)	Similar to that in the trophozoite	
Karyosome (stained)	Similar to that in the trophozoite	
Cytoplasm: chromatoidal bodies, glycogen, inclusions (stained)	Abundant, angular pointed ends, also threadlike chromatoidals may be present; half of cysts contain spherical or ovoidal inclusion mass	

**Table 8.7** Morphologic criteria used to identify *Blastocystis hominis*

Species	Shape and size	Other features
<i>Blastocystis hominis</i>	Organisms are generally round, measure ca. 6–40 $\mu\text{m}$ , and are usually characterized by a large, central body (looks like a large vacuole) surrounded by small, multiple nuclei; the central body area can stain various colors (trichrome) or remain clear	The more amebic form can be seen in diarrheal fluid but is difficult to identify; due to variation in size, it may be confused with various yeast cells

**Table 8.8** Intestinal protozoa: trophozoites of flagellates

Characteristic	Shape and size	Motility	No. and visibility of nuclei	No. of flagella (usually difficult to see)	Other features
<i>Dientamoeba fragilis</i>	Shaped like amebae; 5–15 $\mu\text{m}$ (usual range, 9–12 $\mu\text{m}$ )	Usually nonprogressive; pseudopodia are angular, serrated, or broad lobed and almost transparent	Percentage may vary, but 40% of organisms have 1 nucleus and 60% have 2 nuclei; not visible in unstained preparations; no peripheral chromatin; karyosome is composed of a cluster of 4–8 granules	Internal flagella, not visible	Cytoplasm finely granular and may be vacuolated with ingested bacteria, yeasts, and other debris; there may be great variation in size and shape on a single smear
<i>Giardia lamblia</i>	Pear shaped; 10–20 $\mu\text{m}$ long, 5–15 $\mu\text{m}$ wide	"Falling-leaf" motility may be difficult to see if organism is in mucus; slight flutter of flagella may be visible under low light (duodenal aspirate or mucus from Entero-Test capsule)	2; not clearly visible in unstained mounts	4 lateral, 2 ventral, 2 caudal	Sucking disc occupying half to three-quarters of ventral surface; pear-shaped front view; spoon-shaped side view
<i>Chilomastix mesnili</i>	Pear shaped; 6–24 $\mu\text{m}$ long (usual range, 10–15 $\mu\text{m}$ ), 4–8 $\mu\text{m}$ wide	Stiff, rotary	1; not visible in unstained mounts	3 anterior, 1 in cytostome	Prominent cytostome extending one-third to half the length of the body; spiral groove across ventral surface <i>(continued on next page)</i>

**Table 8.8** Intestinal protozoa: trophozoites of flagellates (*continued*)

Characteristic	Shape and size	Motility	No. and visibility of nuclei	No. of flagella (usually difficult to see)	Other features
<i>Pentatrichomonas hominis</i>	Pear shaped; 5–15 µm long (usual range, 7–9 µm), 7–10 µm wide	Jerky, rapid	1; not visible in unstained mounts	3–5 anterior, 1 posterior	Undulating membrane extending the length of the body; posterior flagellum extending free beyond the end of the body
<i>Trichomonas tenax</i>	Pear shaped; 5–12 µm long (average, 6.5–7.5 µm), 7–9 µm wide	Jerky, rapid	1; not visible in unstained mounts	4 anterior, 1 posterior	Seen only in preparations from mouth; axostyle (slender rod) protrudes beyond the posterior end and may be visible; posterior flagellum extends only halfway down the body, and there is no free end
<i>Enteromonas hominis</i>	Oval; 4–10 µm long (usual range, 8–9 µm), 5–6 µm wide	Jerky	1; not visible in unstained mounts	3 anterior, 1 posterior	One side of body flattened; posterior flagellum extends free posteriorly or laterally
<i>Retortamonas intestinalis</i>	Pear shaped or oval; 4–9 µm long (usual range, 6–7 µm), 3–4 µm wide	Jerky	1; not visible in unstained mounts	1 anterior, 1 posterior	Prominent cytostome extending approximately half the length of the body

**Table 8.9** Intestinal protozoa: cysts of flagellates

Species	Size	Shape	No. and visibility of nuclei	Other features
<i>Dientamoeba fragilis</i> , <i>Pentatrichomonas hominis</i> , <i>Trichomonas tenax</i>	No cyst stage			
<i>Giardia lamblia</i>	8–19 $\mu\text{m}$ long (usual range, 11–14 $\mu\text{m}$ ), 7–10 $\mu\text{m}$ wide	Oval, ellipsoidal, or may appear round	4; not distinct in unstained preparations; usually located at one end	Longitudinal fibers in cysts may be visible in unstained preparations; deep-staining median bodies usually lie across the longitudinal fibers; there is often shrinkage, and the cytoplasm pulls away from the cyst wall; there may also be a "halo" effect around the outside of the cyst wall due to shrinkage caused by dehydrating reagents
<i>Chilomastix mesnili</i>	6–10 $\mu\text{m}$ long (usual range, 7–9 $\mu\text{m}$ ), 4–6 $\mu\text{m}$ wide	Lemon or pear shaped with anterior hyaline knob	1; not distinct in unstained preparations	Cystostome with supporting fibrils, usually visible in stained preparation; curved fibril along side of cystostome usually referred to as "shepherd's crook"
<i>Enteromonas hominis</i>	4–10 $\mu\text{m}$ long (usual range, 6–8 $\mu\text{m}$ ), 4–6 $\mu\text{m}$ wide	Elongate or oval	1–4; usually 2 lying at opposite ends of cyst; not visible in unstained mounts	Resembles <i>E. nana</i> cyst; fibrils or flagella usually not seen
<i>Retortamonas intestinalis</i>	4–9 $\mu\text{m}$ long (usual range, 4–7 $\mu\text{m}$ ), 5 $\mu\text{m}$ wide	Pear shaped or slightly lemon shaped	1; not visible in unstained mounts	Resembles <i>Chilomastix</i> cyst; shadow outline of cystostome with supporting fibrils extends above nucleus; bird beak fibril arrangement

**Table 8.10** Intestinal protozoa: ciliates

<b>Species</b>	<b>Shape and size</b>	<b>Motility</b>	<b>No. and visibility of nuclei</b>	<b>Other features</b>
<i>Balantidium coli</i> trophozoite	Ovoid with tapering anterior end; 50–100 $\mu\text{m}$ long, 40–70 $\mu\text{m}$ wide; (usual range, 40–50 $\mu\text{m}$ )	Ciliates: rotary, boring; may be rapid	1 large kidney-shaped macronucleus; 1 small round micronucleus, which is difficult to see even in the stained smear; macronucleus may be visible in unstained preparation	Body covered with cilia, which tend to be longer near cytostome; cytoplasm may be vacuolated
<i>Balantidium coli</i> cyst	Spherical or oval, 50–70 $\mu\text{m}$ (usual range, 50–55 $\mu\text{m}$ )		1 large macronucleus visible in unstained preparation; micronucleus is difficult to see	Macronucleus and contractile vacuole are visible in young cysts; in older cysts, internal structure appears granular; cilia are difficult to see within the cyst wall

**Table 8.11** Coccidia

Species	Shape and size	Other features
<i>Cryptosporidium</i> spp.	Oocysts are generally round (4–6 $\mu\text{m}$ ); each mature oocyst contains sporozoites	Oocyst is the usual diagnostic stage in stool; various other stages in the life cycle can be seen in biopsy specimens taken from the gastrointestinal tract (brush border of epithelial cells) (intestinal tract) and possibly other tissues (respiratory tract, biliary tract)
<i>Cyclospora cayetanensis</i>	Organisms are generally round (8–10 $\mu\text{m}$ ); they mimic <i>Cryptosporidium</i> spp. (acid-fast) but are larger	In wet smears they look like nonrefractile spheres; they also autofluoresce with epifluorescence; they are acid-fast variable from no color to light pink to deep red; those that do not stain may appear wrinkled; in a trichrome-stained stool smear they appear as clear, round, somewhat wrinkled objects; they cause diarrhea in both immunocompetent and immunosuppressed patients
<i>Isospora belli</i>	Ellipsoidal oocyst; usual range, 20–30 $\mu\text{m}$ long by 10–19 $\mu\text{m}$ wide; sporocysts rarely seen broken out of oocysts but measure 9–11 $\mu\text{m}$	Mature oocyst contains 2 sporocysts with 4 sporozoites each; the usual diagnostic stage in feces is the immature oocyst containing a spherical mass of protoplasm (diarrhea) (intestinal tract)
<i>Sarcocystis hominis</i> ( <i>S. suisomnis</i> , <i>S. bovihominis</i> )	Oocyst is thin walled and contains 2 mature sporocysts, each containing 4 sporozoites; the thin oocyst wall often ruptures; ovoidal sporocysts each measure 9–16 $\mu\text{m}$ long by 7.5–12 $\mu\text{m}$ wide	Thin-walled oocyst or ovoidal sporocysts occur in stool (intestinal tract)
<i>Sarcocystis</i> “ <i>lindemannii</i> ” <sup>a</sup>	Shapes and sizes of skeletal and cardiac muscle sarcocysts vary considerably	Sarcocysts contain several hundred to several thousand trophozoites, each of which is 4–9 $\mu\text{m}$ wide by 12–16 $\mu\text{m}$ long; the sarcocysts may also be divided into compartments by septa, not seen in <i>Toxoplasma</i> cysts; they cause tissue and muscle infections

<sup>a</sup> This name is no longer commonly used for these muscle stages.

**Table 8.12** Microsporidia, general information

Organism	Body site	Diagnosis	Comments
<i>Brachiola</i> , <i>Vittaforma</i> , <i>Encephalitozoon</i> , <i>Enterocytozoon</i> , <i>Pleistophora</i> , <i>Trachipleistophora</i> , <i>Microsporidium</i>	All body organs, including eyes	Routine histology (fair); acid-fast, PAS stains recommended (spores); for other specimens (stool, urine, etc.) modified trichrome stains, optical brightening agents, poly- or monoclonal antibody-based kits (FA, EIA); animal inoculation not recommended—lab animals may carry occult infections; electron microscopy may be necessary for identification to the genus/species level	These organisms have been found as insect or other animal parasites; route of infection may be ingestion, inhalation, or direct inoculation (eye); well documented as emerging opportunistic infection in AIDS patients; organisms identified as <i>Septata intestinalis</i> have now been reclassified with <i>Encephalitozoon (E. intestinalis)</i>

**Table 8.13** Microsporidia: recommended diagnostic techniques<sup>a</sup>

Technique	Use	Comments
<b>Light microscopy</b>		
Stool specimens		
Modified trichrome	++	Reliable, available; light infections difficult to confirm
Giemsa	-	Not recommended for routine use, hard to read
Optical brightening agents	++	Calcofluor, Fungifluor, Unitex 2B; sensitive but nonspecific
Antigen detection	++	Commercial availability limits use; not FDA approved
Other body fluids		
Modified trichrome	++	Reliable, available; light infections difficult to identify
Giemsa	+	Urine, conjunctival swab, BAL, CSF, duodenal aspirate
Optical brightening agents	++	Calcofluor, Fungifluor, Unitex 2B; sensitive but nonspecific
IF technique	++	Commercial availability limits use; products in development
Routine histology		
Hematoxylin & eosin	+	Sensitivity uncertain with small parasite numbers
PAS	+	Controversy over effectiveness
Modified Gram stains (Brown Brenn, Brown-Hopps)	++	Sensitive, generally recommended
Giemsa	+	Sensitivity uncertain with small parasite numbers
Warthin-Starry	+	Not standardized, may not be necessary
Modified trichrome	++	Reliable, sensitive
IF technique	++	Commercial availability limits use; products in development
<b>Electron microscopy</b>		
Bodily fluid	+	Specific, sensitivity low; used for identification to species level
Tissue sections	++	"Gold standard" for confirmation, but sensitivity lower than detection of spores in stool or urine; used for identification to species level
<b>Serologic antibody detection</b>	-	Reagents not commercially available; preliminary results controversial
<b>Cultures</b>	-	Generally used in the research setting; continued advances in culture options and organism survival and growth
<b>PCR</b>	-	Availability limited to research laboratories; studies ongoing

<sup>a</sup> IF, immunofluorescence detection procedure; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; PAS, periodic acid-Schiff; -, not available or recommended for routine use; +, reported; ++, techniques in general use (probably most widely used).

**Table 8.14** Comparison of *Naegleria fowleri*, *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Sappinia diploidea*

Characteristic	<i>Naegleria fowleri</i>	<i>Acanthamoeba</i> spp.	<i>Balamuthia mandrillaris</i>	<i>Sappinia diploidea</i>
Trophozoites	Biphasic (amebic) and flagellate forms; 8–15 µm; lobate pseudopodia (amebic form)	Large (15–25 µm); no flagella; filiform pseudopodia	Large (15–60 µm); no flagella; extensive branching of pseudopodia	Large (40–70 µm); no flagella; distinctive double nucleus
Cysts	Not present in tissue; small, smooth, rounded	Present in tissue; large with wrinkled double wall	Present in tissue; large (15–30 µm), may be binucleate, irregular double wall	Bicellular cysts; not seen in first reported case of amebic encephalitis with this organism
Growth on media	Require living cells (bacteria or cell culture); do not grow with >0.4% NaCl	May grow without bacteria; not affected by 0.85% NaCl	Do not grow well on bacterium-seeded nonnutritive agar plates; tissue culture recommended	Limited information
Appearance in tissue <sup>a</sup>	Smaller than <i>Acanthamoeba</i> spp.; dense endoplasm; less distinct nuclear staining	Large; rounded; less endoplasm; nucleus more distinct	Large; rounded; difficult to differentiate from <i>Acanthamoeba</i> spp.	Trophozoites contain ingested RBCs and stain brightly with Giemsa and PAS

<sup>a</sup> *Entamoeba histolytica* has a delicate nuclear membrane and a small, pale-staining nucleolus. Freshwater amebae have a distinct nuclear membrane and a large, deep-staining nucleolus.

**Table 8.15** Characteristics of *Trichomonas vaginalis*

Shape and size	Pear shaped, 7–23 $\mu\text{m}$ long (average, 13 $\mu\text{m}$ ) by 5–15 $\mu\text{m}$ wide
Motility	Jerky, rapid
No. of nuclei and visibility	1; not visible in unstained mounts
No. of flagella (usually difficult to see)	3–5 anterior, 1 posterior
Other features	Seen in urine, urethral discharges, and vaginal smears; undulating membrane extends half the length of the body; no free posterior flagellum; axostyle easily seen
Infective stage	Trophozoite
Usual location	Vagina (urethra in males)
Striking clinical findings	Leukorrhea, pruritus vulva (thin white urethral discharge in males)
Other sites of infection	Urethra (prostate in males)
Stage usually recovered during clinical phase	Trophozoite only (no cyst)

**Table 8.16** Normal life spans of the most common intestinal nematodes

Nematode	Life span	Comments
<i>Ascaris lumbricoides</i>	1 yr	Infection may be aborted by spontaneous passage of adult worms
<i>Enterobius vermicularis</i>	Several months to years	Reinfection due to both self-infection and outside sources is extremely common
<i>Trichuris trichiura</i>	Several years	Often accompanies <i>Ascaris</i> infection (both are acquired by egg ingestion from contaminated soil)
Hookworms <i>Necator americanus</i> <i>Ancylostoma duodenale</i>	4–20 yr 5–7 yr	Symptoms are directly related to worm burden; many infections are asymptomatic
<i>Strongyloides stercoralis</i>	30+ yr	Autoinfection capability can lead to dissemination and the hyperinfection syndrome in compromised hosts

**Table 8.17** Characteristics of the most common intestinal nematodes

Characteristic	<i>Ascaris lumbricoides</i>	<i>Enterobius vermicularis</i>	<i>Trichuris trichiura</i>	Hookworms ( <i>Necator americanus</i> , <i>Ancylostoma duodenale</i> )	<i>Trichostrongylus</i> spp.	<i>Strongyloides stercoralis</i>
Usual time to infective stage	2–3 wk in soil; second-stage larva in egg	4–6 hr; first-stage larva in egg	2–3 wk in soil; first-stage larva in egg	5–7 days in soil; free, third-stage larva	3–5 days in soil; free, third-stage larva	5–7 days in soil; free, third-stage larva
Mode of infection	Ingestion of infective egg	Ingestion of infective egg	Ingestion of infective egg	Skin penetration by <i>Necator</i> ; ingestion and skin penetration by <i>Ancylostoma</i>	Ingestion of third-stage larva	Skin penetration
Development and location in human host	Obligatory larval migration through liver and lungs; adults in small intestine	Direct development to adult in intestinal tract; adults in cecum, appendix, colon, and rectum	Direct development to adult in intestinal tract; adults in cecum, appendix, and colon	Larval migration through lungs; adults attached to mucosa of small intestine	Direct development to adult in intestinal tract; adults in small intestine	Larval migration through lungs; adult females in mucosal epithelium of small intestine; autoinfection may occur
Prepatent period	2 mo	3–4 wk	3 mo	6–8 wk	2–3 wk	2–4 wk
Normal life span	Up to 1 yr or slightly longer	1–2 mo	Up to 15 yr or more; usually 5–10 yr	Up to 15 yr or more; usually 5–10 yr	Up to 1 yr or slightly longer	Up to many years (30+ yr)

