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MEMORIAM

John Baker (1931–2013)

It is with great sadness that we report that John Baker, a former editor of *Advances in Parasitology*, died on 23 August 2013.

Many past authors will have known John as a meticulous editor with a keen eye for detail. With a quiet determination and a guiding hand, he worked closely with authors to ensure that their finished manuscript would be of the highest quality. His contribution dates back to 1978 when he joined forces with Ralph Muller and W.H.R. Lumsden as a co-editor of the series. His long-standing editorship spanned from Volume 16 all the way through to Volume 64 in 2007. John ensured that 'Advances' maintained a high standard and he helped to build the outstanding reputation of the series for authoritative and state-of-the-art reviews taking personal responsibility for the majority of the protist papers.

John shared his great interest in the biology of parasites with his many friends and colleagues. He contributed enormously to the discipline with outstanding scientific research particularly on the biology of trypanosomes and authored several textbooks on parasitic protozoa and medical parasitology. His editorial skills were in much demand, and later in his career he worked as the editor of the *Transactions of the Royal Society of Tropical Medicine and Hygiene*.

John was a quiet and most charming man, indeed a gentleman of the old school. Always modest, he would arrive at editorial meetings in London on his trusty old bicycle with enormous enthusiasm for the forthcoming volume and a characteristic smile that could help solve most problems. John will be greatly missed by his family, friends and colleagues and by many parasitologists around the world.

CHAPTER ONE

Joint Infectious Causation of Human Cancers

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Contents

Abstract

Joint infectious causation of cancer has been accepted in a few well-studied instances, including Burkitt's lymphoma and liver cancer. In general, evidence for the involvement of parasitic agents in oncogenesis has expanded, and recent advances in the application of molecular techniques have revealed specific mechanisms by which host cells are transformed. Many parasites evolve to circumvent immune-mediated detection and destruction and to control critical aspects of host cell reproduction and survival: cell proliferation, apoptosis, adhesion, and immortalization. The host has evolved tight regulation of these cellular processes—the control of each represents a barrier to cancer.

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These barriers need to be compromised for oncogenesis to occur. The abrogation of a barrier is therefore referred to as an *essential cause* of cancer. Alternatively, some aspects of cellular regulation restrain but do not block oncogenesis. Relaxation of a restraint is therefore referred to as an *exacerbating cause* of cancer. In this chapter, we explore past and current evidence for joint infectious causation of cancer in the context of essential and exacerbating causes. We stress that discovery of joint infectious causation may provide great improvements in controlling cancer, particularly through the identification of many additional nonhuman targets for synergistic interventions for prevention and treatment.

1. INTRODUCTION

Evidence implicating parasites (defined broadly to include multicellular, cellular, and subcellular agents) as causes of cancer has been accumulating and broadening for over a century. The first major advance was reported in 1910, when Rous showed that a nonfilterable agent caused a cancer of chickens, Rous sarcoma (Rous, 1910, 1911). Although Rous's finding and the subsequent identification of the Rous sarcoma virus (RSV) was slow to influence research on human cancer, it led to discoveries of viral causes of mammalian cancer during the middle third of the twentieth century: a papillomavirus in rabbits (Shope and Hurst, 1933), mouse mammary tumour virus (MMTV) in mice (Bittner, 1942), murine leukaemia virus in mice (Gross, 1951), and simian virus 40 in hamsters (Rabson and Kirschstein, 1962).

In 1964, Epstein reported the first viral cause of a human cancer (Epstein et al., 1964). The virus is now known as the Epstein–Barr virus (EBV) and the cancer as endemic Burkitt's lymphoma. Prior to this discovery, Epstein had been working on RSV but was searching for viral causes of human cancer (Epstein, 2005). In 1962, Epstein attended a talk given by Burkitt, who had recently defined cases of Burkitt's lymphoma (Burkitt, 1958). In the lecture, Burkitt described the epidemiological correlation between Burkitt's lymphoma and mosquito density. Thinking that malaria was a cause of Burkitt's lymphoma, Burkitt suggested *Plasmodium falciparum* as an aetiological agent (Kafuko and Burkitt, 1970). Epstein tested lymphoma samples sent to him by Burkitt for viral infection and soon discovered EBV (Epstein et al., 1964). Since then, the overall trend has been towards acceptance of the idea that these pathogens jointly cause endemic Burkitt's lymphoma. In tropical medicine, joint causation has been accepted by

experts for decades (e.g. Manson-Bahr and Apted, 1982, p. 12), but a causal role for *P. falciparum* has been questioned by some, largely because the incriminating evidence has been epidemiological (Carpenter et al., 2008; Orem et al., 2007). Recent work, however, has demonstrated the co-occurrence of serological positivity for EBV and malaria in Burkitt's lymphoma patients (Carpenter et al., 2008; Mutalima et al., 2008). This finding together with studies documenting activation of EBV by *P. falciparum* has reinforced what is now a widely held conclusion that these two pathogens act as cofactors in the aetiology of endemic Burkitt's lymphoma (Chêne et al., 2009; Molyneux et al., 2012; Rochford et al., 2005).

Over the same decades during which infectious correlates of endemic Burkitt's lymphoma were being recognized, trematode infections were implicated in causing human cancer—opisthorchids for cholangiocarcinoma (liver-associated cancers originating from gall bladder ductal cells) and *Schistosoma haematobium* for bladder cancer (Gelfand et al., 1967; Hou, 1956; Manson-Bahr and Apted, 1982; Mustacchi and Shimkin, 1958). Since then, causal roles for these helminths have been generally accepted, and attention has shifted to an increasing variety of cellular and especially viral agents (Afzan and Suresh, 2012; Bouvard et al., 2009; Ewald, 2009; Ferreri et al., 2009; Lax and Thomas, 2002; Samaras et al., 2010; Thomas et al., 2012; Zhang and Begg, 1994; zur Hausen, 2010). Currently, infectious causation is accepted for about 20% of human cancer (de Martel et al., 2012; zur Hausen, 2008). This percentage may grossly underestimate the true extent of infection-induced cancers because infectious causation has not been adequately evaluated for most human cancers and can be ruled out for only a very small portion.

The growing list of infectious agents associated with human cancer draws attention to the need to clarify their roles and mechanisms. Are the associated parasites contributing to oncogenesis? If so, are they instigating or exacerbating influences, and when more than one parasite is implicated in a particular cancer, are they acting separately or in concert? For most cellular parasites that are associated with cancer, causal roles are still uncertain. For the few that are generally accepted or strongly suspected causes of human cancer, such as typhoid *Salmonella*, *Helicobacter pylori*, *P. falciparum*, and opisthorchid trematodes (Bhandari and Crowe, 2012; McColl, 2010; Nath et al., 2010; Samaras et al., 2010), the exact mechanisms are still not well understood.

Although Burkitt's lymphoma was the first human cancer for which joint infectious causation was accepted, it has been viewed more as an anomaly

than a model for understanding oncogenesis. More generally, joint infectious causation of cancer has not been a central focus of cancer research, perhaps because the involvement of parasites acting individually has been so contentious that scientists have been hesitant to propose joint infectious causation. We believe that this neglected area of research may be critically important for understanding the oncogenesis and the range of feasible options for treatment and prevention.

Targeting parasites tends to be safer and more effective than targeting human biomolecules, because natural selection inevitably leads to interconnectedness of human biochemical processes and because human molecules that are contributing to cancer will tend to have important normal functions (Ewald and Swain Ewald, 2011, 2012). Discovering additional infectious causes for a cancer will generate more nonhuman targets. But the effectiveness of interventions against cancers caused jointly by different parasites depends on the way in which each contributes to oncogenesis.

Inflammation, whether initiated by infection, autoimmunity, or mutation associated with inflammatory pathways, has been implicated in cancer. Once triggered, activation of transcription factors, such as STAT3 and NF-kB, leads to increased production of proinflammatory cytokines and chemokines and recruitment of inflammatory cells such as macrophages and neutrophils. This proinflammatory environment has been associated with increased cell proliferation and survival, inhibition of adaptive immunity, angiogenesis, and metastasis (for an overview, see Mantovani et al., 2008). Mechanisms of cancer promotion may include damage to DNA associated with reactive oxygen species, inflammation-initiated epigenetic modifications, and alteration of signalling pathways involved in cell cycle and apoptosis (Trinchieri, 2012). In support of the role of inflammation and cancer, the use of nonsteroidal anti-inflammatory drugs has been correlated with reduced risk and decreased mortality for certain cancers (Mantovani et al., 2008; Trinchieri, 2012).

These aspects of inflammatory processes can all be initiated by infection, and it is often assumed that parasites exacerbate cancer by stimulating inflammation. Some aspects of this presumption are supported by molecular evidence (e.g. Moss and Blaser, 2005; Nath et al., 2010; Trinchieri, 2012). But the complexity of immunologic interactions associated with infection makes it difficult to determine the actual contributions of inflammation and infection to oncogenesis.

The historical tendency to emphasize inflammation has led to interpretations that are at odds with current evidence. It is still claimed, for example,

that inflammation rather than direct viral induction of cell transformation is the mechanism by which hepatitis viruses foster oncogenesis (Trinchieri, 2012). Molecular evidence, however, shows that hepatitis B and C viruses directly compromise anticancer mechanisms employed by host cells (Ewald and Swain Ewald, 2012). Distinguishing between these alternatives has implications for medical intervention because it bears on the value of immunomodulatory treatment as opposed to targeting of causal pathogens.

In this chapter, we explore past and current evidence for the generation of cancer through the joint activity of two or more parasites. Our goal is to clarify the roles played by coinfecting parasites, facilitate the discovery of joint causation, and suggest implications for treating and preventing cancer.

2. ESSENTIAL AND EXACERBATING CAUSES

Parasites may influence development and progression of cancer by different mechanisms, which may vary in their importance. We suggest that an integrated understanding of these mechanisms can be fostered by the barrier theory of cancer, an evolutionary framework for understanding oncogenesis (Ewald and Swain Ewald, 2013).

This framework is built on the distinction between barriers and restraints (Ewald and Swain Ewald, 2013). Barriers prevent oncogenesis. Important barriers are cell-cycle arrest, apoptosis, repression of telomerase, cell adhesion, and asymmetric cell division. When barriers are in place and functioning, oncogenesis is thwarted. A cell cannot become metastatically cancerous if it is not dividing, is destroyed by apoptosis, has only a limited number of future cell divisions, or remains adherent to adjacent cells. Whether these barriers are in place, however, depends on the cell type (Ewald and Swain Ewald, 2013). Asymmetric division, for example, is a barrier for stem cells but not for most other cell types,

Restraints are mechanisms that impede but do not prevent oncogenesis. Mechanisms that suppress angiogenesis, for example, are restraints because oncogenesis can proceed even without altering control of angiogenesis. The reason is that angiogenesis is normally initiated in response to physiological changes associated with precancerous cell growth, such as reduced oxygen concentration. Similarly, regulation of the rate of proliferation in dividing cells is a restraint because oncogenesis can still proceed in a slowly dividing cell.

This separation of barriers from restraints provides the basis for a distinction between essential and exacerbating causes of cancer. Oncogenic events

that abrogate barriers are essential causes of cancer. Those that relax restraints are exacerbating causes. Distinguishing essential from exacerbating causes is important because the blocking of essential causes will prevent cancer. In contrast, blocking exacerbating causes will tend to hinder oncogenesis or the damage associated with the cancer.

Three selective processes provide evolutionary structure to this conceptual framework for understanding oncogenesis (Ewald and Swain Ewald, 2013). Natural selection acts on multicellular organisms to create and adjust barriers and restraints. Natural selection may also act on parasites to compromise barriers and restraints when these protective mechanisms inhibit the ability of parasites to multiply and persist within a host. A third selective process, oncogenic selection, causes normal cells to evolve into cancerous cells as well as evolutionary changes in cancer cells.

Parasites may contribute to oncogenesis in two general ways. They may haphazardly disturb the normal functioning of cells and tissues, for example, by altering the presence of mutagenic compounds or proliferative signals during inflammatory reactions. Alternatively, they may have evolved specific mechanisms to compromise barriers and restraints. The former category has generally been presumed to be the main route by which parasites contribute to oncogenesis. This presumption, however, is tenuous because natural selection can generate sophisticated mechanisms by which parasites interfere with barriers or restraints. Indeed, investigations of tumour virus proteins have revealed precise mechanisms for abrogating barriers to oncogenesis. All well-studied viruses that have been accepted as direct causes of human cancer—human papillomavirus (HPV), EBV, Kaposi sarcoma-associated herpesvirus (KSHV), human T-lymphotropic virus type 1 (HTLV1), hepatitis B virus (HBV), and hepatitis C virus (HCV)—are known to encode proteins that directly compromise cell-cycle arrest, apoptosis, telomerase regulation, and cell adhesion (reviewed by Ewald and Swain Ewald, 2012). These barriers to cancer are also barriers to long-term, productive persistence of the viruses within hosts (Ewald and Swain Ewald, 2013). Natural selection acting on viruses to favour persistence therefore appears to cause infected cells to be pushed towards the brink of cancer, with additional mutations being necessary to complete the transformation.