Diagnosis by usual means	Bile-stained, mammillated, thick-shelled eggs (45–75 $\mu\text{m}$ by 35–50 $\mu\text{m}$ ) in 1-cell stage in feces; infertile eggs (85–95 $\mu\text{m}$ by 43–47 $\mu\text{m}$ ) have thinner shells and distorted mammillations; mature or immature adults may be found in feces or may spontaneously migrate out of anus, mouth, or nares	Smooth, thick-shelled eggs (50–60 $\mu\text{m}$ by 20–32 $\mu\text{m}$ ) in cellulose tape preparations; may be seen in feces; adult or immature worms may be found in feces	Unembryonated, bile-stained, thick-shelled eggs (50–54 $\mu\text{m}$ by 20–23 $\mu\text{m}$ ) have mucoid plugs at each end, in feces	Thin-shelled eggs (56–75 $\mu\text{m}$ by 36–40 $\mu\text{m}$ ) in 4- to 8-cell stage in feces	Large, thin-shelled eggs (73–95 $\mu\text{m}$ by 40–50 $\mu\text{m}$ ), tapered at one end, in feces; inner membrane of egg frequently wrinkled; eggs already in advanced cleavage when passed	First-stage larvae (108–380 $\mu\text{m}$ by 14–20 $\mu\text{m}$ ) in feces; rhabditiform larvae have a short buccal chamber and a prominent, conspicuous genital primordium
Diagnostic problems	Fertile eggs may lose outer mammillated layer (decorticate eggs); infertile eggs may be difficult to recognize; also, do not float in usual solution of $\text{ZnSO}_4$ (sp gr 1.18) used for concentration	Eggs not usually seen in feces; cellulose tape method should be used to demonstrate eggs from perianal region	Rarely presents a problem, routine stool examination	Eggs of the two species are indistinguishable; if eggs hatch in feces due to delay in examination, these first-stage rhabditiform larvae must be differentiated from <i>Strongyloides</i> larvae	May be confused with hookworm eggs	Larvae may be passed sporadically and may be found only by concentration procedures or use of Entero-Test or duodenal intubation; agar plate cultures recommended

(continued on next page)

**Table 8.17** Characteristics of the most common intestinal nematodes (*continued*)

Characteristic	<i>Ascaris lumbricoides</i>	<i>Enterobius vermicularis</i>	<i>Trichuris trichiura</i>	Hookworms ( <i>Necator americanus</i> , <i>Ancylostoma duodenale</i> )	<i>Trichostrongylus</i> spp.	<i>Strongyloides stercoralis</i>
Clinical notes	Due to potential migration of adult worms (fever, drugs, anesthetics), all infections should be treated; pulmonary symptoms may be present during larval migration (prior to egg recovery in the stool); eosinophils present but not impressive	Generally, only symptomatic patients treated due to high reinfection rate; eosinophilia may or may not be present	Light infections usually not treated; patients may be asymptomatic, with eggs an incidental finding; <i>Ascaris</i> and <i>Trichuris</i> infections often found together; moderate eosinophilia in heavy infections (usually does not exceed 15%)	Skin penetration by larvae produces allergic reaction (ground itch, cutaneous larva migrans); pulmonary symptoms usually present only in heavy infection; iron deficiency anemia and eosinophilia up to 70% may be present	Rarely seen in U.S., common in the Orient, Europe, Middle East, and Africa; light infections usually not treated	Hyperinfections may lead to death in compromised or immunosuppressed hosts; patient may become symptomatic many years after original infection (without additional exposure); eosinophilia 10–40% or higher

**Table 8.18** Tissue nematodes

Name	How acquired	Location in body	Symptoms	Diagnosis
<i>Trichinella spiralis</i> , <i>Trichinella</i> spp.	Ingestion of raw or rare meats (pork, bear, walrus, other carnivores and/or omnivores)	Active muscles (diaphragm, tongue, larynx, neck, ribs, biceps, gastrocnemius) contain encysted larvae	Diarrhea (larval migration through intestinal mucosa); nausea, abdominal cramps, general malaise; muscle invasion leading to periorbital edema, pain, swelling, weakness, difficulties in swallowing, breathing, etc.; the most severe symptom is myocarditis; high eosinophilia (20–90%)	Biopsy or autopsy specimen (muscle) compression smear or routine histologic tests; artificial digestion of muscle to release larvae (larvae are very infective, and precautions should be taken); serologic tests can be very helpful
<i>Baylisascaris procyonis</i>	Ingestion of viable eggs in the soil (most likely from raccoon feces)	Central nervous system and eyes contain larvae	Eosinophilic meningitis, unilateral neuroretinitis; severe CNS sequelae, often ending in death	Biopsy or autopsy specimen, routine histologic tests; eggs from raccoon are 80 µm long by 65 µm wide, have a thick shell with a finely granulated surface, and resemble <i>Ascaris lumbricoides</i> eggs
<i>Lagochilascaris minor</i>	Life cycle and route of human infection unknown; suspected to involve ingestion of viable eggs in the soil	Adult worms, larvae, and eggs occur in life cycle within human lesions (neck, throat, nasal sinuses, tonsillar tissue, mastoids, brain, lungs)	Pustule, swelling, pus in lesions; chronic granulomatous inflammation	Identification of adult worms, larvae, or eggs from lesions, sinus tracts, or biopsy or autopsy specimens

(continued on next page)

**Table 8.18** Tissue nematodes (*continued*)

Name	How acquired	Location in body	Symptoms	Diagnosis
<i>Toxocara canis</i> and <i>T. cati</i> (visceral larva migrans)	Ingestion of infective eggs (dog and cat ascarids) from fecal material in the soil	Usually the liver; migratory pathway may include the lungs and even back to the intestine	Migration of larvae may cause inflammation and granuloma formation; fever, hepatomegaly, pulmonary infiltrates, cough, and neurologic damage may occur; high eosinophilia (up to 90%; 20–50% common)	Confirmation at autopsy; serologic tests (ocular fluids as well as serum if eyes are involved)
<i>Ancylostoma braziliense</i> or <i>A. caninum</i> (cutaneous larva migrans)	Skin penetration by filariform/infective larvae of dog or cat hookworms; infection can also occur via ingestion of infective larvae	Larval migration in the skin produces linear, raised, vesicular tracts; can be found on any area of the body	Intense itching, pneumonitis (if larvae migrate to deeper tissues)	Picture of linear tracts; possible removal of larva from tunnel
<i>Dracunculus medinensis</i> (fiery serpent)	Ingestion of infected copepod/water flea ( <i>Cyclops</i> )	Adult worms develop in deep connective tissue; gravid female migrates to feet and ankles (can occur anywhere), where blister forms for larval deposition into the water through the ruptured blister on the skin	Before blister formation, there are erythema, tenderness, urticarial rash, intense itching, nausea, vomiting, diarrhea, or asthmatic attacks; if secondary infection occurs, there may be cellulitis, arthritis, myositis, etc.	Formation of cutaneous lesion with appearance of adult female worm depositing larvae into the water; calcified worms can also be found on X rays
<i>Angiostrongylus cantonensis</i> (eosinophilic meningitis)	Accidental ingestion of infective larvae in slugs, snails, or land planarians	Brain tissue, eyes (rare), lung tissue (rare)	Severe headache, convulsions, limb weakness, paresthesia, vomiting, fever, eosinophilia up to 90%	Presumptive: severe headache, meningitis or meningoencephalitis, fever, ocular involvement; definitive: examination of tissues (surgical specimens)

<i>Angiostrongylus costaricensis</i>	Accidental ingestion of slugs, often on contaminated salad vegetables	Bowel wall	Pain, tenderness, palpable tumorlike mass in right lower quadrant, fever, diarrhea, vomiting, eosinophilia (60%), leukocytosis	Worm recovery and clinical history
<i>Gnathostoma spinigerum</i>	Ingestion of raw, poorly cooked, or pickled freshwater fish or chicken (and other birds), frogs, or snakes	Migration of larvae in deep cutaneous or subcutaneous tissues (may appear anywhere), eyes, or cerebrospinal fluid (less common)	Migratory swellings (hard, nonpitting) with inflammation, redness, pain	Worm recovery and clinical history
<i>Anisakis</i> spp., <i>Phocanema</i> spp., <i>Contracaecum</i> spp.	Ingestion of raw, pickled, salted, or smoked saltwater fish	Wall of gastrointestinal tract	Nausea, vomiting; may mimic gastric or duodenal ulcer, carcinoma, or appendicitis; positive occult blood test in stool	Worm recovery and clinical history
<i>Capillaria hepatica</i>	Accidental ingestion of eggs from soil	Liver	May mimic hepatitis, amebic abscess, and other infections involving the liver	Histologic identification
<i>Thelazia</i> spp.	Larval deposition by flies	Conjunctival sacs, migrating over cornea	Excessive lacrimation, itching, pain (feeling of foreign object in the eye)	Worm recovery (from eye) and identification
<i>Dirofilaria</i> spp.	Larval deposition by mosquitoes in human subcutaneous tissues	Pulmonary tissue; dead worms produce "coin" lesions in lungs; some in subcutaneous nodules (orbit, scrotum, breast, arm, leg); subconjunctival	Itching, burning sensation in eye; painless mass in lower eyelid; <i>Dirofilaria</i> should be considered in all cases of subcutaneous inflammatory or tumorlike lesion of unknown etiology	Worm recovery and identification (surgical or autopsy specimens); positive filarial serologic test can be helpful but does not exclude carcinoma

**Table 8.19** *Trichinella spiralis* life cycle stages and clinical conditions<sup>a</sup>

Stage in life cycle	Beginning of symptoms	Clinical condition
Excysted larvae enter intestinal mucosa	2–4 h 24 h	Gastrointestinal symptoms
Worms mature and mate	30 h	
Females deposit larvae in muscle, invasion begins	Day 6 Day 7	Facial edema and fever
Heaviest muscle invasion	Day 10 Day 11	Maximum fever (40–41°C) Muscle inflammation/pain
Decrease in larval deposition	Day 14	Eosinophilia (antibody present)
Larvae differentiated	Day 17 Day 20	Maximum eosinophilia
Encapsulation of larvae	Day 21	Myocarditis and neurologic symptoms
Intestine free of adult worms	Day 23 Day 26	Respiratory symptoms
Encapsulation almost complete	Mo 1 Mo 2	Fever subsides
Adult worms die	Mo 3	Death from myocarditis or encephalitis
Cyst calcification begins	Mo 6 Mo 8	Slow convalescence Myocarditis and neurologic symptoms subside
Cyst calcification usually complete	Yr 1	
Most larvae still viable within calcified cyst	Yr 6	

<sup>a</sup> Adapted from P. C. Beaver, R. C. Jung, and E. W. Cupp, *Clinical Parasitology*, 9th ed., Lea & Febiger, Philadelphia, PA, 1984.

**Table 8.20** Characteristics of human microfilariae

Species	Geographic area(s)	Vector	Location	Features of microfilariae			
				Periodicity	Sheath	Mean length ( $\mu\text{m}$ ) (range)	Tail nuclei
<i>Wuchereria bancrofti</i>	Tropics and subtropics worldwide	Mosquito	Blood, hydrocele fluid	Nocturnal, subperiodic	+ (not stained using Giemsa)	260 (244–296)	Nuclei do not extend to tail tip
<i>Brugia malayi</i>	Southeast Asia	Mosquito	Blood	Nocturnal, subperiodic	+ (stains pink using Giemsa)	220 (177–230)	Subterminal and terminal nuclei
<i>Brugia timori</i>	Islands of Timor and Lesser Sunda in Indonesia	Mosquito	Blood	Nocturnal	+	310 (290–325)	Subterminal and terminal nuclei
<i>Loa loa</i>	Africa	Mango fly	Blood	Diurnal	+	275 (250–300)	Nuclei continuous to tail tip
<i>Mansonella perstans</i>	Africa and South America	Midge	Blood	None	–	195 (190–200)	Nuclei continuous to tail tip
<i>Mansonella ozzardi</i>	Central and South America	Midge	Blood	None	–	200 (173–240)	Nuclei do not extend to tail tip
<i>Mansonella streptocerca</i>	Africa	Midge	Skin	None	–	210 (180–240)	Nuclei in single row to tail tip, tail curved
<i>Onchocerca volvulus</i>	Africa and Central and South America	Blackfly	Skin	None	–	254 (221–287)	Nuclei do not extend to tail tip

**Table 8.21** Characteristics of intestinal cestodes

Characteristic	<i>Diphyllobothrium latum</i>	<i>Taenia saginata</i>	<i>Taenia solium</i>	<i>Hymenolepis nana</i>	<i>Hymenolepis diminuta</i>	<i>Dipylidium caninum</i>
Intermediate host(s)	Two: copepods and fish	One: cattle	One: hog	One: various arthropods (beetles, fleas); or none	One: various arthropods (beetles, fleas)	One: various arthropods (fleas, dog lice)
Mode of infection	Ingestion of plerocercoid (sparganum) in flesh of infected fish	Ingestion of cysticercus in infected beef	Ingestion of cysticercus in infected pork	Ingestion of cysticercoid in infected arthropod or by direct ingestion of egg; autoinfection may also occur	Ingestion of cysticercoid in infected arthropod	Ingestion of cysticercoid in infected arthropod
Prepatent period	3–5 wk	3–5 mo	3–5 mo	2–3 wk	~3 wk	3–4 wk
Normal life span	Up to 25 yr	Up to 25 yr	Up to 25 yr	Perhaps many years due to autoinfection	Usually <1 yr	Usually <1 yr
Length	4–10 m	4–8 m	3–5 m	2.5–4.0 cm	20–60 cm	10–70 cm
Scolex	Spatulate, 3 mm by 1 mm; no rostellum or hooklets; has 2 shallow grooves (bothria)	Quadrangular, 1 to 2 mm in diameter; no rostellum or hooklets; 4 suckers	Quadrangular, 1 mm in diameter; has rostellum and hooklets; 4 suckers	Knoblike but not usually seen; has rostellum and hooklets; 4 suckers	Knoblike but not usually seen; has rostellum but no hooklets; 4 suckers	0.2–0.5 mm in diameter; has conical/retractile rostellum armed with 4–7 rows of small hooklets; 4 suckers

Usual means of diagnosis	Ovoid, operculate yellow-brown eggs (58–75 $\mu\text{m}$ by 40–50 $\mu\text{m}$ ) in feces; egg usually has small knob at abopercular end; proglottids may be passed, usually in chain of segments (few cm to 0.5 m long); proglottids wider than long (3 mm by 11 mm) and have rosette-shaped central uterus	Gravid proglottids in feces; they are longer than wide (19 mm by 17 mm) and have 15–20 lateral branches on each side of central uterine stem; they usually appear singly; spheroidal yellow-brown, thick-shelled eggs (31–43 $\mu\text{m}$ in diameter) containing an oncosphere may be found in feces	Gravid proglottids in feces; they are longer than wide (11 mm by 5 mm) and have 7–13 lateral branches on each side of central uterine stem; they often appear in chain of 5–6 segments; spheroidal, yellow-brown, thick-shelled eggs (31–43 $\mu\text{m}$ ) containing an oncosphere may be found in feces	Nearly spheroidal pale, thin-shelled eggs (30–47 $\mu\text{m}$ in diameter) in feces; oncosphere surrounded by rigid membrane, which has two polar thickenings from which 4–8 filaments extend into the space between the oncosphere and thin, outer shell	Large, ovoid, yellowish, moderately thick-shelled eggs (70–85 $\mu\text{m}$ by 60–80 $\mu\text{m}$ ) in feces; egg contains oncosphere	Gravid proglottids (8–23 mm long) containing compartmented cluster of eggs in feces; proglottids have genital pores at both lateral margins; individual oncospheres (20–33 $\mu\text{m}$ in diameter) occasionally seen in feces
Diagnostic problems or notes	Eggs are sometimes confused with those of <i>Paragonimus</i> ; eggs are unembryonated when passed in feces	Eggs are identical to those of <i>Taenia solium</i> ; ordinarily can distinguish between species only by examination of gravid proglottids; eggs often confused with pollen grains (handle all proglottids with extreme care)	Eggs are identical to those of <i>T. saginata</i> ; one is less likely to find eggs in feces than with <i>T. saginata</i> (handle all proglottids with extreme care as <i>T. solium</i> eggs are infective to humans)	Sometimes confused with eggs of <i>Hymenolepis diminuta</i> ; rodents serve as reservoir hosts	Should not be confused with <i>H. nana</i> as eggs lack polar filaments; rodents serve as reservoir hosts	Gravid proglottids resemble rice grains (dry) or cucumber seeds (moist); dogs and cats serve as reservoir hosts

**Table 8.22** Characteristics of tissue cestodes

Characteristic	<i>Echinococcus granulosus</i>	<i>Echinococcus multilocularis</i>	<i>Echinococcus vogeli</i> , <i>E. oligarthrus</i>	<i>Multiceps</i> spp.	<i>Spirometra</i> and <i>Diphyllobothrium</i> spp.
Disease	Hydatid disease (cystic)	Hydatid disease (alveolar)	Hydatid disease (polycystic)	Coenurosis	Sparganosis
Geographic location of the parasite	Worldwide	North America, northern and central Eurasia	Central and South America (85% in Brazil, Colombia, Ecuador, and Argentina)	Worldwide	Worldwide; more common in China, Japan, and Southeast Asia
Definitive host(s)	Domestic dog, wild canids (coyote, dingo, red fox, etc.)	Red fox, Arctic fox, raccoon, coyote, domestic dog, cat	Bush dog, domestic dog	Dogs, other canids	Dogs, cats
Acquired by	Egg ingestion (dogs)	Egg ingestion (foxes, cats)	Egg ingestion (dogs [ <i>E. vogeli</i> ], felids [ <i>E. oligarthrus</i> ])	Egg ingestion (dogs, other canids)	(i) Ingestion of infected <i>Cyclops</i> spp. (procercoid); (ii) ingestion of raw infected flesh of amphibians, reptiles, birds, and mammals (spargana); (iii) local application of raw infected flesh as a poultice (spargana)
Intermediate host(s)	Primarily ungulates (sheep, cattle, swine, horses), also marsupials	Rodents (voles, lemmings, shrews, mice), other small mammals	Paca, agouti, spiny rat	Sheep, goats, cattle, horses, lagomorphs, rodents	Frogs, mammals

Stage of organism found in tissue	Larval form (fluid-filled unilocular hydatid cyst), contains protoscolices and daughter cysts, limiting membrane; 1–>15 cm; visceral, primarily liver and lungs	Larval form (alveolar hydatid cyst), no limiting membrane; usually sterile with no protoscolices; visceral, primarily liver	Larval form (fluid-filled polycystic hydatid cyst), scolex visible in wet mounts, large (38–46 µm long) and small (30–37 µm long) hooklets; visceral, liver, abdomen, lungs ( <i>E. vogeli</i> ), orbits, heart ( <i>E. oligarthrus</i> ), vesicles partitioned by septa, protoscolices present	Larval form (intermediate between cysticercus and hydatid cyst)	(i) Procercoid; (ii) spargana; (iii) spargana (see above)
Type of growth in humans	Concentric expansion	Exogenous proliferation, tumorlike, similar to metastatic growth	Exogenous and endogenous proliferation ( <i>E. vogeli</i> ); expansive, no indication of exogenous proliferation ( <i>E. oligarthrus</i> )	Multiple scolices but no daughter cysts	More elongate, wormlike structure, no suckers or hooks; resemble narrow tapeworm proglottids; motile
Location in body	Liver (60%), lungs (20%), kidneys (4%), muscles (4%), spleen (3%), soft tissues (3%), brain (3%), bones (2%), other (1%)	All sites as for <i>E. granulosus</i> ; most common site is liver; metastases in lungs, brain, bones, etc.	Liver, lungs (15%) ( <i>E. vogeli</i> ); eyes and heart ( <i>E. oligarthrus</i> )	Most often in CNS	Most tissues have been involved; depends on site of poultice application  <i>(continued on next page)</i>

**Table 8.22** Characteristics of tissue cestodes (*continued*)

Characteristic	<i>Echinococcus granulosus</i>	<i>Echinococcus multilocularis</i>	<i>Echinococcus vogeli, E. oligarthrus</i>	<i>Multiceps</i> spp.	<i>Spirometra</i> and <i>Diphyllobothrium</i> spp.
Symptoms	Depends on cyst location, usually mechanical from enlarging cyst; may also be allergic reactions from cyst fluid leakage	Hepatic disease resembles slow-growing mucoid carcinoma (no fever); hepatomegaly and splenomegaly, jaundice, ascites	Hepatomegaly, palpable peritoneal masses, jaundice ( <i>E. vogeli</i> )	Like space-occupying lesion in the CNS, similar to tumor, rarely muscles or subcutaneous tissues	Edema, pain, irritation, inflammation, toxemia, eye damage, elephantiasis if lymphatics involved; slowly growing, tender, subcutaneous nodules (may be migratory); ocular sparganosis
Treatment	Surgical removal, albendazole, praziquantel	Albendazole, praziquantel, surgery not recommended	Surgery plus albendazole; difficult to treat	Rarely diagnosed preoperatively	Surgical removal and drainage

**Table 8.23** Characteristics of intestinal trematodes

Species	Geographic distribution	Agent of infection	Egg size ( $\mu\text{m}$ )	Egg morphology	Reservoir hosts	Comments
<i>Fasciolopsis buski</i>	Far East	Water chestnut, bamboo shoots, water caltrop	130–140 by 80–85	Unembryonated; operculated	Dogs, pigs, rabbits	The less mature the egg, the more difficult it may be to see the operculum—it blends into the shell outline, and the “breaks” in the shell may be hard to identify; the egg has no opercular shoulders, making it difficult to see where the operculum breaks occur
<i>Echinostoma ilocanum</i>	Far East	Mollusks	86–116 by 59–69	Unembryonated; operculated	Rats, dogs	Usually <i>E. ilocanum</i> eggs are smaller than <i>F. buski</i> eggs; some strains produce eggs that overlap in size; the operculum may be hard to see
<i>Heterophyes heterophyes</i>	Far East, Middle East	Freshwater fish	27–30 by 15–17	Embryonated; operculated with opercular shoulders	Fish-eating mammals	Eggs have very inconspicuous opercular shoulders and, unlike <i>C. sinensis</i> , lack the “seated” operculum and knob at the abopercular end; due to their small size, these eggs may be missed under low power, so high dry power is recommended <i>(continued on next page)</i>

**Table 8.23** Characteristics of intestinal trematodes (*continued*)

Species	Geographic distribution	Agent of infection	Egg size (μm)	Egg morphology	Reservoir hosts	Comments
<i>Metagonimus yokogawai</i>	Far East, former USSR, Israel, Spain	Freshwater fish	26–28 by 15–17	Embryonated; operculated with opercular shoulders	Fish-eating mammals	Eggs have inconspicuous opercular shoulders, but a more obvious operculum than <i>H. heterophyes</i> ; due to their small size, eggs may be missed under low power, so high dry power is recommended
<i>Gastroducooides hominis</i>	Far East, Middle East, former USSR	Freshwater fish	60–70 by 150	Unembryonated; operculated	Pigs, deer mice, rats	Eggs are more slender than <i>F. buski</i> , but they are much alike. There are no opercular shoulders; it is difficult to see where the operculum "breaks" in the shell occur

**Table 8.24** Characteristics of liver and lung trematodes

Species	Geographic distribution	Reservoir hosts	Infection transmitted by ingestion of:	Egg size ( $\mu\text{m}$ )	Egg morphology
<i>Clonorchis sinensis</i>	Far East	Dogs, cats, other fish-eating mammals	Uncooked fish	28–35 by 12–19	Embryonated, operculated; very prominent opercular shoulders; comma appendage at abopercular end
<i>Opisthorchis viverrini</i>	Northern Thailand, Laos	Dogs, cats, other fish-eating mammals	Uncooked fish	19–29 by 12–17	Embryonated, operculated; prominent opercular shoulders; has a "seated" operculum and may or may not have a knob at the abopercular end; eggs tend to be broader with less prominent shoulders than <i>C. sinensis</i> eggs
<i>Opisthorchis felineus</i>	Poland, Germany, Russian Federation, Kazakhstan, western Siberia	Dogs, cats, other fish-eating mammals	Uncooked fish	28–30 by 11–16	Embryonated, operculated; prominent opercular shoulders; has a "seated" operculum and may or may not have a knob at the abopercular end; eggs tend to be broader with less prominent shoulders than <i>C. sinensis</i> eggs
<i>Fasciola hepatica</i>	Worldwide, mixed <i>F. hepatica</i> and <i>F. gigantica</i> infections have been reported from Pakistan	Herbivores	Uncooked water plants	130–150 by 63–90	Unembryonated operculated; the less mature the egg, the more difficult to see the operculum—it blends into the shell outline, and the "breaks" in the shell may be hard to identify; no opercular shoulders, so it is difficult to see where the operculum "breaks" in the shell occur; eggs resemble those of <i>F. buski</i> , <i>F. ilocanum</i> , <i>F. gigantica</i> , and <i>G. hominis</i> ; they may have thickening at abopercular end of the shell (unlike eggs of <i>F. buski</i> ) <i>(continued on next page)</i>

**Table 8.24** Characteristics of liver and lung trematodes (*continued*)

Species	Geographic distribution	Reservoir hosts	Infection transmitted by ingestion of:	Egg size (μm)	Egg morphology
<i>Dicrocoelium dendriticum</i> , <i>D. hospes</i> , <i>Eurytrema pancreaticum</i>	Europe, Turkey, northern Africa, Far East, China, Japan, North and South America	Cattle, sheep, deer, water buffalo	Ants, grasshoppers, crickets	38–45 by 22–30; dark brown	Embryonated, operculated, thick shell; eggs have a thick, dark brown shell and essentially no opercular shoulders; they cannot be differentiated from each other
<i>Paragonimus westermani</i>	Far East, Africa	Dogs, cats, tigers, lions	Crabs, crayfish	80–120 by 45–65	Unembryonated, operculated; opercular shoulders; eggs have a moderately thick, dark golden brown shell, a prominent operculum, opercular shoulders, and a thickened abopercular end; may be confused with <i>D. latum</i> eggs (smaller, abopercular knob; no opercular shoulders)
<i>Paragonimus mexicanus</i>	Central and South America	Opossum, cats, dogs	Crabs	Avg, 79 by 48	Unembryonated, operculated; thin, irregular undulations on outer shell; eggs have prominent operculum, opercular shoulders, and a thickened abopercular end; may be confused with <i>D. latum</i> eggs (smaller, abopercular, no opercular shoulders); eggs smaller than <i>P. westermani</i> ; also golden brown shell
<i>Paragonimus kellicotti</i>	North and South America	Mink, cats, dogs, pigs	Crabs	75–118 by 48–68	Unembryonated, operculated; slight thickening at abopercular end; tapers more sharply than in <i>P. westermani</i>

**Table 8.25** Human paragonimiasis

Species	Geographic distribution	Disease (source)	Avg egg size and morphology
<i>P. westermani</i> <sup>a</sup>	Asia	Pulmonary (crabs, crayfish, freshwater shrimp)	85–100 µm by 47 µm; abopercular thickening
<i>P. heterotremus</i> <sup>a</sup>	China, Laos, Thailand	Pulmonary (crabs, raw shrimp salad in Thailand)	86 µm by 48 µm; uniform thickness
<i>P. mexicanus</i> <sup>a</sup> ( <i>P. peruvianus</i> , <i>P. ecuadorensis</i> )	Central and South America	Pulmonary (crabs)	79 µm by 48 µm; undulated shell
<i>P. africanus</i>	Nigeria, Cameroon	Pulmonary (crabs)	90 µm by 50 µm; abopercular thickening
<i>P. kellicotti</i>	North America	Pulmonary (crabs, crayfish)	75–118 µm by 48–68 µm; abopercular thickening, tapers more than <i>P. westermani</i>
<i>P. miyazakii</i>	Japan	Pleural (crabs, raw juice of crabs, crayfish)	75 µm by 43 µm; uniform thickness, eggs not normally seen
<i>P. philippinensis</i>	Philippines	Pulmonary (crabs)	79 µm by 50 µm; abopercular thickening
<i>P. skrjabini</i>	China	Pleural, subcutaneous nodules (crabs)	75 µm by 48 µm; uniform thickness
<i>P. hueitungensis</i>	China	Migratory, subcutaneous nodules (crabs)	75 µm by 46 µm; thin shell; visible opercular shoulders, small knob at abopercular end
<i>P. uterobilateralis</i>	Cameroon, Guinea, Liberia, Nigeria	Pulmonary (crabs)	70 µm by 45 µm; abopercular thickening of shell

<sup>a</sup> Pathogenic organisms most frequently isolated from humans.

**Table 8.26** Characteristics of blood trematodes

Species	Geographic distribution	Reservoir hosts	Intermediate snail host genus	Diagnostic specimen	Egg size ( $\mu\text{m}$ )	Egg morphology	Comments
<i>Schistosoma mansoni</i>	Africa, Malagasy, West Indies, Suriname, Brazil, Venezuela	Humans, nonhuman primates	<i>Biomphalaria</i>	Stool, rectal biopsy, serologic specimen	114–180 by 45–73	Elongate, prominent lateral spine; acid-fast positive	In wet preparation, egg may be turned so lateral spine is not visible; evidence of flame cell activity is proof of viability; can use hatching test (unpreserved specimens) to confirm; occasionally found in urine
<i>Schistosoma japonicum</i>	China, Indonesia, Japan, Philippines	Dogs, cats, cattle, water buffalo, pigs	<i>Oncomelania</i>	Stool, rectal biopsy, serologic specimen	55–85 by 40–60	Oval, minute lateral spine; acid-fast positive	Eggs can mimic debris in wet preparation; small spine difficult to see; debris often clings to surface of eggshell; less likely to be found in urine, but possible
<i>Schistosoma mekongi</i>	Mekong River basin	Humans, dogs, rodents	<i>Lithoglyphopsis</i>	Stool, rectal biopsy, serologic specimen	30–55 by 50–65	Oval, minute lateral spine	Eggs look very much like <i>S. japonicum</i> ; lateral spine may be hard to see

<i>Schistosoma haematobium</i>	Africa, Middle East, India, Portugal	Humans	<i>Bulinus</i>	Urine, stool (some cases), serologic specimen	112–170 by 40–70	Elongate, terminal spine; acid-fast negative	If present, easy to identify; membrane filter method can be used (more effective than urine sedimentation); eggs occasionally found in stool; hatching test on urine (unpreserved) sediment is relevant
<i>Schistosoma intercalatum</i>	Central and western Africa	Humans	<i>Bulinus</i>	Stool, rectal biopsy, serologic specimen	140–240 by 50–85	Elongate, terminal spine; acid-fast positive	Eggs resemble <i>S. haematobium</i> , but found in stool rather than urine

**Table 8.27** Malaria<sup>a</sup>

<i>Plasmodium vivax</i> (benign tertian malaria)
1. 48-h cycle
2. Tends to infect young cells
3. Enlarged RBCs
4. Schüffner's dots (true stippling) after 8–10 h
5. Delicate ring
6. Very ameboid trophozoite
7. Mature schizont contains 12–24 merozoites
<i>Plasmodium malariae</i> (quartan malaria)
1. 72-h cycle (long incubation period)
2. Tends to infect old cells
3. Normal size RBCs
4. No stippling
5. Thick ring, large nucleus
6. Trophozoite tends to form "bands" across the cell
7. Mature schizont contains 6–12 merozoites
<i>Plasmodium ovale</i>
1. 48-h cycle
2. Tends to infect young cells
3. Enlarged RBCs with fimbriated edges (oval)
4. Schüffner's dots appear early (in RBCs with very young ring forms, in contrast to <i>P. vivax</i> )
5. Smaller ring than <i>P. vivax</i>
6. Trophozoite less ameboid than that of <i>P. vivax</i>
7. Mature schizont contains ca. 8 merozoites
<i>Plasmodium falciparum</i> (malignant tertian malaria)
1. 36–48-h cycle
2. Tends to infect any cell regardless of age; very heavy infection may result
3. All sizes of RBCs
4. No Schüffner's dots (Maurer's dots: may be larger, single dots, bluish)
5. Multiple rings per cell (only young rings, gametocytes, and occasional mature schizonts are seen in peripheral blood)
6. Delicate rings, may have two dots of chromatin per ring, appliqué or accolé forms
7. Crescent-shaped gametocytes

<sup>a</sup> Characteristics with fresh blood or blood collected using EDTA with no extended lag time (preparation of thick and thin blood films within 60 min of collection).