Being intracellular parasites with capabilities to alter cell replication, viruses are the most obvious infectious candidates for encoding multiple essential causes of cancer. Cellular parasites, however, might also benefit by compromising barriers to cancer. If intracellular bacteria or protozoa can replicate along with the host cell, they, like viruses, could benefit from

making host cells proliferate. Typhoid *Salmonella* may be an example of such an intracellular parasite. It is strongly associated with liver and gall bladder cancers and weakly with pancreatic, colorectal, and lung cancers (Nath et al., 2010; Samaras et al., 2010). Similarly, *H. pylori*, which can infect intracellularly or extracellularly (Deen et al., 2013), contributes to two cancers of the stomach: gastric cancer and mucosa-associated lymphoid tissue lymphomas. It enhances telomerase activity (Chung et al., 2002; Hur et al., 2000; Kameshima et al., 2000) but has complex sometimes contradictory effects on other barriers (Cheng et al., 2013; Wang et al., 2012a); for example, it encodes a protein that exerts antiapoptotic effects that counter the host cell's apoptotic responses to another one of its proteins (Oldani et al., 2009). *H. pylori* also relaxes restraints by stimulating proinflammatory and growth factor signalling (Ashktorab et al., 2007; Suzuki et al., 2009). How all of these effects interact and offset each other is still unclear, but on balance, the evidence suggests that *H. pylori* can compromise at least three of the four barriers to oncogenesis that are abrogated by tumour viruses. The curative effects of antibiotic treatment on *H. pylori*-induced stomach cancers (Gisbert and Calvet, 2011; Kuo et al., 2012; Nakamura et al., 2012) indicate that *H. pylori* abrogates barriers rather than just restraints.

This conceptual framework should be useful for developing our understanding of joint infectious causation in two important ways. First, it allows for an assessment of the relative roles of different infections that contribute to a particular cancer and hence the relative value of targeting the different parasites. If one parasite functions as an exacerbating cause and the other as an essential cause, targeting the former will tend to be less effective than targeting the latter. Second, it helps identify important parts of the causal picture that are missing. These missing parts could involve insufficient knowledge about the mechanism of causation for particular parasites or the presence of an additional cause in the oncogenic process. These ideas are discussed later with reference to particular examples of demonstrated or suspected infectious causation.

3. JOINT ESSENTIAL CAUSES

3.1. Background

Although substantial research has implicated joint infectious causation of cancer, surprisingly little research has evaluated whether joint infectious causation is associated with a greater potential for controlling cancer. If two parasites that compromise different barriers infect the same cell, the cell's set of

barriers will be more completely compromised than would be the case if only one parasite infected the cell. But even if two parasites compromise the same barriers, the joint infection may increase oncogenicity because natural selection probably favours parasites that compromise, but do not completely block, barriers to cancer (Ewald and Swain Ewald, 2013).

3.2. Hepatitis B virus and hepatitis C virus

HBV and HCV each cause cancer by compromising all four of the barriers listed earlier for non-stem cells (Ewald and Swain Ewald, 2012). Joint infection of individuals can be fairly common because both viruses can be transmitted by the same routes, particularly by contaminated blood or injection materials, and in some areas by sexual contact (Ewald and Swain Ewald, 2012). Joint infection increases the risk of hepatocellular cancer (HCC) (Cho et al., 2011; Oh et al., 2012). The main uncertainty is whether the risk is additive, subadditive, or supra-additive (Cho et al., 2011). Several studies, including the most recent meta-analysis (Oh et al., 2012), indicate supra-additive effects. The current state of knowledge therefore suggests that control of either virus will provide similarly powerful control of cancer.

4. ESSENTIAL WITH EXACERBATING INFECTIONS

4.1. Overview

Parasites may exacerbate oncogenesis by damaging cells and altering immunologic processes. Immunosuppression induced by one parasite may reduce the control of other oncogenic parasites. Immune activation can increase proproliferative effects and mutational damage through generation of reactive molecules (as discussed in Section 1). This duality is considered later in the context of joint infection with parasites that act as essential causes of cancer.

4.2. Human immunodeficiency virus

Human immunodeficiency virus (HIV) is the most transparent example of a pathogen that increases the risk of cancer through immunosuppression. HIV increases the risk of several cancers for which other infectious agents encode essential causes: KSHV, cervical and anal cancer (caused by HPV), hepatocellular carcinoma (caused by HBV and HCV), and B-cell lymphomas (Burkitt's lymphoma, diffuse large B-cell lymphoma with centroblastic features, and Hodgkin's disease, all probably caused at least in part by EBV) (Bernstein et al., 2006; Chiao and Krown, 2003; Grulich et al., 2007;

Mbulaiteye et al., 2010). HIV is also a risk factor for cholangiocarcinomas, which are associated with HBV and HCV (Shaib et al., 2005). Leiomyosarcoma, a rare smooth muscle cancer generally of adults, is more common in HIV-infected children and is associated with EBV (Bhatia et al., 2012; McClain et al., 1995).

Evidence indicates that immunosuppression is an important mechanism for the increased incidence of cancer in HIV-infected individuals. There is significant overlap between the types of cancer that are increased in HIV-positive individuals and transplant recipients (Grulich et al., 2007). In the case of leiomyosarcomas, for example, incidence is higher in transplant patients and in HIV-infected adults, with all showing greater than 80% EBV positivity (Bhatia et al., 2012). Many cancers associated with HIV infection have declined in incidence in the post-HAART era, suggesting a protective effect of improved immune function (van Leeuwen et al., 2009). With regard to the issue of joint infectious causation, HIV-associated cancers are almost always demonstrated or suspected to be caused by infectious agents (Grulich et al., 2007). These considerations indicate that HIV's contributions to oncogenesis are often immunosuppressive exacerbations of essential infectious causes.

4.3. Hepatitis D and B viruses

The infection cycle of the hepatitis D virus (HDV) appears to be dependent on the presence of HBV infection, because HDV uses HBV's virion capsule for transmission between cells (Taylor, 2006). Although joint infection of HDV and HBV is associated with an elevated risk of HCC relative to that of HBV alone (Fattovich et al., 2000), HDV infection tends to lower overall proliferation of infected cells relative to uninfected cells (Wang et al., 2001). The HDV ribozyme inhibits rather than enhances telomerase activity (Lu et al., 2011), and HDV does not appear to be oncogenic in the absence of hepatitis B infection (Niro and Smedile, 2012). HDV therefore tends to exacerbate liver damage (Taylor, 2006) and oncogenesis of HCC, but does not appear encode essential causes. Its exacerbation of HCC may result from its damage to the cell and the inflammation that this damage stimulates.

4.4. Cancers caused by trematodes

It has been presumed that trematodes induce cancer through a combination of pro-oncogenic inflammatory responses and oncogenic mutations

(Mostafa et al., 1999). In recent years, however, investigations have linked oncogenic viruses with trematode-induced cancers. HBV and HCV have been documented as risk factors for intrahepatic cholangiocarcinoma (Donato et al., 2001; Lee et al., 2008; Shaib et al., 2007; Zhou et al., 2008). HPV and EBV have been associated with bladder cancer (Badawi et al., 2008; Barghi et al., 2012; Gazzaniga et al., 1998; Husain et al., 2009; Kim and Kim, 1995; Moonen et al., 2007; Noel et al., 1994; Panagiotakis et al., 2013) as have other viruses that are suspected of causing human cancer (Badawi et al., 2010; Fioriti et al., 2003). The linkage of viruses with bladder cancer varies greatly among reports, with some investigators failing to find evidence of the most commonly reported viral correlate: oncogenic serotypes of HPV (e.g. Sano et al., 1995; Yavuzer et al., 2011; Youshya et al., 2005). These discrepancies suggest variation in the roles of particular viruses among patient populations or geographical areas or variation in the accuracy of results. Where trematodes are absent, viral associations with bladder cancer tend to occur in patients who are immunocompromised due to advanced age or immunosuppressive treatment (Husain et al., 2009; Noel et al., 1994; Panagiotakis et al., 2013). This tendency mirrors the associations between EBV and nonendemic Burkitt's lymphoma in immunocompromised patients (see later) and raises the possibility that *Schistosoma* infections may be exacerbating the oncogenic effects of viruses through immunosuppression (for immunosuppressive effects of *Schistosoma*, see Hu et al., 2012).

5. JOINT EXACERBATING INFECTIONS

Interestingly, we do not know of any examples of joint infectious causation for which all of the coinfecting parasites compromise only restraints. This absence may be attributable to incomplete knowledge but may be due at least in part to the possibility that parasites that impose only exacerbating effects need to infect jointly with those that compromises barriers. This idea runs contrary to conventional thinking that tends to presume parasites trigger exacerbating causes such as pro-proliferative signals of inflammation. A strong tendency for oncogenic parasites to compromise barriers would imply a high potential for effective interventions—the greater the involvement of parasites in compromising barriers, the greater the effect of antiparasite interventions in controlling cancer.

6. UNCERTAINTIES IN ASSIGNMENT OF EXACERBATING AND ESSENTIAL CAUSATION

6.1. Overview

The incompleteness of knowledge about infectious mechanisms of oncogenesis creates uncertainty over the extent to which infectious etiologies of cancer involve essential causes. Endogenous retroviruses (ERVs) and Burkitt's lymphoma illustrate these issues.

6.2. Endogenous retroviruses

ERVs result from retroviruses that have integrated into host DNA after germ line infection and are thus directly transmissible from parent to offspring across generations. Some of the ERVs have been transmitted in this way for over 80 million years; others are more recent (Romanish et al., 2010). Like many retroviruses, ERVs have been implicated in certain cancers (Stoye, 2012). Human ERVs (HERVs) or their partial sequences make up approximately 8% of the genome, generally carry disabling mutations, and have yet to be linked directly to any cancer (Romanish et al., 2010; Ruprecht et al., 2008; Stoye, 2012). Natural selection should tend to weed out HERVs that are essential causes of cancer unless they provide a compensating fitness benefit. This possibility is illustrated by syncytin proteins, which are of ERV origin and are aberrantly expressed in some breast cancers but contribute to normal placental development (Ruprecht et al., 2008). It is possible that the stability of evolved associations between HERVs and human cells could be upset by other infectious agents. HERVs might contribute to cancer in these situations through immunosuppression, viral protein expression, or effects on 'host' gene regulation (Romanish et al., 2010; Stoye, 2012).

Joint infectious causation of cancer is suggested by reactivation of endogenous murine leukaemia virus (eMLV) in antibody-deficient mice, which has been associated with lymphoma development in mice raised with standard husbandry but not in mice from germ-limiting environments (Young et al., 2012). The eMLV reactivation may have involved recombination with another ERV after both were stimulated by the presence of LPS, which would normally have been cleared by antibodies. The associations of both eMLV- and LPS-induced inflammation with lymphoma suggest that LPS-laden bacteria may contribute an exacerbating cause through

inflammatory effects of LPS (Young et al., 2012). Associations of HERV activation with immunologic changes suggest that a similar mechanism may contribute to human lymphomas (Young et al., 2012).

If ERVs can be transmitted horizontally, oncogenic potential may be maintained indefinitely over time because the success of such exogenous viruses is not entirely dependent on the success of their hosts. In such cases, the exogenous virus and ERV may act jointly. Whether HERVs are providing coessential causes or exacerbating causes depends on the genes they control.

The middle ground between ERVs and exogenous retroviruses is not well understood. MMTV is in this middle ground. It can be transmitted in the germ line as an ERV but the extent to which exogenous MMTV is transmitted to and from germ lines has not been well characterized. Compromising three barriers, MMTV readily causes cancer in certain strains of mice apparently in the absence of other viruses, but perhaps in conjunction with ERVs. A small study of MMTV in liver disease provides some support for joint causation involving MMTV and hepatocellular carcinoma. Of the 21 cases investigated, three of the four MMTV positive tumours were also positive for HBV accounting for 50% of all HBV-infected tumours (Johal et al., 2009).

6.3. Burkitt's lymphoma

P. falciparum apparently exacerbates EBV induction of Burkitt's lymphoma by fostering replication and release of EBV (Chêne et al., 2007). One mechanism appears to be through the decline in immunologic control of EBV virion production (Snider et al., 2012). Another involves activation and survival of infected B lymphocytes (Chêne et al., 2009; Rochford et al., 2005). This proliferation could amplify the number of virus-infected lymphocytes within a person, thereby increasing the probability of oncogenic mutations in the population of infected cells. Although most of the demonstrated or implicated mechanisms involve relaxation of restraints, activation or survival of B lymphocytes might abrogate barriers (i.e. if it compromises cell-cycle arrest or apoptosis), in which case joint infection with *P. falciparum* would contribute to the essential causes. A finer understanding of the mechanism will clarify whether or not the effect of *P. falciparum* is strictly exacerbating the oncogenic process.

Results from a recent study of Ugandan children (Carpenter et al., 2008) accord with synergistic joint causation, with EBV contributing a more

significant role than *P. falciparum*. The associations of Burkitt's lymphoma with EBV titre were much stronger than the association with malaria (as measured by recent treatment for malaria). The joint occurrence of EBV and malaria was associated with a fivefold increased risk of Burkitt's lymphoma.

EBV is also associated with globally distributed B-cell cancers that are now considered to be variants of Burkitt's lymphoma outside of malaria-endemic areas. It is present in about half of the cases among HIV-infected subjects (Bernstein et al., 2006; Mbulaiteye et al., 2013a) and about two-thirds of cases associated with organ transplantation (Mbulaiteye et al., 2013b). The elimination of EBV from Burkitt's lymphoma cells causes them to die by apoptosis, confirming the idea that EBV provides an essential cause of the cancer rather than being simply a bystander (Vereide and Sugden, 2009; Vereide et al., 2013). On balance, this evidence supports the idea that EBV contributes to Burkitt's lymphoma in the absence of *Plasmodium* infection, particularly when immune function is compromised.