**Table 8.28** Potential problems using EDTA anticoagulant for the preparation of thin and thick blood films<sup>a</sup>

Potential problem	Comments
Adhesion to the slide; blood falls off slide during staining	Incorrect ratio of anticoagulant to blood; fill tube completely with blood (7 ml or pediatric draw tube)
Distortion of parasites; same type of distortion can also be seen after blood is refrigerated (not recommended)	Prolonged storage of blood in EDTA may lead to distortion: trophozoites ( <i>P. vivax</i> ) and gametocytes ( <i>P. falciparum</i> ) tend to round up, thus mimicking <i>P. malariae</i>
Change in ring form size	Ring forms of <i>P. falciparum</i> continue to enlarge, thus resembling rings of the other species; typical "small" rings appear larger than usual
Use of EDTA anticoagulant (used primarily by the hematology laboratory because the cellular components and morphology of the blood cells are preserved) 1. Blood smears for differentials from acceptable specimens should be prepared within 2 h of collection 2. Blood counts from acceptable venipuncture specimens should be performed within 6 h of collection 3. For normal parasite morphology, thick and thin blood films for blood parasites (particularly malaria) should be prepared within 60 min or less from the time of blood collection since parasites begin to disappear within 4–6 h from blood held in EDTA	EDTA prevents coagulation of blood by chelating calcium (calcium is necessary in the coagulation cascade, and its removal inhibits and stops a series of events, both intrinsic and extrinsic, which cause clotting); in some individuals, EDTA causes inaccurate platelet results; these anomalies, platelet clumping and platelet satellitism, may be the result of changes in the membrane structure occurring when the calcium ion is removed by the chelating agent, allowing the binding of preformed antibodies; proper mixing of the whole blood specimen ensures that EDTA is dispersed throughout the sample: evacuated blood collection tubes with EDTA should be mixed by 8–10 end-to-end inversions immediately following venipuncture collection; microcollection tubes with EDTA should be mixed by 10 complete end-to-end inversions immediately following collection and should then be inverted an additional 20 times prior to analysis; underfilling the EDTA blood collection tube can lead to erroneously low blood cell counts and hematocrits, morphologic changes to RBCs, and staining alteration; excess EDTA can shrink RBCs (conversely, overfilling the blood collection tube does not allow proper mixing and may lead to platelet clumping and clotting)
Loss of Schüffner's dots (stippling) in <i>P. vivax</i> and <i>P. ovale</i>	Schüffner's dots (true stippling) occur in both <i>P. vivax</i> and <i>P. ovale</i> ; in the absence of stippling, identification to the species level may not be possible; problems may be related to buffer pH and/or storage of blood in EDTA for more than 1 h
Prolonged storage at room temperature with stopper removed	The pH, CO <sub>2</sub> , and temperature changes may reflect conditions within the mosquito, so that exflagellation of the male gametocyte may occur while still in the tube of blood prior to thin- and thick-blood-film preparation; microgametes may be confused with <i>Borrelia</i> or may be ignored as debris
Release of merozoites from the schizonts into the blood (normally, merozoites are not found outside the RBCs, in contrast to <i>Babesia</i> spp., where rings may be seen outside the RBCs)	Small rings may be seen outside the RBCs or appear to be appliqué forms, thus suggesting <i>P. falciparum</i> ; it is important to differentiate these true rings (both cytoplasmic and nuclear colors) from platelets (uniform color)
Incorrect submission of blood in heparin	EDTA has less impact on parasite morphology than heparin

<sup>a</sup> If blood films are prepared within recommended time guidelines (60 min or less from the time of collection in EDTA), both thick and thin films should provide typical parasite morphology; however, if blood film preparation is delayed, many artifacts are seen and parasites begin to disappear from the blood after ~4 to 6 hours.

**Table 8.29** Characteristics of plasmodia in Giemsa-stained thin blood smears<sup>a</sup>

Characteristic	<i>Plasmodium vivax</i>	<i>Plasmodium malariae</i>	<i>Plasmodium falciparum</i>	<i>Plasmodium ovale</i>
Persistence of exoerythrocytic cycle	Yes	No	No	Yes
Relapses	Yes	No, but long-term recrudescences are recognized	No long-term relapses	Possible, but usually spontaneous recovery
Time of cycle	44–48 h	72 h	36–48 h	48 h
Appearance of parasitized RBCs; size and shape	1.5 to 2 times larger than normal; oval to normal; may be normal size until ring fills half of cell	Normal shape; size may be normal or slightly smaller	Both normal	60% of cells larger than normal and oval; 20% have irregular, frayed edges
Schüffner's dots (eosinophilic stippling)	Usually present in all cells except early ring forms	None	None; occasionally comma-like red dots (Maurer's dots)	Present in all stages including early ring forms; dots may be larger and darker than in <i>P. vivax</i>
Color of cytoplasm	Decolorized, pale	Normal	Normal, bluish tinge at times	Decolorized, pale
Multiple rings/cell	Occasional	Rare	Common	Occasional
Developmental stages present in peripheral blood	All stages present	Ring forms few (ring stage is brief); mostly growing and mature trophozoites and schizonts	Young ring forms and no older stages; few gametocytes	All stages present
Appearance of parasite; young trophozoite (early ring form)	Ring is 1/3 diameter of cell; cytoplasmic circle around vacuole; heavy chromatin dot	Ring often smaller than in <i>P. vivax</i> , occupying 1/8 of cell; heavy chromatin dot; vacuole at times "filled in"; pigment forms early	Delicate, small ring with small chromatin dot (frequently 2); scanty cytoplasm around small vacuoles; sometimes at edge of RBC (appliqué form) or filamentous slender form; may have multiple rings per cell	Ring is larger and more ameboid than in <i>P. vivax</i> , otherwise similar

Growing trophozoite	Multishaped irregular ameboid parasite; streamers of cytoplasm close to large chromatin dot; vacuole retained until close to maturity; increasing amounts of brown pigment	Nonameboid rounded or band-shaped solid forms; chromatin may be hidden by coarse dark brown pigment	Heavy ring forms; fine pigment grains	Ring shape maintained until late in development; nonameboid compared to <i>P. vivax</i>
Mature trophozoite	Irregular ameboid mass; 1 or more small vacuoles retained until schizont stage; fills almost entire cell; fine brown pigment	Vacuoles disappear early; cytoplasm compact, oval, band shaped, or nearly round almost filling cell; chromatin may be hidden by peripheral coarse dark brown pigment	Not seen in peripheral blood (except in severe infections); development of all phases following ring form occurs in capillaries of viscera	Compact; vacuoles disappear; pigment dark brown, less than in <i>P. malariae</i>
Schizont (presegmenter)	Progressive chromatin division; cytoplasmic bands containing clumps of brown pigment	Similar to <i>P. vivax</i> except smaller; darker, larger pigment granules peripheral or central	Not seen in peripheral blood (see above)	Smaller and more compact than <i>P. vivax</i>
Mature schizont	16 (12–24) merozoites, each with chromatin and cytoplasm, filling entire RBC, which can hardly be seen	8 (6–12) merozoites in rosettes or irregular clusters filling normal-sized cells, which can hardly be seen; central arrangement of brown-green pigment	Not seen in peripheral blood	3/4 of cells occupied by 8 (8–12) merozoites in rosettes or irregular clusters
Macrogametocyte	Rounded or oval homogeneous cytoplasm; diffuse delicate light brown pigment throughout parasite; eccentric compact chromatin	Similar to <i>P. vivax</i> , but fewer; pigment darker and more coarse	Sex differentiation difficult; "crescent" or "sausage" shapes characteristic; may appear in "showers," black pigment near chromatin dot, which is often central	Smaller than <i>P. vivax</i>
Microgametocyte	Large pink to purple chromatin mass surrounded by pale or colorless halo; evenly distributed pigment	Similar to <i>P. vivax</i> but fewer; pigment darker and more coarse	Same as macrogametocyte (described above)	Smaller than <i>P. vivax</i>

(continued on next page)

**Table 8.29** Characteristics of plasmodia in Giemsa-stained thin blood smears<sup>a</sup> (*continued*)

Characteristic	<i>Plasmodium vivax</i>	<i>Plasmodium malariae</i>	<i>Plasmodium falciparum</i>	<i>Plasmodium ovale</i>
Main criteria	Large pale RBC; trophozoite irregular; pigment usually present; Schüffner's dots not always present; several phases of growth seen in one smear; gametocytes appear as early as day 3	RBC normal in size and color; trophozoites compact, stain usually intense, band forms not always seen; coarse pigment; no stippling of red cells; gametocytes appear after a few weeks	Development following ring stage takes place in blood vessels of internal organs; delicate ring forms and crescent-shaped gametocytes are the only forms normally seen in peripheral blood; gametocytes appear after 7–10 days	RBC enlarged, oval, with fimbriated edges; Schüffner's dots seen in all stages; gametocytes appear after 4 days or as late as 18 days

<sup>a</sup> Other blood stains are perfectly acceptable; if the PMNs look acceptable/normal on the stained blood films, any parasites present also exhibit typical morphology. Other acceptable stains include Wright's stain, Wright-Giemsa combination, Field's stain, and rapid stains (Diff-Quik, American Scientific Products, McGraw Park, IL; Wright's Dip Stat Stain Set, Medical Chemical Corp., Torrance, CA). Color variation is normal, even with Giemsa stain.

**Table 8.30** *Plasmodium knowlesi*, the fifth human malaria agent

Geographic distribution <sup>a</sup>	Timing of life cycle	Parasite appearance	Comments
Thailand, Myanmar, peninsular Malaysia, Malaysian Borneo, Palawan Island in the Philippines <sup>a</sup>	Replicates every 24 h	Ring forms have multiple nuclei; late trophozoites tend to form bands; schizonts and gametocytes similar to <i>P. vivax</i> ; some parasites consistent with <i>P. malariae</i> (parasitemia, 0.03%); stippling present but probably not easily recognized	Patients experienced chills, minor headaches, and daily low-grade fever; patients responded well to chloroquine and primaquine; parasites isolated from local macaques; this form of malaria is considered a zoonotic disease; patients who reside in or travel to Southeast Asia with <i>P. malariae</i> hyperparasitemia diagnosis by microscopy should receive intensive management as appropriate for severe falciparum malaria (assumption that the correct diagnosis is really <i>P. knowlesi</i> )

<sup>a</sup> *P. knowlesi* is transmitted by *Anopheles latens*.

**Table 8.31** Features of human leishmanial infections<sup>a</sup>

Species	Disease type <sup>b</sup>	Humoral antibodies	Delayed hypersensitivity	Parasite quantity	Self-cure	Recommended specimen
<i>L. donovani</i>	VL	Abundant	Absent	Absent	Rare	Bone marrow, spleen
	CL	Variable	Present	Present	Yes	Skin macrophages
	DL	Variable	Variable	Variable	Variable	Skin macrophages
<i>L. tropica</i>	CL	Variable	Present	Present	Yes	Skin macrophages
<i>L. major</i>	CL	Present	Present	Present	Rapid	Skin macrophages
<i>L. aethiopica</i>	CL	Variable	Weak	Present	Slow	Skin macrophages
	DCL	Variable	Absent	Abundant	No	Skin macrophages
<i>L. mexicana</i>	CL	Variable	Present	Present	Yes	Skin macrophages
	DCL	Variable	Absent	Abundant	No	Skin macrophages
<i>L. braziliensis</i>	CL	Present	Present	Present	Yes	Skin macrophages
	MCL	Present	Present	Scant	No	Skin macrophages

<sup>a</sup> For culture, specimens must be collected aseptically; in older lesions, the number of parasites may be scant and difficult to recover. For isolation, hamsters are used, and culture media include Novy, MacNeal, and Nicolle's medium and Schneider's *Drosophila* medium with 30% fetal bovine serum. Serologic testing is most suitable for visceral leishmaniasis but has little value for cutaneous leishmaniasis and limited value for mucocutaneous leishmaniasis. The Montenegro test measures the delayed hypersensitivity reaction to intradermal injection of cultured parasites.

<sup>b</sup> VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; DL, dermal leishmanoid; DCL, diffuse cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis.

**Table 8.32** Characteristics of American trypanosomiasis

Characteristic	<i>Trypanosoma cruzi</i>	<i>Trypanosoma rangeli</i>
Vector	Reduviid bug	Reduviid bug
Primary reservoirs	Opossums, dogs, cats, wild rodents	Wild rodents
Illness	Symptomatic (acute, chronic)	Asymptomatic
Diagnostic stage		
Blood	Trypomastigote	Trypomastigote
Tissue	Amastigote	None
Recommended specimens	Blood, lymph node aspirate, chagoma	Blood

**Table 8.33** Characteristics of East and West African trypanosomiasis

Characteristic	East African	West African
Organism	<i>Trypanosoma brucei rhodesiense</i>	<i>Trypanosoma brucei gambiense</i>
Vector	Tsetse fly, <i>Glossina morsitans</i> group	Tsetse fly, <i>Glossina palpalis</i> group
Primary reservoirs	Animals	Humans
Illness	Acute (early CNS invasion), <9 mo	Chronic (late CNS invasions), months to years
Lymphadenopathy	Minimal	Prominent
Parasitemia	High	Low
Epidemiology	Anthropozoonosis, game parks	Anthroponosis, rural populations
Diagnostic stage	Trypomastigote	Trypomastigote
Recommended specimens	Chancre aspirate, lymph node aspirate, blood, CSF	Chancre aspirate, lymph node aspirate, blood, CSF

**Table 8.34** Key characteristics of intestinal tract and urogenital system protozoa<sup>a</sup>

Amebae	Trophozoite	Cyst	Comments
<i>Entamoeba histolytica</i> (pathogenic)	Cytoplasm clean, presence of RBCs is diagnostic, but it may also contain some ingested bacteria; peripheral nuclear chromatin is evenly distributed with central, compact karyosome	Mature cyst contains 4 nuclei; chromatoidal bars have smooth, rounded ends; <i>E. histolytica</i> and <i>E. dispar</i> cannot be differentiated on the basis of cyst morphology	Considered pathogenic; should be signed out as <i>E. histolytica</i> if trophozoites contain ingested RBCs and should be reported to Department of Public Health; trophozoites can be confused with macrophages and cysts can be confused with WBCs in the stool; cysts and trophozoites containing no ingested RBCs should be signed out as <i>E. histolytica</i> / <i>E. dispar</i>
<i>Entamoeba dispar</i> (nonpathogenic)	Morphology identical to that of <i>E. histolytica</i> (confirmed by presence of RBCs in cytoplasm); if no ingested RBCs, specific fecal immunoassays are available for confirmation of the species designation	Mature cyst has identical morphology to that of <i>E. histolytica</i> ; <i>E. histolytica</i> and <i>E. dispar</i> cannot be differentiated on the basis of cyst morphology	Nonpathogenic; morphology resembles <i>E. histolytica</i> ; these organisms (no ingested RBCs) should be signed out as <i>E. histolytica</i> / <i>E. dispar</i> ; immunoassay reagents are now available to differentiate pathogenic <i>E. histolytica</i> and nonpathogenic <i>E. dispar</i> ; some laboratories may decide to use these reagents on a routine basis, depending on positive rate and cost
<i>Entamoeba hartmanni</i> (nonpathogenic)	Looks identical to <i>E. histolytica</i> but is smaller (<12 µm); RBCs are not ingested	Mature cyst contains 4 nuclei but often stops at 2; chromatoidal bars often present and look like those in <i>E. histolytica</i> ; cyst is <10 µm	Shrinkage occurs on the permanent stain (especially in the cyst form); <i>E. histolytica</i> may actually be below the 12- and 10-µm cutoff limits; they could be as much as 1.5 µm below the limits quoted for wet preparation measurements

<i>Entamoeba coli</i> (nonpathogenic)	Cytoplasm dirty, may contain ingested bacteria/debris; peripheral nuclear chromatin is unevenly distributed with a large, eccentric karyosome	Mature cyst contains 8 nuclei, may contain more; chromatoidal bars (if present) tend to have sharp, pointed ends	If a smear is too thick or thin and if stain is too dark or light, <i>E. histolytica</i> and <i>E. coli</i> are often confused; there is much overlap in morphology
<i>Endolimax nana</i> (nonpathogenic)	Cytoplasm clean, not diagnostic, great deal of nuclear variation, may even be some peripheral nuclear chromatin; normally only karyosomes are visible	Cyst is round to oval, with the 4 nuclear karyosomes being visible	There is more nuclear variation in this ameba than in any others; it can be confused with <i>Dientamoeba fragilis</i> and/or <i>E. hartmanni</i>
<i>Iodamoeba bütschlii</i> (nonpathogenic)	Cytoplasm contains much debris; organisms usually larger than <i>E. nana</i> but may look similar; large karyosome; the "basket nucleus" may appear in the trophozoites	Cyst contains single nucleus (may be "basket nucleus") with bits of nuclear chromatin arranged on the nuclear membrane (the karyosome is the basket, the bits of chromatin are the handle); large glycogen vacuole	Glycogen vacuole stains brown when iodine is added to the wet preparation; "basket nucleus" is more common in cyst but can be seen in trophozoite; the vacuole may be so large that the cyst collapses on itself
Flagellates	Trophozoite	Cyst	Comments
<i>Giardia lamblia</i> (pathogenic)	Trophozoites are teardrop shaped from the front and like a curved spoon from the side; contain nuclei, linear axonemes, and curved median bodies	Cysts are round to oval, containing multiple nuclei, axonemes, and median bodies	Organisms live in the duodenum, and multiple stools may be negative; may have to use additional sampling techniques (aspiration, Entero-Test) or fecal immunoassays (much more sensitive than routine O&P exams); if fecal immunoassays are used, two stool specimens should be tested before indicating that the patient is negative for <i>G. lamblia</i> antigen <i>(continued on next page)</i>

**Table 8.34** Key characteristics of intestinal tract and urogenital system protozoa<sup>a</sup> (*continued*)

Amebae	Trophozoite	Cyst	Comments
<i>Chilomastix mesnili</i> (nonpathogenic)	Trophozoites are teardrop shaped; cytostome must be visible for identification	Cyst is "lemon shaped" with 1 nucleus and curved fibril called "Shepherd's crook"	Cyst can be identified much more easily than trophozoite, which looks like some of the other small flagellates
<i>Dientamoeba fragilis</i> (pathogenic)	Cytoplasm contains debris; may contain 1 or 2 nuclei (chromatin often fragmented into 4 dots); flagella are internal and not seen in normal laboratory stains (electron microscopy required)	No known cyst form	Tremendous size and shape range on a single smear; trophozoites with 1 nucleus can resemble <i>E. nana</i>
<i>Trichomonas vaginalis</i> (pathogenic)	Supporting rod (axostyle) is present; undulating membrane comes halfway down the organism; small dots may be seen in the cytoplasm along the axostyle	No known cyst form	Recovered from genitourinary system; often diagnosed at bedside by wet preparation (motility)
<i>Pentatrichomonas hominis</i> (nonpathogenic)	Supporting rod (axostyle) is present; undulating membrane comes all the way down the organism; small dots may be seen in the cytoplasm along the axostyle	No known cyst form	Recovered in stool; trophozoites may resemble those of other small flagellates
Ciliates	Trophozoite	Cyst	Comments
<i>Balantidium coli</i> (pathogenic)	Very large trophozoites (50–100 µm long) covered with cilia; large bean-shaped nucleus present; small, micronucleus very difficult to see	Morphology not significant with exception of large, bean-shaped nucleus	Rarely seen in the U.S.; causes severe diarrhea with large fluid loss; is seen in proficiency-testing specimens

Coccidia	Trophozoite or tissue stages	Cyst or other stage in specimen	Comments
<i>Cryptosporidium</i> spp. (pathogenic)	Present in intestinal mucosa (edge of brush border), gallbladder, and lungs; seen in biopsy specimens	Oocysts seen in stool and/or sputum; organisms acid fast, measure 4–6 $\mu\text{m}$ ; hard to find if present in small numbers; organism morphology does not differentiate between <i>C. parvum</i> (humans and animals) and <i>C. hominis</i> (humans)	Chronic infection in compromised hosts (internal autoinfective cycle), self-cure in immunocompetent hosts; numbers of oocysts correlate with stool consistency; can cause severe watery diarrhea; oocysts are immediately infective when passed; fecal immunoassays very sensitive (more so than special modified acid-fast stains)—one specimen is sufficient to rule in or out <i>Cryptosporidium</i> antigen in stool
<i>Cyclospora cayetanensis</i> (pathogenic)	Biopsy specimens can be differentiated from those infected with other coccidia; if patients are immunocompetent, biopsy specimens will probably rarely be required or requested	Oocysts seen in stool; ca. 8–10 $\mu\text{m}$ in size; are unsporulated, thus difficult to recognize as coccidia; mimic <i>Cryptosporidium</i> on modified acid-fast stained smears; not all oocysts stain red to purple, some will not retain stain and thus they are considered modified acid-fast variable (1% acid destain recommended [no stronger or will remove too much stain]); oocysts often resemble “wrinkled cellophane” with no internal structure (do not contain any sporozoites when passed)	To date, most of these infections are associated with immunocompetent individuals; they may also be seen in immunosuppressed patients and may be associated with travelers' diarrhea; safranin stains are also recommended; many food-borne outbreaks documented, primarily with raspberries, strawberries, mesclun, basil, and snow peas (all of which were imported into the U.S.); oocysts not infective when passed

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**Table 8.34** Key characteristics of intestinal tract and urogenital system protozoa<sup>a</sup> (*continued*)

Amebae	Trophozoite	Cyst	Comments
<i>Isospora belli</i> (pathogenic)	Present in intestinal mucosal cells; seen in biopsy specimens; does not seem to be as common as <i>Cryptosporidium</i> spp.	Oocysts seen in stool; organisms are acid fast; best technique is concentration, not permanent stained smear	Thought to be the only <i>Isospora</i> sp. that infects humans; oocysts are not immediately infective when passed
Microsporidia	Trophozoite or tissue stages	Cyst or other stage in specimen	Comments
<i>Nosema</i> <i>Vittaforma</i> <i>Brachiola</i> <i>Encephalitozoon</i> <i>Pleistophora</i> <i>Trachipleistophora</i> <i>Enterocytozoon</i> <i>Microsporidium</i> (pathogenic)	Developing stages sometimes difficult to identify; spores can be identified by size, shape, and presence of polar tubules	Depending on the genus involved, spores could be identified in stool or urine using the modified trichrome stain, brightening-reagent stains, or immunoassay reagents (not yet commercially available); tissue stains (tissue Gram stains) are also used for biopsy specimens	Spores are generally quite small (1–1.5 µm for <i>Enterocytozoon</i> ) and can easily be confused with other organisms or artifacts (particularly in stool); these infections have most often been diagnosed in immunosuppressed patients, but due to the lack of commercial fecal immunoassays, data on infections in immunocompetent hosts are relatively sparse

<sup>a</sup> Modified from H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, 2nd ed., ASM Press, Washington, DC, 2004.

**Table 8.35** Key characteristics of helminths<sup>a,b</sup>

Helminths	Diagnostic stage	Comments
<b>Nematodes (roundworms)</b> <i>Ascaris lumbricoides</i> (pathogenic)	Both fertilized (oval to round with thick, mammillated/tuberculated shell) and unfertilized (tend to be more oval/elongate with bumpy shell exaggerated) eggs can be found in stool; adult worms (10–12 in. long) found in stool; rarely (in severe infections), migrating larvae found in sputum	Unfertilized eggs do not float in flotation concentration method; adult worms tend to migrate when irritated (by anesthesia, high fever); thus patients from endemic areas should be checked for infection prior to elective surgery; often dual infections with <i>Trichuris trichiura</i> occur
<i>Trichuris trichiura</i> (whipworm) (pathogenic)	Eggs are barrel shaped with two clear polar plugs; adult worms are rarely seen; eggs should be quantitated (rare, few, etc.), since light infections may not be treated	Dual infections with <i>Ascaris</i> sp. may be seen (both infections acquired from egg ingestion in contaminated soil); in severe infections, rectal prolapse may occur in children or bloody diarrhea can be mistaken for amebiasis (severe cases usually not seen in the U.S.)
<i>Enterobius vermicularis</i> (pinworm) (pathogenic)	Eggs are football shaped with one flattened side; adult worms are ca. 3/8 in. long, white with pointed tail; female migrates from the anus and deposits eggs on the perianal skin	May cause symptoms in some patients (itching); test of choice is Scotch tape preparation; 6 consecutive tapes necessary to rule out infection; symptomatic patient often treated without actual confirmation of infection; eggs become infective within a few hours; reinfection very common, particularly among children
<i>Ancylostoma duodenale</i> (Old World hookworm), <i>Necator americanus</i> (New World hookworm) (pathogenic)	Eggs of these species are identical—oval with broadly rounded ends, thin shell, clear space between shell and developing embryo (8–16-cell stage); adult worms are rarely seen in clinical specimens	May cause symptoms in some patients (blood-loss anemia on the differential smear in heavy infections); if stool remains unpreserved for several hours or days, eggs may continue to develop and hatch; rhabditiform larvae may resemble those of <i>S. stercoralis</i>
<i>Strongyloides stercoralis</i> (pathogenic)	Rhabditiform larvae (noninfective) usually found in the stool (short buccal cavity or capsule with large, genital primordial packet of cells) ("short and sexy"); in very heavy infections, larvae occasionally found in sputum and/or filariform (infective) larvae can be found in stool (slit in the tail)	Unexplained eosinophilia, abdominal pain, unexplained episodes of sepsis and/or meningitis, and pneumonia (migrating larvae) in the compromised patient may occur; potential for internal autoinfection can maintain low-level infections for many years after the patient has left the endemic area (patient will be asymptomatic with an elevated eosinophilia); hyperinfection can occur in compromised patients (leading to disseminated strongyloidiasis and death) <i>(continued on next page)</i>

**Table 8.35** Key characteristics of helminths<sup>a,b</sup> (*continued*)

Helminths	Diagnostic stage	Comments
<i>Ancylostoma braziliensis</i> (dog and cat hookworm) (pathogenic)	Humans are accidental hosts; larvae wander through the outer layer of skin, creating tracks (severe itching, eosinophilia); no practical microbiological diagnostic tests	Cause of cutaneous larva migrans; typical setup for infection involves dogs and cats defecating in sand boxes and hookworm eggs hatching and penetrating human skin when in contact with infected sand or soil
<i>Toxocara canis or cati</i> (dog and cat ascarid) (pathogenic)	Humans are accidental hosts; ingestion of dog or cat ascarid eggs in contaminated soil; larvae wander through deep tissues (including the eyes); can be mistaken for cancer of the eye; serologic tests helpful for confirmation; eosinophilia is found	Cause of visceral larva migrans and ocular larva migrans; requests for laboratory services often originate in the ophthalmology clinic
<b>Cestodes (tapeworms)</b> <i>Taenia saginata</i> (beef tapeworm) (pathogenic)	Scolex (4 suckers, no hooklets), gravid proglottid (>12 branches on a single side) are diagnostic; eggs indicate <i>Taenia</i> spp. only (thick, striated shell, containing a 6-hooked embryo or oncosphere); worm usually around 12 ft long	Adult worms cause symptoms in some individuals; ingestion of raw or poorly cooked beef; usually only a single worm per patient; individual proglottids may crawl from the anus; proglottids can be injected with India ink to see the uterine branches for identification
<i>Taenia solium</i> (pork tapeworm) (pathogenic)	Scolex (4 suckers with hooklets), gravid proglottid (<12 branches on a single side) are diagnostic; eggs indicate <i>Taenia</i> spp. only (thick, striated shell, containing a 6-hooked embryo or oncosphere); worm usually around 12 ft long	Adult worm causes gastrointestinal complaints in some individuals; cysticercosis (accidental ingestion of eggs) can cause severe symptoms in the CNS; ingestion of raw or poorly cooked pork; usually only a single worm per patient; occasionally 2 or 3 proglottids (hooked together) are passed; proglottids can be injected with India ink to see the uterine branches for identification; cysticerci are normally small and contained within an enclosing membrane; occasionally they develop as the "racemose" type where the worm tissue grows in the body like a metastatic cancer
<i>Diphyllobothrium latum</i> (broad fish tapeworm) (pathogenic)	Scolex (lateral sucking grooves), gravid proglottid (wider than long, with reproductive structures in the center "rosette"); eggs are operculated but have no opercular shoulders	Causes gastrointestinal complaints in some individuals; ingestion of raw or poorly cooked freshwater fish; life cycle has 2 intermediate hosts (copepod, fish); worm may reach 30 ft long; associated with vitamin B <sub>12</sub> deficiency in genetically susceptible groups (Scandinavians)

<i>Hymenolepis nana</i> (dwarf tapeworm) (pathogenic)	Adult worm not normally seen; eggs round to oval with thin shell, containing a 6-hooked embryo or oncosphere with polar filaments lying between the embryo and eggshell	Causes gastrointestinal complaints in some individuals; ingestion of eggs (this is the only life cycle where the intermediate host [grain beetle] can be bypassed); life cycle of egg to larval form to adult can be completed in the human; most common tapeworm in the world
<i>Hymenolepis diminuta</i> (rat tapeworm) (pathogenic)	Adult worm not normally seen; eggs round to oval with thin shell, containing a 6-hooked embryo or oncosphere with no polar filaments lying between the embryo and eggshell	Uncommon; eggs can be confused with <i>H. nana</i> ; eggs will be submitted in proficiency-testing specimens and must be differentiated from <i>H. nana</i>
<i>Echinococcus granulosus</i> (pathogenic)	Adult worm found only in the carnivore (dog); hydatid cysts develop (primarily in the liver) when humans accidentally ingest eggs from the dog tapeworms; cyst contains daughter cysts and many scolices; laboratory should examine fluid aspirated from cyst at surgery	Humans are accidental intermediate hosts; normal life cycle involves sheep and dog, with the hydatid cysts developing in the liver, lungs, etc. of the sheep; the human host may be unaware of the infection unless fluid leaks from the cyst (can trigger an anaphylactic reaction) or pain is felt from the cyst location
<i>Echinococcus multilocularis</i> (pathogenic)	Adult worm found only in the carnivore (fox, wolf); hydatid cysts develop (primarily in the liver) when humans accidentally ingest eggs from the carnivore tapeworms; cyst grows like a metastatic cancer with no limiting membrane	Humans are accidental intermediate hosts; prognosis in this infection is poor; surgical removal of the tapeworm tissue is very difficult; found in Canada, Alaska, and less frequently in the northern U.S., although the geographic range is moving further south and the infection is being seen more often
<b>Trematodes (flukes)</b> <i>Fasciolopsis buski</i> (giant intestinal fluke) (pathogenic)	Eggs found in stool; very large and operculated (morphology like that of <i>F. hepatica</i> eggs)	Symptoms depend on worm burden; acquired from ingestion of plant material on which metacercariae have encysted (water chestnuts); worms hermaphroditic
<i>Fasciola hepatica</i> (sheep liver fluke) (pathogenic)	Eggs found in stool; cannot be differentiated from those of <i>F. buski</i>	Symptoms depend on worm burden; acquired from ingestion of plant material on which metacercariae have encysted (watercress); worms hermaphroditic
<i>Clonorchis sinensis</i> ( <i>Opisthorchis</i> ) (Chinese liver fluke) (pathogenic)	Eggs found in stool; very small (<35 µm); operculated, with shoulders into which the operculum fits	Symptoms depend on worm burden; acquired from ingestion of raw fish; eggs can be missed unless 400× power is used for examination; eggs can resemble those of <i>Metagonimus yokogawai</i> and <i>Heterophyes heterophyes</i> (small intestinal flukes); worms hermaphroditic

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**Table 8.35** Key characteristics of helminths<sup>a,b</sup> (*continued*)

Helminths	Diagnostic stage	Comments
<i>Paragonimus westermani</i> (lung fluke) (pathogenic)	Eggs are coughed up in sputum (brownish "iron filings" are egg packets); can be recovered in sputum or stool (if swallowed); are operculated with shoulders into which operculum fits with a thickened abopercular end	Symptoms depend on worm burden and egg deposition; acquired from ingestion of raw crabs or crayfish; eggs can be confused with those of <i>D. latum</i> ; infections seen in the Orient— <i>infections with P. mexicanus</i> found in Central and South America while infections with <i>P. kellicotti</i> found in North America; worms hermaphroditic but often cross fertilize with another worm if present
<i>Schistosoma mansoni</i> (blood fluke) (pathogenic)	Eggs recovered in stool (large lateral spine); specimens should be collected with no preservatives (to detect egg viability); worms found in veins of large intestine	Acquired from skin penetration by single cercariae from the freshwater snail; pathology caused by body's immune response to eggs in tissues; adult worms in veins cause no problems; adult worms are separate sexes; occasionally, eggs found in urine; both urine and stool should be examined in any patient suspected of having schistosomiasis
<i>Schistosoma haematobium</i> (blood fluke) (pathogenic)	Eggs recovered in urine (large terminal spine); specimens should be collected with no preservatives (to detect egg viability); found in veins of bladder	Acquired from skin penetration of single cercariae from the freshwater snail; pathology worms as with <i>S. mansoni</i> ; 24-h and spot urine samples should be collected; chronic infection has association with bladder cancer; adult worms are separate sexes; occasionally, eggs found in stool; both urine and stool should be examined in any patient suspected of having schistosomiasis
<i>Schistosoma japonicum</i> (blood fluke) (pathogenic)	Eggs recovered in stool (very small lateral spine); specimens should be collected with no preservatives (to detect egg viability); worms found in veins of small intestine	Acquired from skin penetration by multiple cercariae from the freshwater snail; pathology as with <i>S. mansoni</i> ; infection usually the most severe of the three due to original loading infective dose of cercariae from the freshwater snail (multiple cercariae stick together); pathology associated with egg production, which is greatest in <i>S. japonicum</i> infections

<sup>a</sup> Modified from H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, 2nd ed., ASM Press, Washington, DC, 2004.