7. IMPLICATIONS FOR CANCERS OF UNCERTAIN CAUSE

7.1. Overview

The focus on barriers can help indicate whether a candidate parasite needs to infect jointly with another candidate parasite to cause a particular cancer. It also provides a sense of what studies need to be conducted to facilitate a more complete understanding of joint infectious causation of cancer. Breast cancer offers illustrations.

7.2. Breast cancer

Five pathogens have been linked to breast cancer through comparisons with normal tissue: MMTV (also referred to as mouse mammary tumour-like virus or HMTV to designate isolation from humans), EBV, HPV, bovine leukaemia virus (BLV), and JC virus (JCV) (Buehring et al., 2007; Glenn et al., 2012; Hachana et al., 2012; Joshi and Buehring, 2012; Lawson and Heng, 2010; Simoes et al., 2012; Wang et al., 2012b). For the first three of these viruses, associations with breast cancer have been documented by two or more different research groups, but the association has not been found by at least one additional research group (Glenn et al., 2012; Joshi and Buehring, 2012; Salmons and Gunzburg, 2013; Wang et al., 2012b). Studies that report associations tend to find an elevated prevalence

in breast cancer samples (or breast cancer patients) of roughly 20–50%. If each virus caused breast cancer independently, they could collectively account for nearly all human breast cancer. If joint infection is required, however, this set of viruses might not be sufficient to explain the spectrum of breast cancer.

Evidence indicates that MMTV compromises apoptosis and cell adhesion (Katz et al., 2005; Lawson et al., 2010; Ouatas et al., 2002; Ross, 2010; Ross et al., 2006). MMTV oncogenesis involves activation of the Wnt-1 pathway (Kim et al., 2012), which compromises cell-cycle arrest (Niehrs and Acebron, 2012) though feasible mechanisms seem more complicated than originally thought (Bearss et al., 2002; Michaelson and Leder, 2001; Rowlands et al., 2004). There has been no report of the abrogation of telomerase regulation by MMTV. Evolutionary considerations suggest that it is unlikely that MMTV would have this capability because its normal host, *Mus domesticus*, does not regulate telomerase. Apparently, this barrier to cellular immortalization regulation has not been favoured by natural selection because fewer barriers to cancer are needed in mice; mice are small and short-lived relative to humans and therefore have a lower probability of acquiring oncogenic mutations during a normal lifespan (Caulin and Maley, 2011; DeGregori, 2011; Seluanov et al., 2008).

These considerations suggest that research investigating the link between MMTV and breast cancer would benefit especially from investigations of joint infectious causation. The low positivity for MMTV in normal tissue suggests that any complementary joint infection would need to be common so that MMTV would be infecting in a background with a high prevalence of one or more viruses that compromise the regulation of telomerase. EBV would be the most obvious candidate among the four viruses that have been correlated with breast cancer.

Glenn et al. (2012) evaluated whether these viruses tend to co-occur in invasive breast cancer. They tested 50 invasive breast cancer samples for DNA from EBV, MMTV, and oncogenic serotypes of HPV and found positivity in 68%, 78%, and 50%, respectively (compared with 35%, 32%, and 20%, respectively, for cell samples from normal breasts). Seventy-two per cent of the samples were positive for two or more of these viruses. EBV and HPV were found jointly in 38% of cancers but in only 10% of samples from normal breasts. The analogous figures for MMTV were not given in their report, but unpublished results from this research group showed that joint infections with MMTV in the cancer specimens were greater than expected by chance (J. S. Lawson, personal communication).

In addition to complementing each other's oncogenicity, these viral agents may more directly exacerbate oncogenic infections. Synergistic interactions between EBV and HPV have also been reported (Hagensee et al., 2011 cited by Glenn et al., 2012). MMTV is known to exacerbate infections, including oncogenic viral infection, an effect attributable at least in part to its Sag protein (Bhadra et al., 2006).

Underlying this evidence for viral associations with breast cancer is uncertainty about whether the correlations reflect causation. Aside from the widely recognized problems associated with causal interpretations of correlative evidence, some aspects of the correlations themselves raise concerns about causation. Calculations, for example, indicate a very low ratio of EBV genomes relative to cellular genomes derived from the tumour (Perkins et al., 2006; Perrigoue et al., 2005). Such low ratios could reflect infiltration of tumour samples with EBV-infected cells rather than an oncogenic role for EBV or infiltration of tumours by uninfected nontransformed cells (e.g. leukocytes or fibroblasts). Variation in techniques among research groups may have important effects on quantification of EBV load (Kimura et al., 2008); insensitivity of measurement of viral genomes may therefore also be a contributing factor. Comparison with ratios for cancers that are known to be caused by the particular virus (e.g. nasopharyngeal cancers for EBV) may be useful (De Paoli et al., 2007; Perrigoue et al., 2005) but not definitive, because a virus may be more abundant in cell types that are part of the normal transmission process than in those that are not.

8. IMPLICATIONS FOR THE CONTROL OF CANCER

The examples of demonstrated, probable, and possible joint infectious causation discussed in this chapter have practical implications because the greater the number of parasites that contribute to a particular cancer, the greater the number of nonhuman targets for prevention and treatment. The value of the parasite as a target is related to the number of essential causes it compromises. It is therefore important to assess the extent to which known or suspected causes of cancer compromise barriers.

Interfering with joint infectious causes that compromise only restraints will tend to have less impact but still may be valuable because eliminating exacerbating causes of cancer might retard oncogenesis or contribute to control of the aggressiveness of a cancer. Burkitt's lymphoma provides an illustration. Household use of insecticide is associated with an 80% reduction in the risk of developing Burkitt's lymphoma (Carpenter et al., 2008), and

Burkitt's lymphoma has declined in the wake of malaria control. The incidence of Burkitt's lymphoma is about 5- to 10-fold greater in areas where *P. falciparum* is endemic than where it is absent (Orem et al., 2007). This difference provides a sense of the extent to which Burkitt's lymphoma could be reduced through malaria eradication. Still, the more predominant role of EBV and its ability to compromise four barriers to cancer suggest that prevention of EBV infection (e.g. through vaccination) should provide more complete control of Burkitt's lymphoma than would a comparable level of prevention of *P. falciparum* infection. One caveat is that the current evidence is not sufficient to rule out the possibility that *P. falciparum* could compromise barriers to cancer. Although *P. falciparum* does not infect the cancerous cell type of Burkitt's lymphoma (B lymphocytes), this possibility still needs to be considered because *P. falciparum* could have extracellular effects.

Cervical cancer provides a variation on this theme for a cancer that is already being controlled by targeting a pathogen (HPV) that compromises four barriers and causes virtually all cases. Substantial evidence indicates that *Trichomonas* species contribute to cervical cancer (Afzan and Suresh, 2012; Zhang and Begg, 1994). The aetiological role of *Trichomonas* has attracted relatively little attention in the biomedical community, probably because its contribution appears to be relatively small and it probably exacerbates the oncogenesis of cervical cancer through inflammation. If, however, *Trichomonas* compromises one or more barriers to cervical cancer, then its importance may be greater than is generally presumed, and efforts to control *Trichomonas* correspondingly more significant.

With regard to bladder cancer, infection control efforts have generally focused on trematodes. The theoretical framework proposed in this chapter suggests, however, that other infectious correlates of bladder cancer may prove to be important targets for intervention. The correlations with HPV (Badawi et al., 2008; Barghi et al., 2012; Husain et al., 2009; Kim and Kim, 1995; Moonen et al., 2007; Noel et al., 1994), for example, suggest that vaccination against HPV may prevent bladder cancer even in people infected with *S. haematobium*. The focus on barriers suggests that trematode-induced bladder cancer might as a rule require still other oncogenic pathogens, which might be controlled similarly by vaccination. The reported associations of bladder cancer with polyomaviruses and herpesviruses (Badawi et al., 2010; Fioriti et al., 2003; Gazzaniga et al., 1998) have generated an array of candidate pathogens for such control. An analogous argument applies to HBV and HCV relative to opisthorchid

trematodes in cholangiocarcinomas. Prevention of hepatitis viruses by vaccination and blood screening may provide especially effective control of cholangiocarcinoma in trematode endemic regions.

Although these considerations suggest that more effort should be devoted to identifying and controlling pathogens that contribute to these cancers, they do not negate the value of controlling the trematodes. Viral vaccines and antiworm treatment may act powerfully in concert, especially if the trematode infections compromise one or more barriers. The effectiveness of treatment or prevention of schistosome infections will depend in part on the extent to which schistosome-induced mutations compromise barriers relative to restraints. Treating schistosomiasis with anti-helminthic drugs is associated with a decline in the number of bladder cells that show chromosomal damage, perhaps because of reduced mutation-inducing inflammation (Anwar, 1994). Some of these mutations may be abrogating barriers. The combination of viral vaccination, inhibition of viral transmission (e.g. by blood screening or condom use), and anti-helminthic drugs may therefore act powerfully and synergistically to control cholangiocarcinoma and bladder cancer.

When a parasite compromises several barriers to cancer and infects different cell types, the barrier theory of cancer suggests that it may be acting as a packet of essential causes of different cancers (Ewald and Swain Ewald, 2013). Vaccinating against such parasites may therefore exert far greater control of cancer than would be assumed on the basis of infection-induced inflammation or mutation (Ewald and Swain Ewald, 2013). The broadening recognition of the spectrum of cancers caused by a particular pathogen coincides with expansions in the number of candidate pathogens that have been identified for particular cancers. These developments may reflect a widespread occurrence of joint infectious causation and thus a pervasive benefit of controlling any particular pathogen. HPV illustrates this point. HPV attracted attention when it was shown to be a cause of cervical cancer. This attention eventually led to approval of a vaccine against HPV to protect against cervical cancer. Since then, it has been generally accepted that HPV serotypes that cause cervical cancer also cause anal cancer, penile cancer, and some oropharyngeal cancer (Chaux and Cubilla, 2012; Frisch, 2002; Geissler et al., 2013; Marty et al., 2013; No et al., 2011; Rosenquist, 2005). It is now expected that the HPV vaccines will protect against these cancers directly and through herd immunity (e.g. Marty et al., 2013). The same oncogenic serotypes have been associated with other cancers, such as those of the breast (Damin et al., 2004; Glenn et al., 2012;

Hennig et al., 1999; Kroupis et al., 2006) and urinary bladder (Badawi et al., 2008; Barghi et al., 2012; Kim and Kim, 1995; Moonen et al., 2007). As mentioned earlier, these cancers have been associated with other parasites and are therefore prime candidates for joint infectious causation.

The value of controlling parasites that exacerbate oncogenesis is also related to the number of cancers they exacerbate. HIV is perhaps the most extreme example of a pathogen that is important to control even though its effects on cancer are exacerbating rather than essential. By compromising immunologic restraints, it exacerbates oncogenesis when other pathogens function as essential causes. Preventing HIV infection would therefore have major beneficial effects on control of cancer even though it would not prevent any particular kind of cancer.

We can expect that recognition of joint infectious causation of cancer will be increasingly important in the upcoming decades because cancers caused by a single pathogen are more conspicuously infectious and the most conspicuous examples of infectious cancers will tend to be discovered first. Epidemiological evidence of joint infectious causation may be more ambiguous because epidemiological patterns of disease will not accord neatly with the geographical distribution of a causal pathogen. Similarly, clinical evidence may be ambiguous because a candidate pathogen may be associated with the disease in one study population but not in another if the joint infectious cause is absent, because of differences in geography or patient selection (e.g. a sexually transmitted pathogen may be of little importance in a patient population that is not sexually active).

The treatment and control of infectious disease is one of the greatest achievements of the health sciences. Historically, the discovery of an infectious cause has almost always led to major advancement in the control of the disease. The chances of failing to counter in some way one infectious cause of cancer are therefore low. If two parasites are involved, the probability of failing to counter at least one of the two is vastly lower. For this reason alone, the search for joint infectious causes of cancers should be a priority. This idea seems fairly obvious when the discovered causes cannot explain all of the risk, as was the case with transfusion-associated HCC before HCV was discovered. But such efforts need to continue even when a known infectious cause explains much of the disease, in which case the roles of contributing pathogens need to be understood in terms of essential and exacerbating causes. Clarity of hindsight makes this point apparent for AIDS-associated cancers, in which HIV acts as an exacerbating cause, but this point is also illustrated by trematode-associated cancers, in which the essential causes

may have been overlooked, and by endemic Burkitt's lymphoma and cervical cancer, in which joint infectious causes appear to be exacerbating but may also contribute in an essential way. In each case, determining the roles of the jointly infecting parasitic causes has important implications for interventions.

Past experience demonstrates that even after an effective intervention against an infection-induced cancer has been developed and enacted, its effectiveness will generally fall short of 100%. Vaccine coverage, for example, becomes increasingly difficult as it approaches 100% because of difficulties with access and compliance. Even when coverage is close to 100%, effectiveness will often be limited for reasons specific to each vaccine. Prevention of hepatocellular carcinoma by the current hepatitis B vaccine, for example, is limited to about a 70% reduction largely because babies can become infected prior to the earliest administration of the vaccine (Chang et al., 2009). Prevention of cervical cancer by current HPV vaccines is limited by the serotypes that have been included in the vaccines. Having two different infectious targets will ameliorate such limits on maximal effectiveness. If cholangiosarcoma is caused jointly by HBV and opisthorchid trematodes, targeting both parasites will compensate for the incompleteness of each approach. The same argument applies to viruses and schistosomes in control of bladder cancer and HPV serotypes and *Trichomonas* in the control of cervical cancer. An emphasis on joint causation of cancers might thus provide a spectrum of possibilities that brings control of infection-induced cancers into the range of effectiveness that has been achieved in the control of acute infectious diseases.

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CHAPTER TWO

Neurological and Ocular Fascioliasis in Humans

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Abstract

Fascioliasis is a food-borne parasitic disease caused by the trematode species *Fasciola hepatica*, distributed worldwide, and *Fasciola gigantica*, restricted to given regions of Africa and Asia. This disease in humans shows an increasing importance, which relies on its recent widespread emergence related to climate and global changes and also on its pathogenicity in the invasive, biliary, and advanced chronic phases in the human endemic areas, mainly of developing countries. In spite of the large neurological affection capacity of *Fasciola*, this important pathogenic aspect of the disease has been pronouncedly overlooked in the past decades and has not even appear within the numerous reviews on the parasitic diseases of the central nervous system. The aim of this wide retrospective review is an in-depth analysis of the characteristics of neurological and ocular fascioliasis caused by these two fasciolid species. The terms of neurofascioliasis and ophthalmofascioliasis are restricted to cases in which the direct affection of the central nervous system or the eye by a migrant ectopic fasciolid fluke is demonstrated by an aetiological diagnosis of recovered flukes after surgery or spontaneous moving-out of the fluke through the orbit. Cases in which the ectopic fluke is not recovered and the symptoms cannot be explained by an indirect affection at distance may also be included in these terms. Neurofascioliasis and ophthalmofascioliasis cases are reviewed and discussed. With regard to fascioliasis infection giving an indirect rise to neurological affection, the distribution and frequency of cases are analysed according to geography, sex, and age. Minor symptoms and major manifestations

are discussed. Three main types of cases are distinguished depending on the characteristics of their manifestations: genuine neurological, meningeal, and psychiatric or neuropsychic. The impressive symptoms and signs appearing in each type of these cases are included. Brain examination techniques and neuroimaging useful for the diagnosis of neurological cases are exposed. Within fascioliasis infection indirectly causing ocular manifestations, case distribution and frequency are similarly analysed. A short analysis is devoted to clarify the first reports of a human eye infection. The affection of related and close organs is discussed by differentiating between cases of the dorsal spine, pulmonary manifestations, heart and vessel affection, findings in blood vessels, skin and dermatologic reactions, cases of ectopic mature flukes, and upper body locations. The clinical complexity of the puzzling polymorphisms, the disconcerting multifocality of the manifestations, and their changes along the evolution of the disease in the same patient, as well as the differences between the clinical pictures shown by different patients, are highlighted. The many syndromes involved are enumerated. The pathogenic and physiological mechanisms underlying neurofascioliasis and ophthalmofascioliasis caused by ectopic flukes and the physiopathogenic processes indirectly affecting the central nervous system and causing genuine neurological, meningeal, psychiatric, and ocular manifestations are discussed. The diagnosis of neurological and ophthalmologic fascioliasis is analysed in depth, including clinical and paraclinical diagnosis, eosinophilia in the blood and cerebrospinal fluid, differential diagnosis from other parasitic infections such as helminthiases and myiases, an update of human fascioliasis diagnosis, and fluke and/or fluke egg recovery by surgery. Diagnostic analyses with faecal and blood samples for fascioliasis patients are updated. Therapy for patients with major neurological manifestations includes both antiparasitic treatments and anti-inflammatory therapeutics. Prognosis in fascioliasis patients with neurological manifestations is discussed, with emphasis on sequelae and fatal cases, and the care of patients with ophthalmologic manifestations is added. Conclusions indicate that neurological cases are overlooked in human fascioliasis endemic areas and also in developing countries in general. In remote zones, rural health centres and small hospitals in or near the human endemic areas do not dispose of the appropriate equipments for neurological analyses. Moreover, physicians may not be aware about the potential relationship between liver fluke infection and neurological implications, and such cases may therefore remain misdiagnosed, even in developed countries. Priority should henceforth be given to the consideration of neurological and ocular affection in human endemic areas, and efforts should be implemented to assess their characteristics and frequency. Their impact should also be considered when estimating the global burden of fascioliasis.

1. INTRODUCTION

The central nervous system and the eye of mammals present characteristics that do not make them suitable for parasites. Thus, only very few parasite species have developed strategies for the colonization of these

two locations throughout evolution and use them as their final microhabitats. However, many parasites may be located in the meningeal spaces or may penetrate into the tissues of the brain and spinal cord, including protozoans, helminths, and arthropods infecting both humans and animals. Many of these parasites wander into those microhabitats aberrantly, mainly when infecting host species other than those they specifically or usually infect in their normal life cycles. In spite of this, the negative repercussions of neurological manifestations in health and normal life explain the importance of their studies and the great number of publications dealing with parasitic infections affecting the central nervous system (Abdel Razek et al., 2011; Bia and Barry, 1986; Brown and Voge, 1982; Chacko, 2010; Cook and Zunmia, 2009; Graeff-Teixeira et al., 2009; Hughes and Biggs, 2002; Kristensson et al., 2002; Lowichik and Ruff, 1995a,b; Lowichik and Siegel, 1995; Lv et al., 2010; Vercruysse et al., 1988). Similar observations may be made when referring to the eye (François et al., 1985; Grüntzig, 1988; Otranto and Eberhard, 2011; Sabrosa et al., 2010). Therefore, results of such neurological and ophthalmologic studies acquire a great weight in the assessments of pathogenicity and morbidity when analysing the global burden of each one of the parasitic diseases involved.

In humans, among helminthiases, mainly schistosomiasis and secondarily paragonimiasis are the only diseases usually included in the aforementioned reviews dealing with parasitic affections of the central nervous system. Old references mention cerebral infection by *Heterophyes heterophyes* (Africa et al., 1936; Collomb and Bert, 1957, 1959; Collomb et al., 1960) only as a rarity, and *Dicrocoelium dendriticum* (syn. *D. lanceolatum*) (Siguier et al., 1952) remain usually completely overlooked. However, if overlooking neurological heterophyiasis and dicrocoeliasis may be explained by their rarity, that of fascioliasis should be emphasized given the numerous clinical records on neurological manifestations caused by this trematodiasis in many countries of different continents. Only very recently has the importance of fascioliasis in the affection of the central nervous system appropriately been dealt with, although only summarized (Mas-Coma et al., 2013), in a general review on this subject (García et al., 2013).

With regard to helminthiases affecting the human eye, besides *Alaria* mesocercariae and *Philophthalmus lacrimosus*, only one case report on ocular fascioliasis was included in the most recent review on ophthalmologic trematodiases (Otranto and Eberhard, 2011).

Fascioliasis is a food-borne parasitic disease caused by two trematode species of the genus *Fasciola*: *Fasciola hepatica*, distributed throughout all

continents except the two poles, and *Fasciola gigantica*, only present in given regions of Africa and Asia (Mas-Coma et al., 2009a). This helminthiasis is a zoonotic, waterborne, snail-transmitted disease whose impact has been well known in veterinary medicine for a long time (Spithill et al., 1999; Torgerson and Claxton, 1999).

Human fascioliasis was considered a secondary disease for decades. However, the description of many human endemic areas in the Americas, Europe, Africa, and Asia and the emergence or reemergence of human infection in many countries, including both prevalence and intensity increases and geographic expansion, as well as recent human epidemic situations, have drastically changed the global scenario from the mid-1990s. Fascioliasis is at present the vector-borne disease presenting the widest latitudinal, longitudinal, and altitudinal distribution known (Mas-Coma, 2005; Mas-Coma et al., 2005, 2009a). A global estimation of up to 17 million people infected throughout has been made (Hopkins, 1992), and an underestimation of the real frame due to the hitherto unknown situations in many countries, mainly of Asia and Africa, has been highlighted (Mas-Coma, 2004).

This disease in humans shows an increasing importance, which does rely not only on the recent widespread emergence it has shown but also on the results obtained in studies on pathogenicity (Mas-Coma et al., 1999a; Valero et al., 2003, 2006, 2008) and immunity (Brady et al., 1999; Gironés et al., 2007), according to which this disease appears to be pronouncedly more complicated and with a greater impact in long-term infection than what was believed until the 1990s. In human endemic areas, fascioliasis chronicity and superimposed repetitive infections pose additional pathological complications (Valero et al., 2003). The origin of the emergence of fascioliasis in recent years has been argued to be related to climate change, at least in part and in given countries (Mas-Coma et al., 2008, 2009b), as a consequence of the high dependence of fascioliasis transmission and freshwater lymnaeid snails on climate and environmental characteristics (Fuentes et al., 1999, 2001; Ollerenshaw and Smith, 1969).

Emergence, long-term pathogenicity, and immunologic interactions are in the background of the decision taken by the World Health Organization (WHO) to include this disease among the so-called neglected tropical diseases (WHO, 1995). The great concern related to the epidemiological situations in many countries led WHO to launch a worldwide initiative against this disease already from the end of the 1980s (WHO, 2007, 2008), taking into account the great heterogeneity of epidemiological situations and transmission patterns of this disease (Mas-Coma, 2005;

Mas-Coma et al., 2009a). Control action and measures undertaken in human hyperendemic areas are at present showing promising results (Valero et al., 2012a; Villegas et al., 2012).

However, several crucial aspects still need to be clarified, such as elucidating morbidity and assessing its impact in humans worldwide. The present in-depth analytic review of fascioliasis affecting the central nervous system and the eye is intended to be one of the main steps in this endeavour. The importance of the neurological and ophthalmologic manifestations caused by fascioliasis in humans, and mainly the frequency and geographic distribution of the former, highlights the extensive pathogenicity and potential morbidity of this food-borne trematodiasis.

2. *FASCIOLA* INFECTION IN HUMANS

Due to the increasing public health importance of this disease, research efforts in the past two decades have shed a great deal of light on human fascioliasis, showing that many aspects differ pronouncedly from the information previously considered, which was mainly based on the characteristics of the disease in domestic animals (Mas-Coma et al., 2009a). A summary of selected aspects of the disease is presented to conform the updated baseline to facilitate the analysis of the affection of the central nervous system and the eye by *Fasciola* in humans.

In both species, the adult stages, with a body length/width of up to 29/14 mm in *F. hepatica* and 52/12 mm in *F. gigantica* (Periago et al., 2006), infect the large biliary passages and gall bladder. *F. hepatica* is a common parasite of ruminants, including sheep, goats, cattle, horses, donkeys, and mules. Alternate definitive hosts are buffaloes, pigs, camelids, and many wild herbivorous mammals. *F. gigantica* usually infects sheep, goat, cattle and buffalo, and also camel, pig, horse, donkey, and several wild animals (Mas-Coma, 2004; Mas-Coma and Bargues 1997).

The two *Fasciola* species use specific freshwater snail species of the family Lymnaeidae as intermediate hosts or vectors in their life cycles. *F. hepatica* and *F. gigantica* show different lymnaeid specificity, which is epidemiologically very important, because of the different ecological requirements of their respective amphibious *Galba/Fossaria* and aquatic *Radix* vector species (Bargues et al., 2001; Mas-Coma, 2004).

Their two-host life cycle is similar and takes about 14–23 weeks, comprising four phases (Mas-Coma, 2004; Mas-Coma and Bargues 1997): (A) the presence of fluke adults in the mammal liver and production of eggs

reaching the external milieu via the bile and intestine; the definitive host is infected by ingestion of metacercariae; in humans, the flukes attain sexual maturity in 3–4 months; (B) the long resistance phase of the egg and the short active phase of the miracidium allow for the transit between definitive host and snail vector; eggs shed with mammal faeces continue their development in freshwater; (C) after miracidium penetration, sporocyst, redial generations, and cercariae develop inside the snail, until cercarial shedding into water; the prepatent period, of around 38–86 days, depends on temperature, higher temperatures reducing the period; (D) a short swimming phase of cercaria and a long resistance phase of metacercaria allow for the transit between snail and mammal; cercariae swim until contacting a solid surface, mostly leaves of water plants above or below the waterline, to attach and encyst; metacercarial cysts become infective within only 1 day.

2.1. Sources of human infection, intraorganic migration, and lifespan

Human infection takes place by ingestion of infective metacercariae. Metacercarial infectivity is dependent upon storage time, being lower when metacercariae are older (Valero and Mas-Coma 2000). Climatic factors as temperature and rainfall pronouncedly determine the incidence. Human infection is more frequently observed in years with heavy rainfall (Mas-Coma, 2004). A seasonal distribution is typical in many areas, although human infections may occur throughout the year, as in areas where lymnaeids inhabit permanent water bodies.

There are several sources of infection for humans (Ashrafi et al., 2006; Mas-Coma, 2004):

- *Ingestion of freshwater wild plants*: This is the main human infection source. Freshwater vegetables incriminated differ according to geographic zones and human dietary habits. Most human reports are related to common or wild watercress, secondarily to dandelion leaves, lamb's lettuce, spearmint, and others.
- *Ingestion of freshwater cultivated plants*: Given metacercariae-carrying species may even be so important in the human diet as to be grown by humans (at family or even industrial level) and commercially sold in public markets, thus explaining infection of subjects living far away from the endemic area.
- *Ingestion of terrestrial wild plants*: The long survival capacity and dryness resistance of metacercariae explain the infection by consumption of wild terrestrial plants picked in dry habitats but that were submerged in water before.