<sup>b</sup> 1 in. = 2.54 cm; 1 ft = 30.48 cm.

**Table 8.36** Key characteristics of blood parasites<sup>a</sup>

Parasite	Diagnostic stage	Comments
<b>Malaria parasites</b> <i>Plasmodium vivax</i> (benign tertian malaria)	Ameboid rings; presence of Schüffner's dots, beginning in older rings (appear later than in <i>Plasmodium ovale</i> ); all stages seen in peripheral blood; enlarged RBCs; mature schizont contains 16–18 merozoites	Infects young cells; 48-h cycle; large geographic range; tends to have "true relapse" from the residual liver stages (hypnozoites)
<i>Plasmodium ovale</i> (ovale malaria)	Nonameboid rings; presence of Schüffner's dots, beginning in young rings (appear earlier than in <i>Plasmodium vivax</i> ); all stages seen in peripheral blood; enlarged RBCs; mature schizont contains 8–10 merozoites; RBCs may be oval and have fimbriated edges	Infects young cells; 48-h cycle; narrow geographic range; tends to have "true relapse" from residual liver stages (hypnozoites)
<i>Plasmodium malariae</i> (quartan malaria)	Rings are thick; no stippling; all stages seen in peripheral blood; presence of "band forms" and "rosette"-shaped mature schizont containing ca. 8 merozoites; normal to small RBCs; abundant malarial pigment	Infects old cells; 72-h cycle; narrow geographic range; associated with recrudescence and "nephrotic syndrome"; no true relapse
<i>Plasmodium falciparum</i> (malignant tertian malaria)	Multiple rings; appliquéd/accolé forms; no stippling (rare Maurer's clefts); rings and crescent-shaped gametocytes seen in peripheral blood (no other developing stages with the rare exception of the mature schizont); no limits on number of RBCs that can be infected	Infects all cells, 36–48-h cycle; large geographic range; no true relapse; most pathogenic of the four species; plugged capillaries can cause severe symptoms and sequelae (cerebral malaria, lysis of RBCs, etc.)
<i>Babesia</i> spp.	Ring forms only (resemble <i>P. falciparum</i> rings); seen in splenectomized patients; endemic in the U.S. and Europe (no travel history necessary); if present, "Maltese cross" configuration is diagnostic; parasitemia may be higher than that seen with cases of malaria	Tick-borne infection; associated with Nantucket Island; infection mimics malaria; ring forms more pleiomorphic than malaria; more rings/cell (usually) than in malaria; endemic in several areas in the U.S.; organisms occasionally seen outside the RBCs (unlike malaria merozoites); more severe infections seen with European <i>B. divergens</i> and some of species in the western U.S.; less severe infections seen with <i>B. microti</i> in the eastern U.S. (except in compromised patients)
<b>Trypanosomes</b> <i>Trypanosoma brucei gambiense</i> (West African sleeping sickness)	Trypomastigotes long and slender, with typical undulating membrane; lymph nodes and blood can be sampled; microhematocrit tube concentration helpful; CSF sampled in later stages of infection	Tsetse fly vector; tends to be chronic infection, exhibiting the real symptoms of sleeping sickness, often over several months

(continued on next page)

**Table 8.36** Key characteristics of blood parasites<sup>a</sup> (*continued*)

Parasite	Diagnostic stage	Comments
<i>Trypanosoma brucei rhodesiense</i> (East African sleeping sickness)	Trypomastigotes long and slender, with typical undulating membrane; lymph nodes and blood can be sampled; microhematocrit tube concentration helpful; CSF sampled in later stages of infection	Tsetse fly vector; tends to be more severe, short-lived infection (particularly in children); patient may die before progressive symptoms of sleeping sickness appear over weeks rather than months
<i>Trypanosoma cruzi</i> (Chagas' disease) (American trypanosomiasis)	Trypomastigotes short, stumpy, often curved in "C" shape; blood sampled early in infection; trypomastigotes enter striated muscle (heart, gastrointestinal tract) and transform into the amastigote form	Reduviid bug vector ("kissing bug"); chronic in adults, severe in young children; great morbidity associated with cardiac failure and loss of muscle contractility in heart and gastrointestinal tract; organisms and disease now endemic in Texas
<i>Leishmania</i> spp. (cutaneous) <sup>b</sup>	Amastigotes found in macrophages of skin; presence of intracellular forms containing nucleus and kinetoplast diagnostic	Sand fly vector; organisms recovered from site of lesion only; specimens can be stained or cultured in NNN and/or Schneider's medium; animal inoculation (hamster) rarely used
<i>Leishmania braziliensis</i> (mucocutaneous) <sup>b</sup>	Amastigotes found in macrophages of skin and mucous membranes; presence of intracellular forms containing nucleus and kinetoplast diagnostic	Sand fly vector; organisms recovered from site of lesion only; specimens can be stained or cultured in NNN and/or Schneider's medium; animal inoculation (hamster) rarely used
<i>Leishmania donovani</i> (visceral)	Amastigotes found throughout the reticuloendothelial system (spleen, liver, bone marrow, etc.); presence of intracellular forms containing nucleus and kinetoplast diagnostic	Sand fly vector; organisms recovered from buffy coat (rarely found), bone marrow aspirate, spleen or liver puncture (rarely performed); specimens can be stained or cultured in NNN and/or Schneider's medium; animal inoculation (hamster) rarely used; cause of kala azar
<b>Helminths</b> <i>Wuchereria bancrofti</i> (pathogenicity due to adult worms)	Microfilaria sheathed, clear space at end of tail; nocturnal periodicity seen; elephantiasis seen in chronic infections	Mosquito vector; microfilariae recovered in blood (membrane filtration, Knott concentrate, thick films); hematoxylin stains sheath (Giemsa does not stain sheath)
<i>Brugia malayi</i> (pathogenicity due to adult worms)	Microfilaria sheathed, subterminal and terminal nuclei at end of tail; nocturnal periodicity seen; elephantiasis seen in chronic infections	Mosquito vector; microfilariae recovered in blood (membrane filtration, Knott concentrate, thick films); hematoxylin stains sheath (Giemsa stains sheath pink)

<i>Loa loa</i> (African eye worm) (pathogenicity due to adult worms)	Microfilaria sheathed, nuclei continuous to tip of tail; diurnal periodicity; adult worm may cross the conjunctiva of the eye	Mango fly vector; history of calabar swellings; worms in the eye; microfilariae difficult to recover from blood; hematoxylin stains sheath
<i>Mansonella</i> spp. (pathogenicity mild and due to adult worms)	Microfilaria unsheathed, nuclei may or may not extend to tip of tail (depending on species); nonperiodic; symptoms usually absent or mild	Midge or blackfly vector; microfilariae recovered in blood (membrane filtration, Knott concentrate, thick films)
<i>Mansonella streptocerca</i> (pathogenicity mild and due to adult worms and/or microfilariae)	Microfilaria unsheathed, nuclei extend to tip of tail; when immobile, curved like "shepherd's crook"; adults found in dermal tissues	Midge vector; microfilariae found in skin snips; microfilarial tails are split rather than blunt
<i>Onchocerca volvulus</i> (pathogenicity due to microfilariae)	Microfilaria unsheathed, nuclei do not extend to tip of tail; adults found in nodules	Blackfly vector; microfilariae found in skin snips; microfilariae migrate to optic nerve; cause of "river blindness"

<sup>a</sup> Modified from H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, 2nd ed., ASM Press, Washington, DC, 2004.

<sup>b</sup> This is not actually a blood parasite but is presented for comparison with *L. donovani*.

**Table 8.37** Rapid diagnostic procedures

Traditional procedures	Additional options	Comments
<p>Fecal specimens (permanent stained smear)</p> <p>Microscopic examination of stained fecal smear using oil immersion objectives</p> <p><b>Note:</b> The total O&amp;P exam is recommended when the fecal immunoassays (limited to specific organisms) are negative and the patient remains symptomatic.</p>	<p>Methods for fecal specimens include fecal immunoassays (fluorescence/microscopy, EIA/ELISA plates, lateral-membrane-flow immunochromatographic assay/cartridge).</p> <p>Fecal immunoassays are generally simple to perform and allow a large number of tests to be performed at one time, thereby reducing overall costs.</p> <p><i>A major disadvantage of using these assays with stool specimens is that the method can detect only one or two pathogens at a time. A routine O&amp;P exam must be performed to detect other parasitic pathogens.</i></p> <p>The current commercially available antigen tests (DFA, EIA, or lateral-flow cartridges) are more sensitive and more specific than routine microscopy.</p> <p>Current testing is available for <i>E. histolytica</i>, the <i>E. histolytica/E. dispar</i> group, <i>G. lamblia</i>, and <i>Cryptosporidium</i> spp. Diagnostic reagents are also in development for some of the other intestinal protozoa.</p>	<p>Batch testing is possible, the methods use very few steps, and the visual assessment of positive or negative is quite easy.</p> <p>These tests can be very beneficial in the absence of trained microscopists. However, for patients who remain symptomatic after a negative result, the O&amp;P exam should always remain as an option. <i>Both the O&amp;P exam and fecal immunoassays should be part of the laboratory test menu; specific patient histories will dictate correct ordering of the most relevant test options.</i></p> <p>Although tests for <i>E. histolytica</i> or the <i>E. histolytica/E. dispar</i> group are limited to the use of fresh or frozen fecal specimens, they can be helpful in the absence of trained microscopists. Tests for <i>Giardia</i> and/or <i>Cryptosporidium</i> can be performed on fresh, frozen, unfixed, or formalin-fixed (5%, 10%, SAF) fecal specimens. Cary Blair and some of the single vial fecal parasite fixatives can also be used; check with the manufacturer.</p>

<p>Blood specimens (stained blood films)</p> <p>Microscopic examination of stained blood films using oil immersion objectives</p> <p><b>Note:</b> A minimum of 300 oil immersion fields (using the 100× oil immersion objective) must be examined on both thick and thin blood films.</p>	<p>The BinaxNOW malaria test is a rapid immunodiagnostic assay for differentiation and detection of circulating <i>Plasmodium falciparum</i> antigen and the antigen common to all other malarial species (<i>Plasmodium vivax</i>, <i>Plasmodium ovale</i>, and <i>Plasmodium malariae</i>) in whole blood. It was FDA approved in June 2007.</p> <p>Test line 1 positive = <i>P. falciparum</i>      Test line 2 positive = <i>P. vivax</i>, <i>P. malariae</i>, or <i>P. ovale</i>      Test lines 1 and 2 positive = <i>P. falciparum</i> and possible mixed infection</p>	<p>This product is positive at 0.1% (5,000/<math>\mu</math>l) sensitivity and specificity data according to the manufacturer (Inverness<sup>a</sup>):</p> <p>Sensitivity          99.7% (<i>P. falciparum</i>)          93.5% (<i>P. vivax</i>)</p> <p>Specificity          94.2% (<i>P. falciparum</i>)          99.8% (<i>P. vivax</i>)</p> <p>It is important to remember that many patients may be seen who have a much lower parasitemia than the level of detection of the malaria rapid test. Their parasitemia may be as low as 0.01%. Other tests are in use throughout the world but are not yet FDA approved.</p>
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<sup>a</sup> Inverness Medical Innovations, Inc., 51 Sawyer Road, Suite 200, Waltham, MA 02451.

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**Identification Key 8.1** Key to the identification of intestinal amebae  
(permanent stained smear)

1. Trophozoites present Cysts present	2 7	
2. Trophozoites measure $>12 \mu\text{m}$ Trophozoites measure $<12 \mu\text{m}$	3 4	
3. Karyosome central, compact; peripheral nuclear chromatin evenly arranged; "clean" cytoplasm (RBCs present or absent) Karyosome eccentric, spread out; peripheral nuclear chromatin unevenly arranged; "dirty" cytoplasm		<i>Entamoeba histolytica</i> <sup>a</sup>
		<i>Entamoeba coli</i>
4. Peripheral nuclear chromatin Other than above	5 6	
5. Karyosome central, compact; peripheral nuclear chromatin evenly arranged; "clean" cytoplasm Karyosome large, blot-like; extensive nuclear variation		<i>Entamoeba hartmanni</i>
		<i>Endolimax nana</i>
6. No peripheral chromatin, karyosome large, junky cytoplasm No peripheral chromatin, karyosome variable, clean cytoplasm		<i>Iodamoeba bütschlii</i>
		<i>Endolimax nana</i>
7. Cysts measure $>10 \mu\text{m}$ (including any shrinkage "halo") Cysts measure $<10 \mu\text{m}$ (including any shrinkage "halo")	8 10	
8. Single <i>Entamoeba</i> -like nucleus with large inclusion mass Multiple nuclei		<i>Entamoeba polecki</i> <sup>b</sup>
		9
9. 4 <i>Entamoeba</i> -like nuclei, chromatoidal bars have smooth, rounded ends $\geq 5$ <i>Entamoeba</i> -like nuclei, chromatoidal bars have sharp, pointed ends		<i>Entamoeba histolytica</i> <sup>a</sup>
		<i>Entamoeba coli</i>
10. Single nucleus (may be "basket" nucleus), large glycogen vacuole Multiple nuclei		<i>Iodamoeba bütschlii</i>
		11
11. 4 <i>Entamoeba</i> -like nuclei, chromatoidal bars have smooth, rounded ends (nuclei may also number only 2) 4 karyosomes, no peripheral chromatin, round to oval shape		<i>Entamoeba hartmanni</i>
		<i>Endolimax nana</i>

<sup>a</sup> *Entamoeba histolytica* = *Entamoeba histolytica*/*Entamoeba dispar*. *E. histolytica* (pathogen) can be determined by finding RBCs in the cytoplasm of the trophozoites. Otherwise, on the basis of morphological grounds *E. histolytica* (pathogen) and *E. dispar* (nonpathogen) cannot be differentiated and should be reported as *Entamoeba histolytica*/*E. dispar*.

<sup>b</sup> It is very difficult to differentiate *Entamoeba polecki* trophozoites from *E. histolytica* or *E. coli*.