- *Ingestion of terrestrial cultivated plants*: Metacercariae have been found in nonaquatic vegetables whose plantations need frequent irrigation. Due to transport of vegetables (both aquatic and terrestrial) from rural endemic zones to cities, plants carrying metacercariae can be sold in noncontrolled city markets giving rise to urban infection.
- *Ingestion of traditional foods and beverages made from local wild plants*: Appetizers in the Near East, alfalfa juice in Andean countries, and local beverages in Cape Verde are good examples.
- *Drinking of contaminated water*: Water has sometimes been cited as a source of human infection.
- *Ingestion of dishes and soups made with contaminated water*: Water containing metacercariae may also contaminate food.
- *Washing of kitchen utensils or other objects with contaminated water*: Washing with contaminated water may be the source of fortuitous infection.
- *Ingestion of raw liver*: Humans consuming dishes prepared from raw livers infected with immature flukes may also become infected.

After ingestion, a proportion of metacercariae die in the gastrointestinal tract and relatively few eventually develop into adults. Once in the small intestine, metacercariae excyst within an hour, penetrate the host's intestine wall, and appear in the abdominal cavity by about 2 h. Most reach the liver within 6 days. Juvenile flukes migrate to the liver parenchyma for 5–6 weeks, preferentially feeding directly on the liver tissue. They eventually penetrate into the bile ducts where they become sexually mature (Mas-Coma and Bargues, 1997). It has also been speculated that immature flukes may enter the bloodstream and be carried to various parts of the body or may reach the liver by travelling up the bile duct (Chen and Mott, 1990).

The prepatent period (from ingestion of metacercariae to the first appearance of eggs in faeces) varies according to the host species and also depends on the number of the adult flukes in the liver, so that the greater the fluke number, the longer the time to mature and to initiate egg laying. In humans, at least 3–4 months are necessary for the flukes to attain sexual maturity. The lifespan of *Fasciola* in humans may reach up to 13.5 years (Mas-Coma and Bargues, 1997).

Once in their final hepatic microhabitat, the endocrine system of the host provides reliable signals that are part of the perceptual world of the blood-feeding *Fasciola* adult. Whilst feeding, the adults attach to the mucosa of the bile duct by their ventral suckers (Sukhdeo et al., 1988). When the host eats, the gastrointestinal hormone cholecystokinin–pancreozymin (CCK–PZ) is released, which stimulates the bile duct to contract and expel its contents

including the worms. However, CCK–PZ stimulates increased contractions of the ventral suckers to hold the trematode fast during the expulsive bile duct activity (Sukhdeo and Sukhdeo, 1989).

2.2. Pathology, symptomatology, and disease periods

Metacercarial penetration through the duodenum or jejunum wall may give rise to focal haemorrhage and inflammation. During fluke migration, extensive parenchymal destruction with intensive haemorrhagic lesions and immunologic and inflammatory reactions occur. Migratory parasites become sometimes trapped in the liver parenchyma and die without reaching the bile ducts, leaving cavities filled with necrotic debris. Considerable liver areas may subsequently be replaced by scar tissue.

The disease is mainly confined to the liver, including hepatic lesions, fibrosis, and chronic bile duct inflammation. The liver usually enlarges, showing a smooth or uneven surface. Macroscopic lesions include multiple soft, yellowish or greyish-white nodules ranging from 2 to 30 mm in diameter, corresponding to eosinophilic abscesses. Nodules are also observed in the parietal peritoneum close to the liver and on the round liver ligament. These nodules appear accompanied by marginal haemorrhagic stippling. Yellow and opalescent ascites has been recorded in cases with marked involvement of peritoneal wall and liver surfaces. The adult flukes cause pronounced tissue reaction and calcification of the bile passages. The common bile ducts appear large and dilated and the wall is thickened on palpation. Ultrastructurally, there is bile ductular hyperplasia, fibrosis of the portal tracts, widening of the interhepatic spaces by many microvilli, and dilated Disse space with collagen fibres. Multiple, greyish-white subserous nodules are present. Lithiasis is very frequent (Mera y Sierra et al., 2011; Valero et al., 2003).

The gall bladder wall appears pronouncedly oedematous and thickened due to muscular hypertrophy and perimuscular fibrosis and contains patchy infiltrates with lymphocytes, plasma cells, and eosinophils.

In the liver, Charcot–Leyden crystals and eosinophils are often found on the walls of migration tracks surrounded by a considerable eosinophilic infiltrate. Track cavities are filled with necrotic cellular debris, including hepatocytes, fibrin, and red cells. In older lesions, macrophages, lymphocytes, eosinophils, and fibrous tissue are observed. Focal calcification is sometimes seen in the margin of the necrotic debris. Necrotizing arterial vasculitis and portal venous thrombosis are frequent. Nevertheless, fluke egg granulomas

have only been sporadically reported. For additional details on pathology, see reviews (Arjona et al., 1995; Chen and Mott, 1990; Mas-Coma and Bargues, 1997; Mas-Coma et al., 1999a, 2000).

Important sonographic imaging features in the differential diagnosis are multiplicity, ill-defined borders of confluent nodules, absence of a halo, and absence of contrast enhancement. Periportal lymph node enlargement or lymphadenopathy is helpful in the diagnosis. In humans, parenchymal and biliary ductal calcifications seem to be rare whilst occurring frequently in animals (Kabaalioglu et al., 2007).

Clinically, different periods may be distinguished. The incubation period comprises from the ingestion of metacercariae to the appearance of the first symptoms. This initial period varies considerably depending on the number of metacercariae ingested and the host's response. In humans, it has not yet been accurately determined: only "a few" days, 6 weeks, 2–3 months, or even more. The infection periods include (i) the invasive period or acute phase (fluke migration up to the bile ducts; 2–4 months) and (ii) the biliary period in which there may be first a latent phase (maturation of the parasites and starting of oviposition; months or years) and a subsequent chronic or obstructive phase (after months to years of infection) (Mas-Coma et al., 1999a, 2000).

2.2.1 Invasive period or acute phase

The clinical picture is related to the mechanical destruction of the liver tissue and abdominal peritoneum by the migrating flukes causing localized or generalized toxic and allergic reactions lasting 2–4 months. However, in endemic areas, liver fluke infection is usually repetitive and the acute lesions are superimposed in chronic disease. Thus, the acute phase may be prolonged and overlap with the latent or the obstructive phase.

Fever, abdominal pain, gastrointestinal disturbances, urticaria, and respiratory symptoms are among the major symptoms. Fever is usually the first symptom, which may be generally low or moderate but may reach 40 °C or even 42 °C; it may be remittent, intermittent, or irregular, increasing in the evening; a low, recurrent fever may sometimes last for 4–18 months. Abdominal pain appears from mild to excruciating, sometimes vague; it may be generalized at the outset but is usually localized below the xiphoid or in the right hypochondrium (right upper-quadrant pain or "Murphy sign"). Gastrointestinal disturbances including loss of appetite, abdominal flatulence, nausea, and diarrhoea are common, whereas vomiting and constipation are infrequent. Urticaria becomes a distinctive feature in the early stage

and may be accompanied by bouts of bronchial asthma. Respiratory symptoms comprise cough, dyspnoea, haemoptysis, and chest pain occurring occasionally, but in some cases, they are the first manifestations of the infection.

On physical examination, different signs may appear, such as hepatomegaly and splenomegaly, ascites, anaemia, chest signs, and jaundice. Hepatomegaly includes a usually enlarged and tender (never hard) liver, sometimes reaching down to the right iliac fossa; the degree of hepatomegaly seems to increase during the course of the disease; hepatic abscess may be detected. Splenomegaly is not common and, if present, only mild and transient. Ascites has been reported several times as yellow with a high leucocyte count, with eosinophils predominating; the pathogenesis is considered to be an inflammatory response to numerous metacercariae penetrating the intestinal wall, irritation of the peritoneum, and penetration through the liver capsule. Anaemia appears mild to moderate; pallor of the skin and mucosa is commonly associated with lassitude, dizziness, palpitation, and weakness. Chest signs include dry or moist rales that may, on auscultation, occasionally be elicited upon coughing at the base of the right lung, probably due to migration of the juvenile flukes. Pleural rub with effusion and even spontaneous pneumothorax have been reported. Parenchymal infiltrates resembling the Loeffler syndrome and pleural effusion are common radiological manifestations. Pyopneumothorax has also been reported. Mild jaundice may appear, although it is infrequent.

2.2.2 Biliary period

The proportion of asymptomatic subjects in the latent phase is unknown. They are often discovered during family screenings after a patient is diagnosed, confirmed after clinical suspicion or in epidemiological surveys. A prominent eosinophilia may be suggestive of infection. Individuals may have gastrointestinal complaints or relapses of acute symptoms during this phase.

In the chronic or obstructive phase, cholangitis and cholecystitis are the results of inflammation, epithelial hyperplasia, and thickening and dilatation of the bile ducts and gall bladder walls. Combined with the large body of the flukes, this is sufficient to cause mechanical obstruction of the ducts. The most frequent site of obstruction is the common bile duct. In the case of obstruction, the gall bladder is usually oedematous and enlarged, with its lower edge reaching the umbilicus and common fibrous adhesions to adjacent organs. The obstruction caused by advanced chronic fascioliasis appears

related to biliary sepsis, bacterobilia appearing associated with the duration of *Fasciola* infection, its intensity, and liver damage (Valero et al., 2006).

Clinical manifestations such as biliary colic, epigastric pain, fatty food intolerance, nausea, jaundice, pruritus, and right upper-quadrant abdominal tenderness pose the problem of being indistinguishable from cholangitis, cholecystitis, and cholelithiasis of other origins. Hepatic enlargement may appear associated with ascites or an enlarged spleen.

The gall bladder and bile ducts may contain blood mixed with bile (haemobilia), blood clots, and fibrinous plugs. Cirrhosis due to fascioliasis has rarely been noted in patients of developed countries (Heredia et al., 1984; Mas-Coma et al., 1999a) but may be relatively frequent in human endemic areas (Marcos et al., 2009a). Lithiasis of the bile duct and/or gall bladder is frequent and the stones are usually small and multiple. Gallstone disease depends on factors that favour bile duct obstruction (cholangitis and fluke body development vs. time and intensity of infection). Thus, a high gallstone risk may be expected in subjects inhabiting human hyperendemic areas where liver fluke burdens may be very high (Valero et al., 2003). Other complications reported are bleeding and multiple extrahepatic venous thrombosis (Mas-Coma et al., 1999a).

Anaemia may be important in human endemic areas where children are the most affected (Curtale et al., 2003), although the numerous nutritional deficiencies and coexisting infectious diseases also related to anaemia did not allow for a cause–effect demonstration. However, experimental results have shown that anaemia may even be considered a biomarker of the fascioliasis chronicity period, changing from normocytic, to macrocytic in the early chronic phase, to microcytic in the advanced chronic phase and, likewise, changing from normochromic in the early chronic phase to hypochromic in the advanced chronic phase (Valero et al., 2008). Among the different factors associated with anaemia, fluke burden showed the highest risk. Thus, a high risk of anaemia may be expected in heavily infected subjects inhabiting human hyperendemic areas (Valero et al., 2008).

Diffuse pain in the right hypochondrium, persisting 2–6 years after treatment, and weight loss, appearing during treatment and persisting afterwards, are probably due to the presence of persistent hepatic lesions following fascioliasis treatment (Rondelaud et al., 2006). Residual fibrotic or necrotic foci may remain for years after cure. Even after 1 year, some patients had sonographic hypoechoic and hypodense nodules in the liver, reflecting necrotic granulomas and fibrosis. Shrinkage of the enlarged periportal lymph nodes may also take months or even years. In the long term, patients may have

symptoms and laboratory signs of recurrent cholangitis and sonographic evidence of biliary ductal oedema and dilatation. Prolonged cholangitis due to fascioliasis is rare after effective treatment but may be important in human endemic areas of developing countries where infected subjects, mainly children, may remain undiagnosed. Fibrotic liver tissues after necrosis may be imaged as hypoechoic or hypodense nodules for years after the infection. In a few patients, recurrent cholangitis, gallstones, or asymptomatic viability of the flukes may be observed in the long term (Kabaalioglu et al., 2007).

For details on symptomatology and clinical aspects, see reviews (Arjona et al., 1995; Chen and Mott, 1990; Mas-Coma et al., 1999a, 2000).

2.3. Laboratory analyses

With regard to haematologic characteristics, leucocyte counts are usually above 10,000/mm^3 and up to 43,000/mm^3 in the acute phase. The eosinophil count is nearly always greater than 5% of the total leucocytes and may be as high as 83%, although unusual cases without eosinophilia have been reported. Anaemia is common, but usually not very severe, mostly between 7.0 and 13.5 g dl^{-1} haemoglobin. Levels as low as 2.8 and 4.0 g dl^{-1} have been reported. The erythrocyte sedimentation rate may be high in the acute phase, reaching 165 mm in an hour, normal in the latent phase, and normal or only moderately high in the chronic phase.