### Identification Key 8.2 Key to the identification of intestinal flagellates

1. Trophozoites present Cysts present	2 7
2. Pear shaped Other shape	3 6
3. 2 nuclei, sucking disk present 1 nucleus present	<i>Giardia lamblia</i> 4
4. Costa length of body No costa	<i>Pentatrichomonas hominis</i> 5
5. Cystostome present, >10 $\mu\text{m}$ Cystostome present, <10 $\mu\text{m}$	<i>Chilomastix mesnili</i> <i>Retortamonas intestinalis</i> or <i>Enteromonas hominis</i>
6. Amoeba shaped, 1 or 2 fragmented nuclei Oval shaped, 1 nucleus	<i>Dientamoeba fragilis</i> <i>Enteromonas hominis</i>
7. Oval- or round-shaped cyst Lemon-shaped cyst	8 9
8. 4 nuclei, median bodies, axoneme, >10 $\mu\text{m}$ 2 nuclei, no fibrils, <10 $\mu\text{m}$	<i>Giardia lamblia</i> <i>Enteromonas hominis</i>
9. 1 nucleus, "shepherd's crook" fibril 1 nucleus, "bird's beak" fibril	<i>Chilomastix mesnili</i> <i>Retortamonas intestinalis</i>

### Identification Key 8.3 Key to the identification of helminth eggs<sup>a</sup>

1. Eggs nonoperculate (no "trap door"), spherical or subspherical, containing a 6-hooked embryo (oncosphere); thick or thin shell Eggs other than described above	2 5
2. Eggs passed separately Eggs passed in packets of 12 or more	3 <i>Dipylidium caninum</i> (dog tapeworm)
3. Thick, radially striated shell (6-hooked oncosphere may not be visible in every egg from formalinized fecal specimens) (eggs cannot be identified to species level without special stains)  Thin shell, clear space between shell and developing embryo	<i>Taenia</i> spp. ( <i>T. saginata</i> , beef tapeworm; <i>T. solium</i> , pork tapeworm) 4
4. Polar filaments (filamentous strands) present between thin shell and 6-hooked embryo No polar filaments (filamentous strands) present between shell and embryo; somewhat larger	<i>Hymenolepis nana</i> (dwarf tapeworm) <i>Hymenolepis diminuta</i> (rat tapeworm)
5. Egg operculate, generally oval ("trap door" at one end of egg) Egg nonoperculate, generally oval	6 10
6. Egg <35 $\mu\text{m}$ long  Egg $\geq 38 \mu\text{m}$ long	<i>Clonorchis</i> ( <i>Opisthorchis</i> ) spp. (Chinese liver fluke), <i>Heterophyes</i> <i>heterophyes</i> , <i>Metagonimus yokogawai</i> 7 (continued on next page)

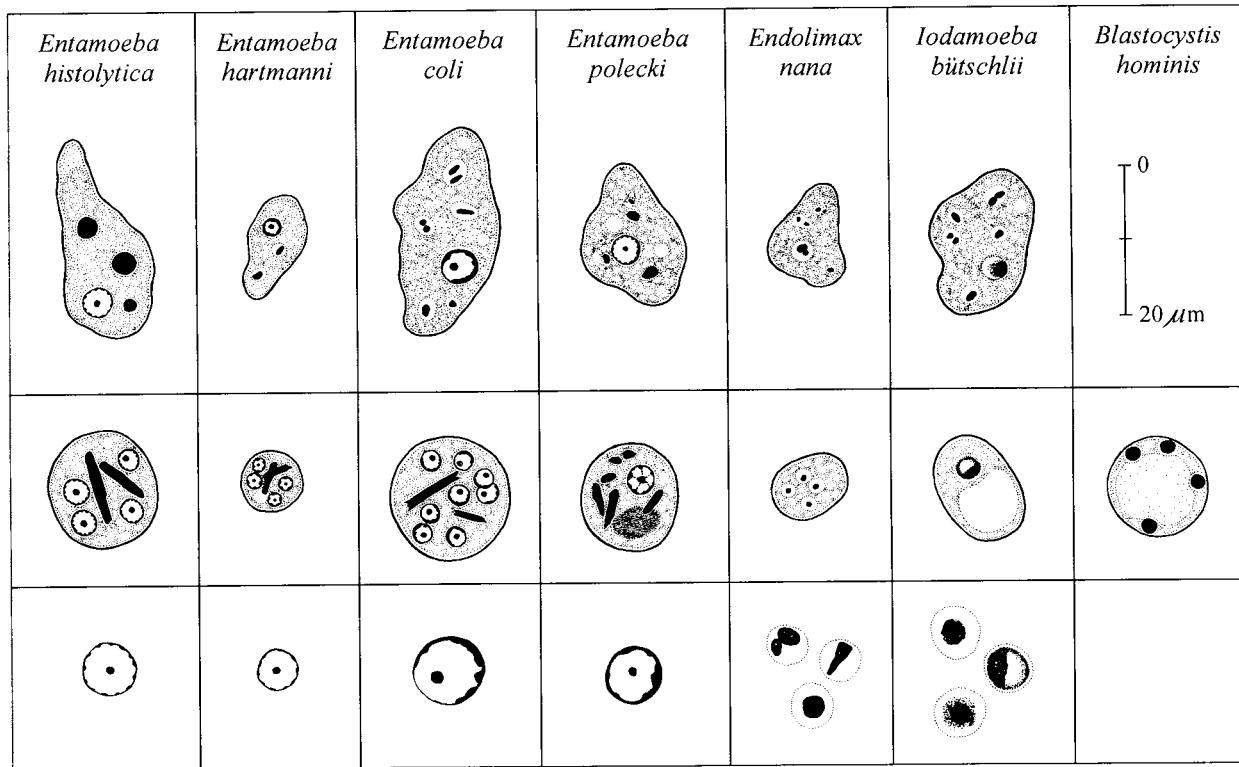
### Identification Key 8.3 (continued)

7. Egg 38–45 $\mu\text{m}$ long	<i>Dicrocoelium dendriticum</i>
Egg >60 $\mu\text{m}$ long	8
8. Egg with opercular shoulders into which the operculum fits (looks like teapot lid and flange into which lid fits), abopercular end thickened (sometimes hard to see)	<i>Paragonimus</i> spp. (lung fluke)
Egg without opercular shoulders	9
9. Egg >85 $\mu\text{m}$ long, operculum break in shell sometimes hard to see—smooth transition from shell to operculum	<i>Fasciolopsis buski</i> (giant intestinal fluke), <i>Fasciola hepatica</i> (sheep liver fluke), <i>Echinostoma</i> spp.
Egg <75 $\mu\text{m}$ long, operculum break in shell sometimes hard to see—smooth transition from shell to operculum	<i>Diphyllobothrium latum</i> (broad fish tapeworm)
10. Egg $\geq 75 \mu\text{m}$ long, spined, ciliated miracidium larva may be seen	11
Egg <75 $\mu\text{m}$ long, not spined	13
11. Spine terminal (check for egg viability)	<i>Schistosoma haematobium</i> (blood fluke, from urine)
Spine lateral	12
12. Lateral spine very short (hard to see) (check for egg viability)	<i>Schistosoma japonicum</i> (blood fluke, from stool)
Lateral spine prominent and easily seen (check for egg viability)	<i>Schistosoma mansoni</i> (blood fluke, from stool)
13. Egg with thick, tuberculated (mammilated/bumpy) capsule (in decorticate egg, capsule is absent—occurs in both fertilized and unfertilized eggs)	<i>Ascaris lumbricoides</i> (large roundworm)
Egg without thick, tuberculated capsule	14
14. Egg barrel shaped, with clear polar plugs	15
Egg not barrel shaped, no polar plugs	16
15. Shell nonstriated	<i>Trichuris trichiura</i> (whipworm)
Shell striated	<i>Capillaria hepatica</i>
16. Egg flattened on one side, may contain larva	<i>Enterobius vermicularis</i> (pinworm)
Egg symmetrical	17
17. Egg with large blue-green globules at poles	<i>Heterodera marioni</i>
Egg without polar globules	18
18. Egg bluntly rounded at ends, 56–76 $\mu\text{m}$ long, thin shell (contains developing embryo at 8–16-cell stage of development)	Hookworm
Egg pointed at one or both ends, 73–95 $\mu\text{m}$ long	<i>Trichostrongylus</i> spp.

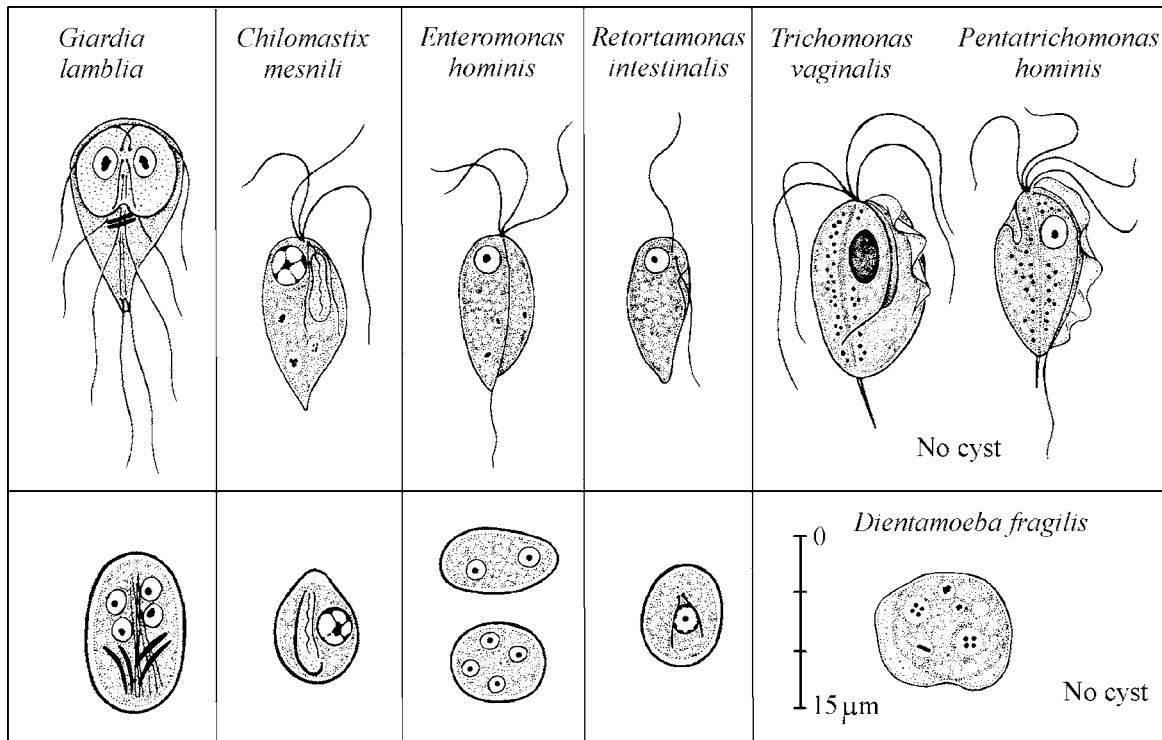
<sup>a</sup> Adapted from E. K. Markell, M. Voge, and D. T. John, *Medical Parasitology*, 7th ed., The W. B. Saunders Co., Philadelphia, PA, 1992.

**Identification Key 8.4** Key to the identification of microfilariae

1. Larvae sheathed Larvae unsheathed	2 <i>Mansonella ozzardi</i> , <i>Mansonella perstans</i>
2. Tail nuclei do not extend to the tip of the tail Tail nuclei extend to the tip of the tail	3 <i>Wuchereria bancrofti</i>
3. Nuclei not continuous (two nuclei at the tip of the tail) Nuclei form continuous row in the tail	<i>Brugia malayi</i> <i>Loa loa</i>

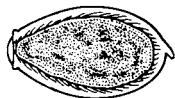


**Figure 8.1** Intestinal amebae of humans. Top row: Trophozoites. *Entamoeba histolytica* shown with ingested RBCs. This is the only microscopic finding allowing differentiation of pathogenic *E. histolytica* from nonpathogenic *E. dispar*; all cysts and trophozoites containing no RBCs must be reported as *Entamoeba histolytica/E. dispar*. An ameboid form of *Blastocystis hominis* is rarely seen and difficult to identify. Middle row: Cysts. For *B. hominis* the central-body form is depicted. Bottom row: Trophozoite nuclei, shown in relative proportion. (From A. L. Leber and S. M. Novak, in P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaffer, ed., *Manual of Clinical Microbiology*, 9th ed., ASM Press, Washington, DC, 2007.)



**Figure 8.2** Intestinal and urogenital flagellates of humans. Top row: Trophozoites. *Trichomonas vaginalis* is found in urogenital sites; all other flagellates are intestinal. Bottom row: Cysts. *Dientamoeba fragilis* trophozoites shown; no cyst stage. (From A. L. Leber and S. M. Novak, in P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller, ed., *Manual of Clinical Microbiology*, 9th ed., ASM Press, Washington, DC, 2007.) (Illustration by Sharon Belkin.)

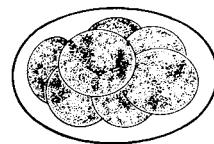
**Figure 8.3** Helminth eggs depicted in the order of size (smallest to largest)—see *following two pages*.



*Clonorchis sinensis*

27-35  $\mu\text{m}$  long

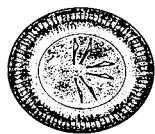
12-19  $\mu\text{m}$  wide



Hookworm

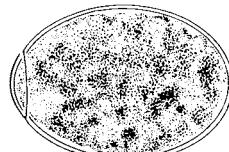
56-75  $\mu\text{m}$  long

36-40  $\mu\text{m}$  wide



*Taenia* spp.

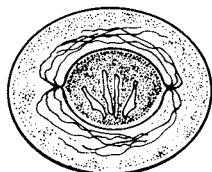
31-43  $\mu\text{m}$  diameter



*Diphyllobothrium latum*

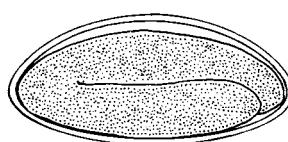
58-75  $\mu\text{m}$  long

40-50  $\mu\text{m}$  wide



*Hymenolepis nana*

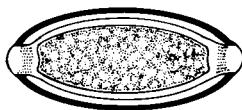
30-47  $\mu\text{m}$  diameter



*Enterobius vermicularis*

70-85  $\mu\text{m}$  long

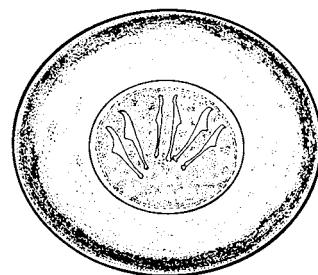
60-80  $\mu\text{m}$  wide



*Trichuris trichiura*

50-54  $\mu\text{m}$  long

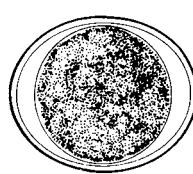
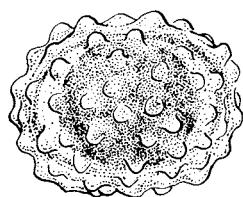
20-23  $\mu\text{m}$  wide



*Hymenolepis diminuta*

70-85  $\mu\text{m}$  long

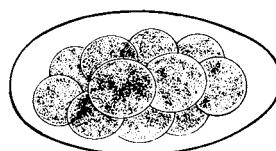
60-80  $\mu\text{m}$  wide



*Ascaris lumbricoides* (fertile egg)

45-75  $\mu\text{m}$  long

35-50  $\mu\text{m}$  wide



*Trichostrongylus*

73-95  $\mu\text{m}$  long

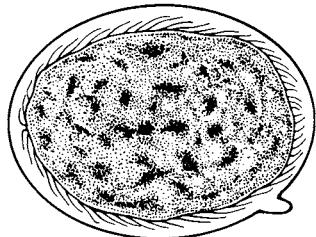
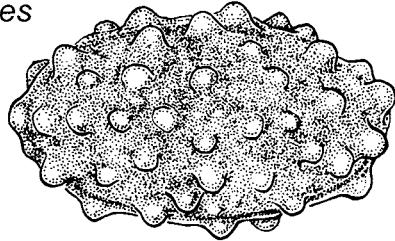
40-50  $\mu\text{m}$  wide

*Ascaris lumbricoides*

(unfertilized egg)