Abnormal results may be obtained in hepatic function tests. In the acute phase, results sometimes include a rise of the two aminotransferases (formerly transaminases) most frequently utilized, namely, alanine aminotransferase (ALT, formerly serum glutamic-pyruvic transaminase) and aspartate aminotransferase (AST, formerly serum glutamic-oxaloacetic transaminase), and elevated thymol turbidity, zinc sulphate turbidity, serum globulin, and serum bilirubin. In other cases, tests give normal results, with the exception of alkaline phosphatase (AKP or ALP). Serum electrophoresis may show an increase of α_2- and γ-globulins. Serum triglycerides and very-low-density lipoproteins have been seen to increase, whilst total serum cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol exhibit a significant decrease. These changes were due to the degenerative necrotic damage of the hepatocytes. Other reported findings include abnormally high levels of β-glucuronidase.

Jaundice is a prominent feature in the chronic phase. Serum bilirubin levels between 2.0 and 8.6 have been reported. Biliary colic is usually

followed by a higher level of serum bilirubin and dark urine positive for bilirubin. Serum bilirubin may be normal in this phase and between attacks of biliary colic. Whilst albumin is decreased, AKP, ALT, AST, and serum globulin (mainly γ-globulin) appear often elevated (Mas-Coma et al., 1999a).

Concerning immunoglobulins, IgG, IgM, and IgE levels are usually increased. Specific IgE antibodies were reported in 48% of the patients. Total and specific IgE levels have been shown to be positively correlated with egg burden, age, clinical features, and degree of eosinophilia. IgA levels are usually normal (Chen and Mott, 1990; Mas-Coma et al., 1999a).

2.4. Final microhabitat finding and ectopic infections

Host site finding by trematodes migrating within their definitive hosts is accomplished through the release of innate patterns of behaviour adapted to the worm's environment (Sukhdeo and Sukhdeo, 2004). Migrating trematodes express innate behaviour programmes that lead them to their next site, and these often rely on topological features of the host (e.g. intraocular ducts and systemic blood vessels) as guideposts or highways for their migration. The very complex migrations can be explained by behaviours that are programmed to direct them in time to the space where the likelihood of encountering their final site is highest. Thus, migrations in the host often include distinct developmental stages (time) that coincide with their arrival at the final site (space) (Sukhdeo and Sukhdeo, 2002). Host tissues and organs are physicochemically and topologically consistent and always occur in the same relative positions, and the entire body is interlaced with ducts and blood vessels in anatomically predictable patterns (Sukhdeo 1990; Sukhdeo and Sukhdeo 1994). Thus, it is likely that the behavioural strategies of the migrating worms will also take advantage of the host's predictability (Sukhdeo and Sukhdeo, 2004).

In *Fasciola*, the tiny worms (250 μm) show an incredible success rate (40–80%) in finding the liver (Doy and Hughes, 1984; Montgomerie, 1928). The worms do not take a direct route to the liver, but first migrate to the abdominal wall and then crawl with a sliding motion that keeps them adhered to the abdominal wall whilst approaching the liver (Sukhdeo et al., 1987). Penetration usually occurs on the liver surface against the diaphragm and body wall. The indirect migration route explains why >80% of the worms penetrate the liver on the surface against the diaphragm, rather than on the internal surfaces of the liver against the viscera (Doy and Hughes, 1984). For parasites crawling on the abdominal wall, the inside of the

abdominal cavity is like the inside of an egg, and any direction taken will bring the worms to the liver/diaphragm junction.

The migration patterns in *Fasciola* appear to be released by signals contained within ingested tissue. A sequential study demonstrated the appearance of the visceral tissue, abdominal wall muscle tissue, and then liver tissue in fluke caeca during migration (Dawes, 1963). Thus, (i) worms feeding on visceral tissues exhibit a kind of pinching behaviour that takes them to the body wall, (ii) worms feeding on the abdominal wall tend to creep along the wall, and (iii) worms feeding on liver tissue will enter the organ. These worms will make mistakes because they have to feed on the tissue before recognizing it. After reaching the liver/diaphragm junction, 25% of the worms penetrate accidentally into the diaphragm and leave holes as big as poppy seeds before turning back and penetrating into the liver (Dawes and Hughes, 1964).

Ectopic fascioliasis refers to cases in which juvenile flukes deviate during migration and enter other organs. Ectopic lesions most frequently reported are in the gastrointestinal tract and subcutaneous tissue. Other ectopic locations described include the heart, blood vessels, and lung and pleural cavity; brain; orbit; abdominal wall; stomach, caecum, and appendix; pancreas; spleen; peritoneum; inguinal nodes; cervical node; skeletal muscle; and epididymis (Mas-Coma and Bargues, 1997; Mas-Coma et al., 1999a, 2000). Ectopic manifestations usually appear shortly after infection (Arjona et al., 1995), although exceptions have been reported (Beaudoing et al., 1970). Such ectopic flukes achieve maturity only very rarely.

In ectopic lesions, the usual pathological effects are due to the migratory tracks that cause tissue damage with inflammation and fibrosis. The suspicion that many juvenile flukes perish in human tissues before reaching the biliary system was experimentally confirmed in other primate hosts (Tomimura et al., 1975). Parasites may then be calcified or incorporated in a granuloma. The precise route of migration towards ectopic sites is unknown, and various theories have been formulated, including the migration through blood vessels or through soft tissue during the acute phase (Catchepole and Snow, 1952; Neghme and Ossandon, 1943; Rushton and Murray, 1978).

Most recorded cases with ectopic locations are caused by *F. hepatica*. Only a few ectopic cases have been reported to be caused by *F. gigantica* (Doby and Beaucournu, 1970; Fain et al., 1973; Le et al., 2007; Ongom, 1980; Paraf et al., 1967; Ragab and Farag, 1978; Stemmermann, 1953; Xuan et al., 2005), in spite of having been noted to be able to give rise to ectopic lesions more commonly than *F. hepatica* (Boray, 1966).

3. NEUROLOGICAL FASCIOLIASIS

3.1. Distribution and frequency

An analysis of the reports of fascioliasis patients presenting with neurological manifestations shows that such cases have already been described from all continents and from numerous countries (Tables 2.1 and 2.2). Although a complete review cannot be assured, given the very wide literature on human fascioliasis cases worldwide, the global picture obtained after the exhaustive retrospective analysis performed may be assumed to be sufficiently accurate. Certain publications of cases with minor symptoms might have been overlooked, but this should hardly have happened in reports including major neurological manifestations. Overlooking of cases with major manifestations may, however, have happened in unpublished cases (as suggested by several ones included in many theses), reports published in local journals in different languages, hospital journals, or secondary journals not available in the Internet. In fact, many of those reports were published in such journals throughout many decades of the past century. Another fact sometimes hindering the detection of such reports is the title and abstract of the article not clearly referring to the clinical characteristics of the patient (Frances et al., 1994; Lemoine, 1954; Pelletier et al., 1995). A special effort was made to collect old literature, mainly regarding the period when there was still no specific treatment for fascioliasis available, because many of those old reports include long-term follow-ups of patients.

Europe stands out due to the highest number of neurological cases published, mainly in France and secondarily in Spain. Patients with neurological implications but being infected by the liver fluke in other European countries such as Portugal and Belgium were also diagnosed in France (Table 2.1). In other continents, reports on neurological fascioliasis appear to be highly dispersed, with the exception of Argentina where several patients have been diagnosed presenting with minor neurological manifestations and Egypt and Peru where clinical analyses performed within large surveys also allowed for the detection of minor neurological symptoms (Table 2.2).

Some interesting conclusions may be obtained from this analysis. First, for the moment, nothing suggests the existence of different geographic strains of the liver fluke presenting different capacities to affect the central nervous system. This could a priori be argued when considering that the existence of strains with different biochemical characteristics, including marked variation in end-product formation, was already highlighted as a

Table 2.1 Human fascioliasis reports including cases showing minor or major neurological manifestations and/or ocular manifestations in Europe

		No. of cases presenting with[a]				
Country of infection	**No. of cases**	**Minor neurological manifestations**	**Major neurological manifestations**	**Ocular manifestations**	**Studies and analyses (no. of articles)**	**Studies and analyses (references)**
France	47	27	38	10	35	Prunac (1878 in Bürgi, 1936), Vézeaux de Lavergne (1916 and 1918 in Bürgi, 1936), Mauriac (1922 in Bürgi, 1936), Dunet (1924), Meerssemann et al. (1933 in Bürgi, 1936), Meerssemann et al. (1933 in Bürgi, 1936), Stieffel and Chatron (1934 in Bürgi, 1936), Martin et al. (1944), Brouet et al. (1951), Cattan et al. (1953), Lemoine (1954), Bernheim et al. (1958), Girard et al. (1959), Leger (1959, unpubl. in Berenger, 1984), Dejean (1960), Garde et al. (1961), Boissiere et al. (1961), Guyot (1962), Leng-Levy et al. (1965), Arlet et al. (1966), Aubertin et al. (1966), Domart et al. (1967), Bothier et al. (1968), Coulaud et al. (1970), Saimot et al. (1971), Gil et al. (1970), Lefevre et al. (1970), Domart et al. (1971), Mignot

Continued

Table 2.1 Human fascioliasis reports including cases showing minor or major neurological manifestations and/or ocular manifestations in Europe—cont'd

Country of infection	No. of cases	No. of cases presenting with			Studies and analyses (no. of articles)	Studies and analyses (references)
		Minor neurological manifestations	Major neurological manifestations	Ocular manifestations		
						et al. (1971), Saimot et al. (1971), Lesecq et al. (1972), Giroud et al. (1979), Aimard et al. (1984), Berenger (1984), Oujamaa et al. (2003), Dauchy et al. (2006)
Spain	16	7	5	1	9	Aguirre Errasti et al. (1981), Campo et al. (1984a), Aliaga et al. (1984), Anton Aranada et al. (1985), Arias et al. (1986), Alcoba et al. (1988), Arjona et al. (1995), Nuñez Fernández et al. (2001), Linares et al. (2006)
Portugal (diagnosed in France)	1	0	1	0	2	Pelletier et al. (1995), Frances et al. (1994)
Italy	3	1	3	1	3	Frank (1823 in Bürgi, 1936), Lunedei and Roselli del Turco (1934), Caturelli et al. (2008)
Switzerland	3	2	1	0	2	Blanchod (1909 in Bürgi, 1936), Schussele and Laperrouza (1971a)

Germany	2	1	1	0	2	Mehlis (1825 in Bürgi, 1936), Bürgi (1936)
Austria	1	0	1	0	1	Paul (1927)
Austria (original from Chile)[b]	1	1	1	1	2	Auer et al. (1982), Kristoferitsch et al. (1982)
Belgium (diagnosed in France)	1	0	1	1	1	Becquet and Delassus (1961)
United Kingdom	5	3	2	2	4	Humble and Lush (1881 in Bürgi, 1936), Ward (1911 in Bürgi, 1936), Biggart (1937), Facey and Marsden (1960)

[a]Numbers on cases with neurological and/or ocular manifestations refer to minimum case numbers (i.e. sometimes data were not sufficiently explicit as to reach a conclusion).
[b]Patient born in Chile but living in Austria after 5 years.

Table 2.2 Human fascioliasis reports including cases showing minor or major neurological manifestations and/or ocular manifestations in Africa, Asia, the Americas, and Oceania

Country of infection	No. of cases	No. of cases presenting with[a]			Studies and analyses (no. of articles)	Studies and analyses (references)
		Minor neurological manifestations	Major neurological manifestations	Ocular manifestations		
Africa						
Morocco	2	1	2	0	2	Dunet (1924 in Bürgi, 1936), Ait Ali et al. (2002)
Algeria	3	2	1	1	3	Desage (1926), Bereni and Duboureau (1963), Coumbaras (1966)
Tunisia	1	1	1	1	1	Ayadi et al. (1991)
Egypt	24	22	2	0	3	Ragab and Farag (1978), Yassien et al. (1996), Wahib et al. (2006)
Cameroon (finally diagnosed in France)	1	1	1	0	1	Paraf et al. (1967)
Lebanon	1	1	0	0	1	Birjawi et al. (2002)
Asia						
Turkey	13	13	0	0	3	Aksoy et al. (2005), Karahocagil et al. (2011), Tezer et al. (2013)
Iran	1 (+8[b])	1 (+8[b])	0	1	2	Mansour-Ghanaei et al. (2003), Dalimi and Jabarvand (2005)

Uzbekistan	1	0	1	1	1	Cheng et al. (2007)
India	1	0	1	0	1	Vatsal et al. (2006)
Japan	4	0	4	0	1	Murase et al. (1998)
Korea	2	1	2	1	2	Cho et al. (1994), Park and Sohn (2010)
China	3	2	3	2	5	Pan and Huang (1954), Chen (1991), Ying et al. (2007), Wang et al. (2007), Zhou et al. (2008)
Vietnam	1	1	0	0	1	Andresen et al. (2000)
Sumatra (diagnosed in Switzerland)	1	0	1	0	1	Biermer (1863 in Bürgi, 1936)
The Americas						
Argentina	10	10	1	1	11	Del Valle and Donovan (1928), Cames et al. (1947), Cid (1947), Ahualli and Arias (1961), Strada (1961), Ruggieri et al. (1967), Correa et al. (1969), Padilla Antoni et al. (1970), Carena et al. (1972), Sonzini Astudillo et al. (1973), Giffoniello et al. (1983)
Chile	2	1	1	1	2	Pesse and Atias (1956), Llanos et al. (2006)

Continued

Table 2.2 Human fascioliasis reports including cases showing minor or major neurological manifestations and/or ocular manifestations in Africa, Asia, the Americas, and Oceania—cont'd

Country of infection	No. of cases	No. of cases presenting with			Studies and analyses (no. of articles)	Studies and analyses (references)
		Minor neurological manifestations	Major neurological manifestations	Ocular manifestations		
Peru	150[c]	150[c]	4	1	6	Cosme and Burga (1971), Raymundo et al. (2002), Blancas et al. (2004), Terashima (2004, unpubl. in Blancas et al., 2004), Marcos et al. (2005), Marcos et al. (2006a)
Venezuela	3	3	1	0	3	Bello (1916), Mendoza (1922), Torrealba (1922)
Brazil	3	3	0	1	2	Pereira Igreja et al. (2004), Coral et al. (2007)
Mexico	1	0	1	0	1	Sanchez Vega et al. (2001)
Guatemala	1	0	1	1	2	Aguilar et al. (1967, 1968)
Cuba	2	0	2	0	1	Kouri et al. (1938)
United States	1	1	0	0	1	MacLean and Graeme-Cook (2002)
Hawaii	2	1	1	0	1	Stemmermann (1953)
Oceania						
Australia	2	1	1	0	2	Unpubl. in Prociv et al. (1992), Patrick and Isaac-Renton (1992)

[a]Numbers on cases with neurological and/or ocular manifestations refer to minimum case numbers (i.e. sometimes data were not sufficiently explicit as to reach a conclusion).
[b]Eight cases with only minor neurological symptoms after metronidazole posttreatments.
[c]Data from surveys.

complicating factor in helminths (Barrett, 1984) and that a pronounced genetic heterogeneity of fasciolids has been recently demonstrated throughout (Mas-Coma et al., 2009a). Indeed, although most of the reports are from countries where *F. hepatica* is the only fasciolid present, a few reports from countries where *F. hepatica* is absent, as in parts of Africa (Cameroon), or markedly minority, as in the Far East (China) and Southeast Asia (Vietnam) (Mas-Coma et al., 2009a), indicate that infection by *F. gigantica* may also give rise to neurological manifestations.

The relatively high number of neurological fascioliasis case reports in France, followed by Spain, when compared to the rest of Europe, fits with the higher infection reports in these two countries (Mas-Coma et al., 1999a).

The absence of reports on neurological cases in Bolivia should be highlighted, as indeed the fascioliasis hyperendemic area presenting the highest prevalences and intensities in humans is the northern Bolivian altiplano (Esteban et al., 1999; Hillyer et al., 1992; Mas-Coma et al., 1999b). The sporadic neurological reports in Peru, when excluding minor neurological symptoms found in surveys, are also surprising when considering that this country is the one estimated to present the higher number of people affected by this disease (Esteban et al., 2002; Gonzalez et al., 2011; WHO, 1995). A similar comment should be noted for Egypt according to the very few reports of this kind in that country, whereas estimations indicate hundreds of thousands or even more people infected in the Nile delta human hyperendemic area (Esteban et al., 2003; WHO, 1995).

A chronological analysis of the neurological reports, after excluding the decades of the two world wars, shows a progressive increase of the number of reports, with only a slight decrease of this trend in 1980–1989 and 1990–1999 (Fig. 2.1). Moreover, the chronology of the reports also indicates that, in France, a kind of hospital reporting trend on neurological findings in fascioliasis patients appears evident between the end of the 1950s and the mid-1980s, markedly declining thereafter. Additionally, this fact chronologically parallels the production of many theses on this subject by hospital physicians (Berenger, 1984; Bothorel, 1953; Dejean, 1960; Guyot, 1962; Lesecq, 1972; Massey, 1964; Rigaud, 1957). This is also in agreement with the period in which several thousands of fascioliasis patients were diagnosed in French hospitals (Gaillet, 1983). The description of neurological symptomatology in fascioliasis patients in Spain seems to follow the aforementioned trend in France a few years later. Summing up, the concentration of fascioliasis patients with neurological implications in France should be considered a consequence of a temporary reporting trend rather than

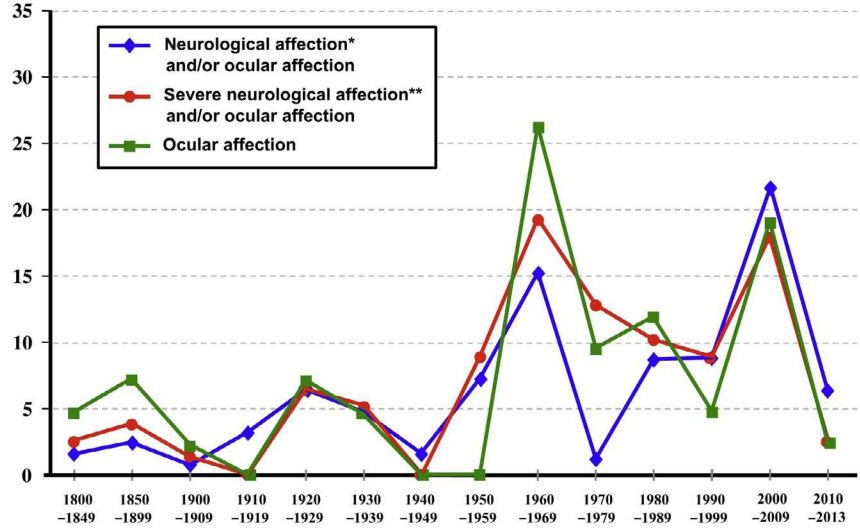

Figure 2.1 Distribution of articles on patients (number of articles in %) suffering from neurological and ocular manifestations, according to year period of publication. Total of articles referring to neurological affection* and/or ocular affection = 125; total of articles referring to both severe neurological affection** and ocular affection = 78; total of articles referring to ocular affection = 42. *including minor and/or major neurological manifestations; **including only major neurological manifestations.

anything related to peculiar neurogenic characteristics of western European liver fluke strains.

In France, in the 1950s, patients reported to show major neurological manifestations were described as being exceptional. Only a decade later, such cases were elevated to the rank of rare (Aubertin et al., 1966; Garde et al, 1961; Guyot, 1962). And finally, several years later, French clinicians began to refer to the possibility of neurological cases being indeed more frequent than initially suspected and, given the difficulties to reach the correct final diagnosis of fascioliasis, to note that the fascioliasis aetiology of many such neurological patients was most probably overlooked, even in France. Several reports dealing with more than one such patient in the same article suggest that specialist expertise is decisive in establishing the cause–effect between fascioliasis and such neurological clinical pictures. Hence, the lack of such knowledge among the physician community may be underlying the overlooking of fascioliasis as causal agent of neurological disease in other countries. Even in France, the authors of the articles on neurological case

reports recognize having been fully disconcerted in front of such long-term puzzling clinical polymorphisms and emphasize the final decisive help of the finding of a previous report on another neurological fascioliasis patient that guided them to the correct diagnosis (Coulaud et al., 1970; Saimot et al., 1971).

All in all, it is easy to conclude that sanitary infrastructure capacities play a crucial role in the analytic feasibility when dealing with patients presenting with such complicated, heterogeneous, and variable clinical neurological pictures. Thus, results indicate that reports on major neurological cases have been simply more numerous in those countries where hospitals were used to analyse and diagnose fascioliasis patients. Infrastructures and personal expertise of neurologists in well-equipped hospitals, usually of a large city, made the difference when comparing with rural hospitals and small health centres in remote poor areas of human endemic regions.

Neither minor nor major neurological manifestations appear to differentiate between the two sexes (Table 2.3). Similarly, all ages seem to be affected, although the highest case records in the 30–39-year-old group should be noted (Fig. 2.2). The neurological affection in children should be highlighted (Table 2.4), because this disease shows clear trends, in both prevalences and intensities, for children at least in human endemic areas (Mas-Coma, 2004; Mas-Coma et al., 2005). The possibility of neurological affection in children may be a serious problem in remote rural areas of developing countries.

Table 2.3 Distribution of cases with neurological and/or ophthalmologic manifestations according to gender

	Gender			
Cases	**Male**	**Female**	**Indet.**	**Total det.**
Cases with only minor neurological manifestations[a]	26 (49.0%)	27 (50.9%)	161	53 det. (100%)
Cases with major neurological manifestations	40 (51.94%)	37 (48.0%)	7	77 det. (100%)
Cases with ocular manifestations	19 (67.8%)	9 (32.1%)	0	28 (100%)
Cases with neurological and/or ophthalmologic manifestations	69 (51.5%)	65 (48.5%)	201	134 det. (100%)

[a]Posttreatment cases excluded.

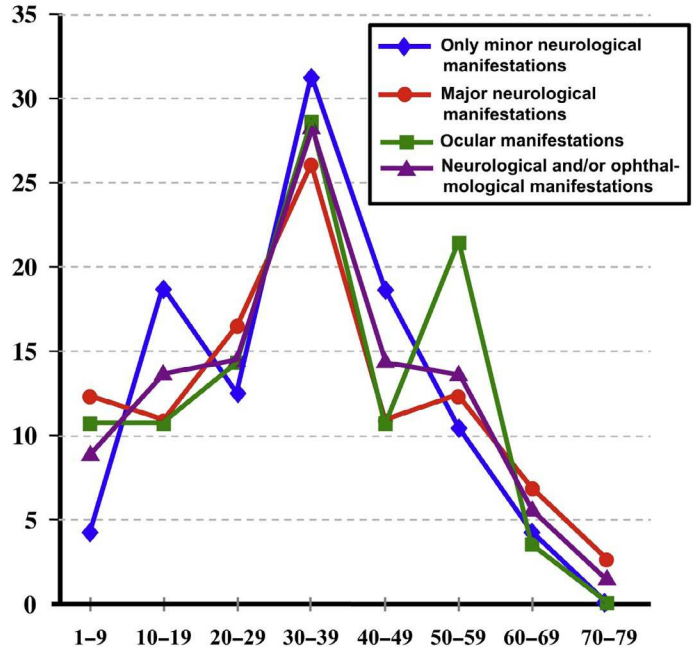

Figure 2.2 Distribution of patients (number of patients in %) suffering from neurological and ocular manifestations, according to age groups (in years). Total of patients with only minor neurological manifestations = 48; total of patients with major neurological manifestations = 73; total of patients with ocular manifestations = 28; total of patients with neurological and/or ophthalmologic manifestations = 124.

Table 2.4 Distribution of cases with neurological and/or ocular manifestations in children according to age groups

	Children age groups			
Cases	**1–4**	**5–9**	**10–14**	**15–19**
Cases with only minor neurological manifestations[a]	0 (0.0%)	2 (4.2%)	4 (8.3%)	5 (10.4%)
Cases with major neurological manifestations	4 (5.5%)	5 (6.8%)	5 (6.8%)	3 (4.1%)
Cases with ocular manifestations	1 (3.6%)	2 (7.1%)	2 (7.1%)	1 (3.6%)
Cases with neurological and/or ophthalmologic manifestations	4 (3.2%)	7 (5.6%)	9 (7.2%)	8 (6.4%)

[a]Postreatment cases excluded.

3.2. Types of cases

Fascioliasis patients may be distinguished into two main types according to the location of the fluke parasites causing the neurological manifestations:

- *Neurofascioliasis*: Cases in which the neurological symptoms are due to the direct effects of a migrating juvenile fluke present in the brain or present in a neighbouring organ and with cerebral lesions suggesting having migrated to the brain. According to the few number of reports on cases of this type, it appears that this modality may be rare. However, it is evident that such an ectopic intracranial parasitization is not easy to diagnose, may be confused with other types of cerebral disturbances, or may remain without diagnosis in remote rural areas where the poor people are only able to attend rural local health centres that are usually devoid of the necessary equipments for appropriate analyses and diagnosis. In such cases of direct affection of the central nervous system, the migrating fluke may remain undetected, and the lack of the typical symptomatology of fascioliasis, due to a low number of infecting flukes in the liver or even their total absence and consequent absence of faecal egg shedding, leads to fascioliasis overlook. In fact, the frequency of such intracranial migrating flukes may be higher than the one suggested by the few published reports.
- *Fascioliasis with neurological implications*: Cases in which the neurological manifestations appear to be due to indirect effects at distance, from flukes present in the liver (hepatic fascioliasis) or in other organs (ectopic fascioliasis) and without apparent damage or "mechanic" lesions in the brain or organs close to the brain. This type of cases constitutes the great majority of neurological fascioliasis reports, including from minor to major symptoms within a large variety of clinical pictures. In patients showing such indirect neurological effects, liver fluke infection may be evident showing typical fascioliasis symptoms. But sometimes, it may be almost asymptomatic or only show mild symptoms, leading to *Fasciola* infection overlook, mainly in patients in whom parasite eggs are not found in stools and a specific serological test is not applied because fascioliasis was not suspected. In such cases, neurological symptoms may easily be linked to any other aetiology.

The term of neurofascioliasis should be restricted to patients in whom an intracranial invasion by migrating flukes has been proved. All other patients showing neurological manifestations caused by fasciolids located in the liver or other organs should be referred to fascioliasis with neurological implications, despite sometimes clinically appearing as impressive and similarly severe to those in neurofascioliasis.

Detailed accounts of the two aforementioned modalities of neurological *Fasciola* infection, together with their diagnosis and treatment, are given in the following.