85-95  $\mu\text{m}$  long

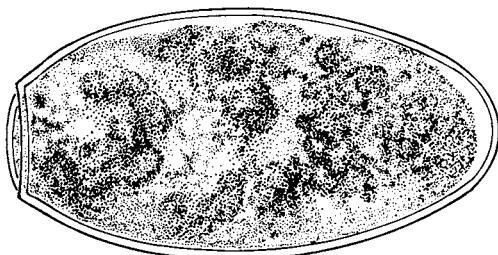
43-47  $\mu\text{m}$  wide



*Schistosoma japonicum*

70-100  $\mu\text{m}$  long

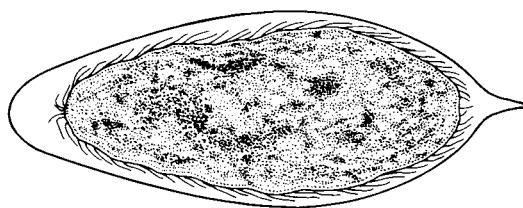
55-65  $\mu\text{m}$  wide



*Paragonimus westermani*

80-120  $\mu\text{m}$  long

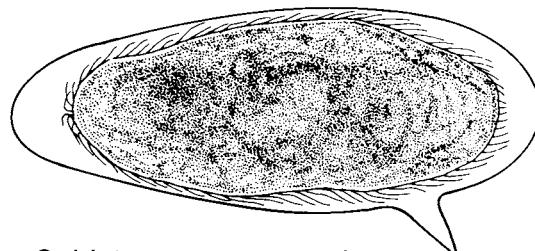
48-60  $\mu\text{m}$  wide



*Schistosoma haematobium*

112-170  $\mu\text{m}$  long

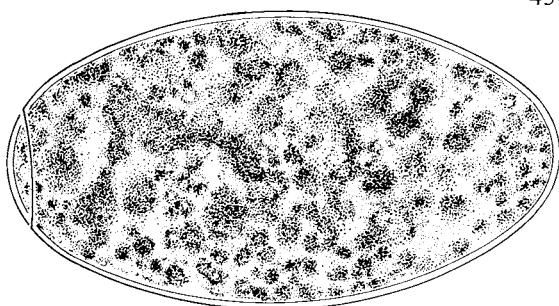
40-70  $\mu\text{m}$  wide



*Schistosoma mansoni*

114-180  $\mu\text{m}$  long

45-70  $\mu\text{m}$  wide

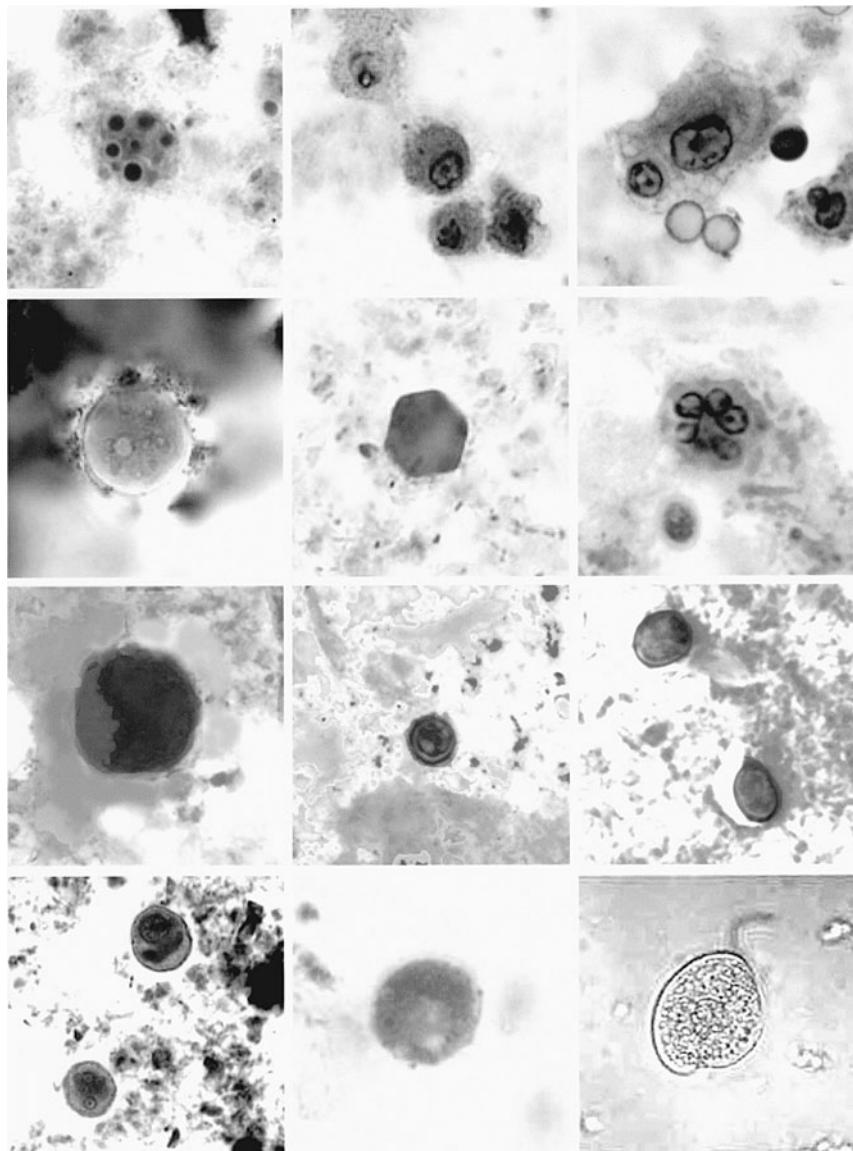


*Fasciola hepatica*  
or *Fasciolopsis buski*

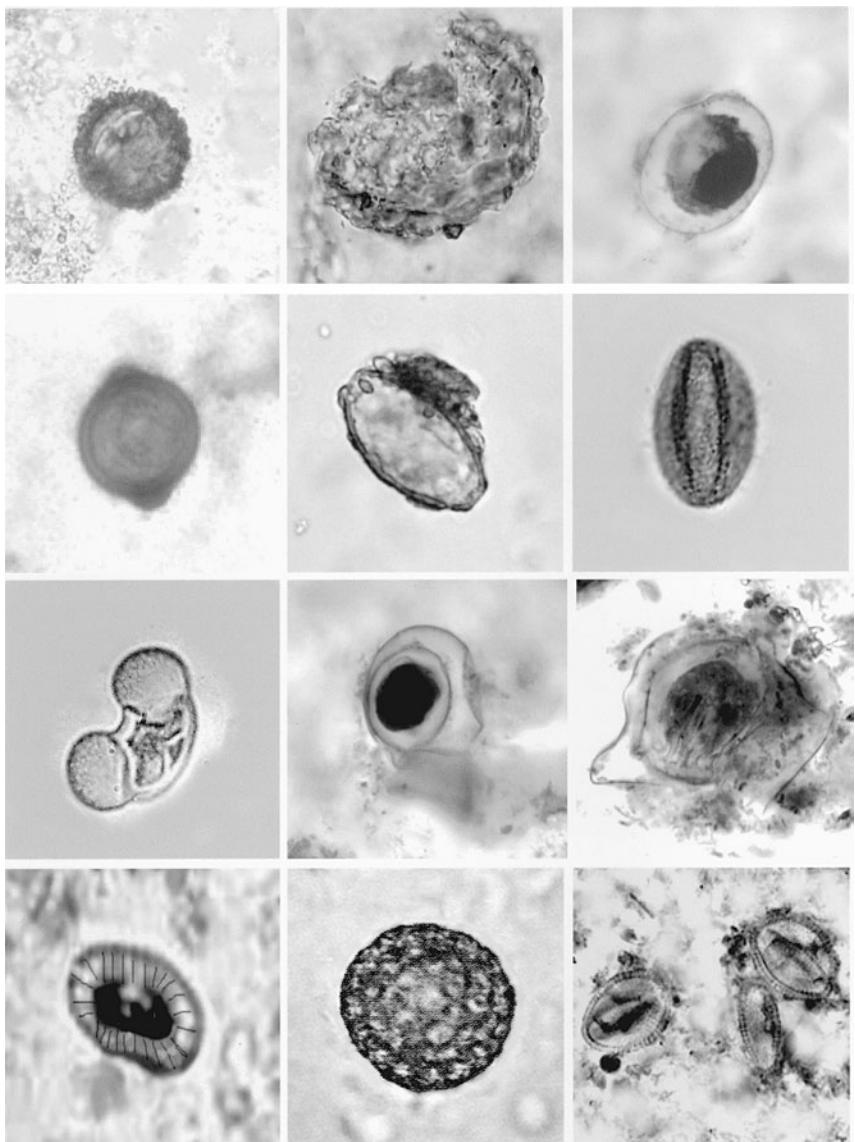
130-140  $\mu\text{m}$  long

80-85  $\mu\text{m}$  wide

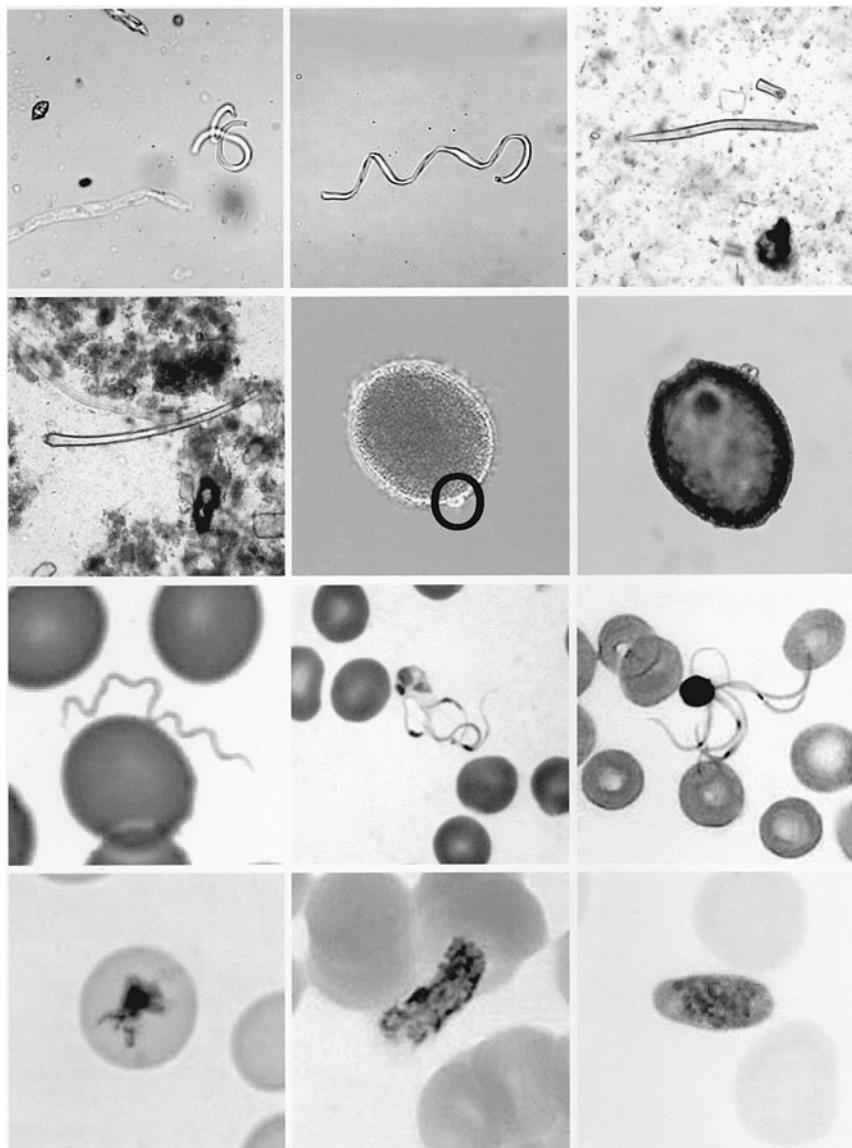
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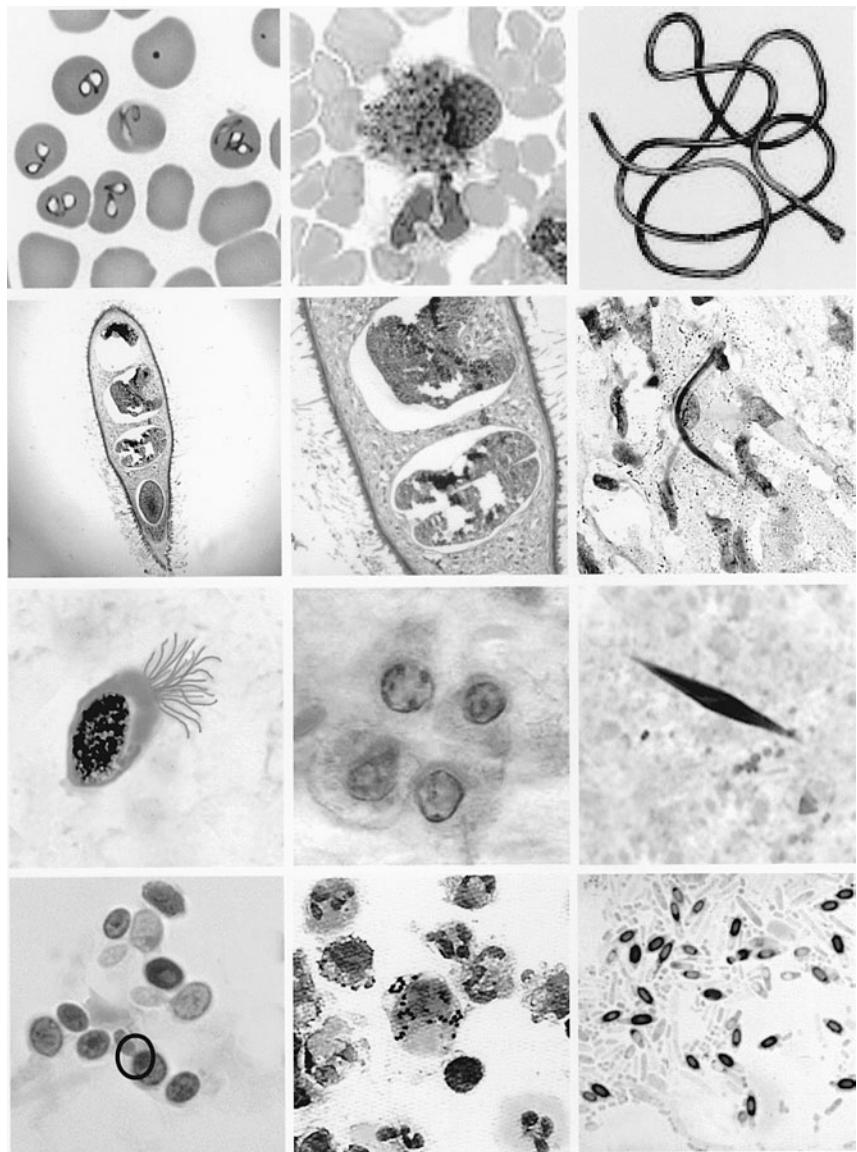
**Plate 8.1** Row 1: artifact, human macrophages (the artifact and human cells mimic intestinal amebae); row 2: artifacts tend to mimic intestinal amebae, possibly *Entamoeba coli*; row 3: artifact (*Cyclospora cayetanensis* oocyst), artifact (*Cryptosporidium* spp. oocyst), spores (*Cryptosporidium* spp. oocysts); row 4: amebic precysts (can be confused with artifacts), pale "organism" (appears to have a nucleus), epithelial cell (can mimic a larger protozoan like *Balantidium coli*).



**Plate 8.2** Row 1: first two images are artifacts that mimic *Ascaris lumbricoides* eggs, third image is an artifact that mimics a hookworm egg; row 2: artifacts tend to mimic *Trichuris trichiura* eggs; row 3: first image is pollen (if turned correctly, can mimic *Trichuris trichiura* egg), next two images are artifacts (suggestive of *Hymenolepis nana* eggs); row 4: artifact (can be misidentified as *Taenia* egg), pollen (also mimics *Taenia* egg), artifacts (can mimic helminth eggs).



**Plate 8.3** Row 1: threads (mimic small worms or helminth larvae), root hair (mimics *Strongyloides stercoralis* larva); row 2: root hair (mimics *Strongyloides stercoralis* larvae), artifact (mimics *Diphyllobothrium latum* egg), artifact (mimics helminth egg, possibly trematode egg with operculum missing); row 3: the first image is an artifact (mimics organism in blood), the next two images represent exflagellation of male *Plasmodium* gametocytes (can mimic spirochetes in blood); row 4: artifact (stain deposition can mimic *Plasmodium* spp.), flattened *Plasmodium vivax* trophozoite (shape can mimic crescent-shaped gametocyte of *Plasmodium falciparum*), immature *Plasmodium falciparum* gametocyte (may not be recognized as an actual parasite).



**Plate 8.4** Row 1: artifacts (mimic *Babesia* spp.), *Histoplasma capsulatum* (mimics *Leishmania donovani* amastigotes), *Gordius* (can mimic actual parasitic worms); row 2: first two images are seeds (mimic helminth histologic sections), third image is thread in tissue (mimics microfilarial nematode); row 3: ciliated human cell (mimics “unknown” flagellate), human cells (can mimic protozoa or amebae), Charcot-Leyden crystal (related to breakdown products of eosinophils—may not be recognized); row 4: yeast (if budding is not recognized, can be misidentified as small protozoa or coccidia), Gram stain showing intracellular microsporidial spores (can be misidentified as bacteria), artifact that may be bacterial spores (may be misidentified as microsporidial spores).

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