4. NEUROFASCIOLIASIS OR INTRACRANIAL FASCIOLIASIS

Case reports in which an intracranial invasion by migrating fasciolids has been demonstrated are very few. Their clinical pictures show common surprising characteristics, including a sudden appearance of symptoms, a pronounced heterogeneity of neurological manifestations, a disconcerting clinical evolution, and an unexpected appearance–disappearance of manifestations. Given that a summary becomes impossible and would easily give rise to misinterpretations, the clinical pictures of selected patients are briefly described in the following to furnish an appropriate overview.

A typical clinical fascioliasis picture without complications developed in a 35-year-old man in mid-September, including increasing abdominal pain, fever, eosinophilia, and appearance of *F. hepatica* eggs in stools (Lunedei and Roselli del Turco, 1934). Unexpectedly, on the 5th of January, pronounced pain at the right side of the shoulder block appeared and disappeared 2 days later. On the 13th of January, strong pain on the frontal region and left back of the neck, together with hemiparalysis of the left midbody and marked left hemihypoesthesia, more evident on upper limbs, appeared and again disappeared 2 h later, except mild cephalalgias, neck back stiffness, and vomits that remained until the 15th of January to slowly disappear during the following days. On the 20th of January, neck cephalalgia returned accompanied by strength decrease in the left limbs. A violent cephalalgia appeared, followed by hemiparalysis of the left body and right face, speech power loss, and ophthalmologic symptoms, increasing 4 h later with peripheral paralysis of the VII cranial nerve affecting both the upper and lower limbs similarly; left hemiplegia of the tongue and limbs with pronounced left tendinous areflexia (neither Babinski nor Oppenheim); and strong decrease of tactile, thermic, and pain sensibility in the left midbody. In the following 2 days, the nervous system conditions did not change, whereas the general status experienced a progressive fall down. Patient's conditions kept almost invariable until 10 months later when he showed an evident hypoacousia and mild left vestibular hypoexcitability at ear examination and a 72% rachidian eosinophilia. A small oval-shaped, greenish, motionless form of around 4.10 mm, with a small protuberance at its upper extremity,

was observed in his right eye. The sudden appearance of an inferior pontine syndrome of the Millard–Gubler type with VI cranial nerve involvement, the absence of other symptoms justifying an embolism, the absence of crisis and haemorrhagic manifestation trends, the high rachidian eosinophilia, the many serious manifestations suggesting a direct affection of the central nervous system, the complexity of the pontine syndromes mainly regarding sensibility disorders, and the recovery after four emetine treatment courses suggested a cerebral location of an *F. hepatica* worm and subsequent eye involvement, evoking a possible consequence of reinfection. Six months after treatment, the patient gradually recovered. Only slight hypoesthesia on the left upper limb and reduced paresis persistence of abducens and right upper facial remained 3 months thereafter.

A case to be highlighted is that of a 44-year-old woman in Argentina admitted with presumptive diagnosis of intracranial tumour (Correa et al., 1969; Ruggieri et al., 1967). Past history revealed episodes of sharp pain in the right upper abdomen quadrant spreading to both the epigastrium and shoulder of the same side and periodic episodes of jaundice, usually accompanied by fever, nausea, and vomiting, together with episodes of severe generalized itching for about 7 years. She had become listless and irritable and complained of an intense headache in the past 2 years. These symptoms progressed, and she had an episode of temporary aphasia with opisthotonos and deviation of the head and eyes towards the left. On examination, the patient appeared apathetic and showed yellowish skin, with a painful abdomen upon deep palpation and percussion. The remainder of the neurological examination was normal except for right hyperreflexia. Parasitosis was not considered, due to only slight anaemia without leucocytosis and eosinophilia. An electroencephalogram revealed a left frontotemporal focus. An arteriogram showed a shift of the left anterior cerebral artery, Sylvian vessels displaced downwards, and a vascular shell with clearly delineated lesion margins in the parietal region. On operation, two cysts containing dark green liquid, each approximately 2×3 cm, were removed. The remarkable soft consistency of the brain and extensive oedema necessitated closure without replacement of the bone flap. The patient died 24 h later. Gross examination of the surgical specimen revealed abundant, yellow-white material; part of this was solid and another part resembled a cystic wall. On microscopic examination, several pieces of this material were meninges and brain. Numerous *F. hepatica* eggs surrounded by intense lymphocytic infiltration were found, but the parasite was not. Since the parasite had been present for a considerable period, it could have become

liquefied inside one of the cysts, when not inadvertently suctioned during operation. The bloodstream was evoked to be the most reasonable route.

The Spanish case of a 26-year-old male was highlighted because of the coexistence of multiple meningoencephalic, orbital, and pulmonary involvements of the disease caused by an ectopic *Fasciola* (Arias et al., 1986). On admission, there was permanent headache after 1 week, accompanied by dysphasia and unilateral facial paraesthesia. Fever, jaundice, hepatomegaly, and splenomegaly were already detected 1 month before. Transient pulmonary infiltrates agreeing with the Loeffler manifestations were found on thorax radiography. There was neck back stiffness, 70% eosinophilia in the cerebrospinal fluid, and only 16% eosinophilia in the blood, with normal results in hepatic tests and no eggs in stools. After the disappearance of cephalalgias and normalization of the cerebrospinal fluid, dysphagia and ocular disorders appeared unexpectedly 2 months later, increasing progressively thereafter. Additionally to the ocular problems, paresis of the soft palate with abolition of the homolateral gag reflex appeared. There was neither eosinophilia in the cerebrospinal fluid and blood nor hepatosplenomegaly, but there was presence of numerous eggs in stools. After a 2-week treatment with dehydroemetine, dysphagia and soft palate paralysis disappeared, but right eye amaurosis was still present. This patient suffering from eosinophilic meningitis fully recovered, except for eye blindness. An intracranial fasciolid fluke was not found nor observed, but it was concluded that the soft palate paralysis was indicative of a mononeuritis due to direct compressive pathology, although immunologic pathogenicity could not be dismissed. The cerebrospinal fluid eosinophilia, the retro-ocular pain, and subsequent ocular disorders support an ectopic *Fasciola* infection.

In a woman admitted to a Sydney hospital in 1974, with patent *Fasciola* infection and a cerebral abscess, ectopic fascioliasis could not be confirmed by finding trematode structures in tissue sections or by using a specific antibody test applied to the sections (Prociv et al., 1992, unpublished observation, p. 351).

A 28-year-old man suffered from intermittent right frontal headache for a month followed by left-sided hemiparesis 10 days before admission (Cho et al., 1994). Brain computed tomography (CT) showed a resolving haematoma extending from the genu to the body of the corpus callosum (Fig. 2.3). A neurological examination revealed finger agnosia and slight dysarthria left hemiparesis of grade IV and hemihypoesthesia on the left side. Blood counts showed 18,200/μl leucocytosis with 83% neutrophils. Stool

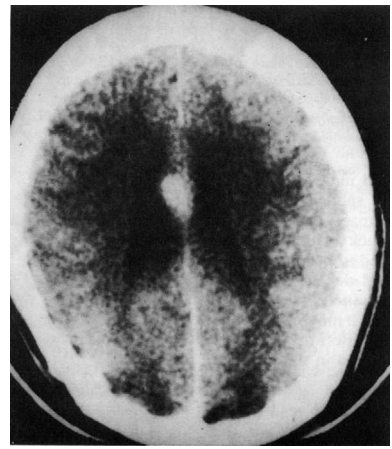

Figure 2.3 Computed tomography (CT) of the brain showing a resolving haematoma in the corpus callosum of a 28-year-old man presenting with left-sided hemiparesis, slight dysarthria, and finger agnosia. *From Cho et al. (1994); © by the American Society of Tropical Medicine and Hygiene.*

examination revealed no helminth eggs. Under an impression of subarachnoid haemorrhage, conservative treatment was given. Angiography was done, but no pathological findings were recognized. On the eighth day, frontal headache, aggravated by steroid therapy, continued. The next day, disorders in the right eye appeared. An intraocular *F. hepatica* juvenile was recovered at enucleation at 11th day post-admission. The neurological symptoms disappeared gradually within 6 months after the surgery and the patient did not exhibit clinical problems in a 3-year follow-up.

A left frontal lesion and two cystic lesions were revealed by brain CT scan and liver ultrasound, respectively, in a 6-year-old boy presenting with facial swelling and monocular blindness (Cheng et al., 2007). Initial clinical findings and radiological investigation had suggested sinusitis, and the patient underwent a sinus drainage procedure. Following surgery, the patient was found to have proptosis, chemosis, dilated episcleral vessels, no perception of light in the left eye, a dispersed hyphaema, and a cataractous lens. A leaf-shaped, undulating "stingray"-like organism with cephalic cone was visible in the left eye.

Significant cerebral and ophthalmic tissue damage as results of worm migration was detected in an 8-year-old boy suffering episodes of sudden onset and persistent headache, nausea, and vomiting, indicating haemorrhage of the right occipital lobe of the brain and meningitis (Wang et al., 2007; Ying et al., 2007). Upon admission, eosinophilia was only 6.7%. Headache,

nausea, and vomiting recurred on the fourth day. CT showed haemorrhage of the left parietal lobe, and digital subtraction angiography (DSA) showed aneurysms in the right middle cerebral artery and left vertebral artery. After the 19th post-admission day, during a period of ocular disorders, the patient was found to have subdural haemorrhage of the left frontal, temporal, parietal, and occipital lobes. Haemorrhagic location was not consistent with that of aneurysms. Ultrasonography revealed mild hepatomegaly, but liver function tests were normal. No eggs were found in stools. On the 26th day, a juvenile *F. hepatica* was recovered from the eye.

Another case showed multiple brain haemorrhages and haematomas that were also associated with ectopic fascioliasis in the brain and eye. A 10-year-old boy suffered from intermittent headache, vomiting, repeated fever, and several monocular symptoms (Zhou et al., 2008). Upon admission, there was, however, no neurological symptom. A blood test revealed leucocytosis (5.19×10^9/l) and slight eosinophilia (6.7%), but liver function and blood coagulation were normal. CT and ultrasonography showed mild liver enlargement, and CT scan showed a haematoma in the right occipital lobe and a subacute subdural haematoma in the left temporoparietal lobe. On the third hospital day, the patient began to have a headache and to vomit. CT scan showed a new haemorrhage in the left parietal lobe (Fig. 2.4). Head DSA revealed two unruptured intracranial saccular aneurysms located in the right middle cerebral artery and the left vertebral artery. On the 11th day, after progressive headache, another CT scan showed a new haematoma in the right lateral ventricle. On the 17th day, the patient developed ophthalmologic disorders and fever. At the 20th day, magnetic resonance imaging (MRI) revealed a large new subdural haematoma in the left frontotemporoparietooccipital lobe. On the 29rd day, a small leaf-shaped immature *F. hepatica* was recovered when moving out of the right eye. Serological tests were positive, despite egg absence in stools. After 1 year post-treatment, recovery was total, CT scan showed cure of brain haematomas, and DSA revealed that the left vertebral artery aneurysm disappeared, but the aneurysm in the M1 segment of the right middle cerebral artery remained unchanged.

Two other patients were reported to have been most probably caused by a direct intracranial affection, one with impressive manifestations (Garde et al., 1961) and another interesting apyretic one who finally died (case 1 of Berenger, 1984). However, given that the involvement of an ectopic fluke could not be found nor demonstrated, their manifestations are not analysed here within the neurofascioliasis cases.

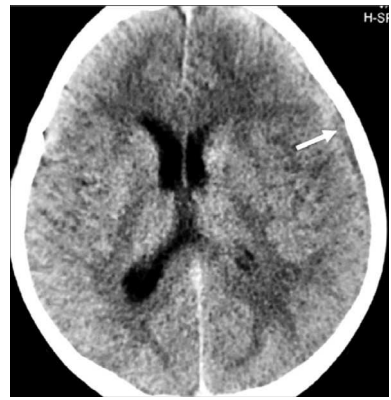

Figure 2.4 Head computed tomography (CT) of a 10-year-old boy from China affected by varying brain lesions caused by a migrating small immature *Fasciola hepatica* juvenile, including five consecutive episodes of intracranial haemorrhages and haematomas: CT scan on the third hospital day showing a subacute subdural haematoma (second haematoma) in the left temporoparietal lobe. *Modified from Zhou et al. (2008); © by Elsevier Ltd.*

5. FASCIOLIASIS WITH NEUROLOGICAL IMPLICATIONS

5.1. Minor symptoms

In patients presenting neurological effects related to *Fasciola* infection, two types of manifestations may be distinguished according to their importance and frequency: minor symptoms and major manifestations.

Minor symptoms include cephalalgias, character disorders (instability and irritability), vertigo, nightmares, delusional disorders, and/or insomnia (Aubertin et al., 1966; Dejean, 1960; Domart et al., 1967; Schussele and Laperrouza, 1971a,b). These manifestations are rather frequent and cannot be related to fascioliasis unless they are of recent appearance and/or regress after fasciolicide treatment.

Cephalalgia (headache), more or less intense, is by far the neurological manifestation most frequently noted in studies performed in different, both developed and developing, countries. Surveys of 5–14-year-old schoolchildren in human hyperendemic rural Andean areas of Peru have shown that cephalalgias are usual in liver fluke-infected subjects shedding eggs in stools, such as the 60% of children presenting with cephalalgias in Cajamarca (Cosme and Burga, 1971), 63% in Mantaro Valley (Terashima, 1970 in Blancas et al., 2004), and 59% in the Peruvian altiplano

(Raymundo et al., 2002). Unfortunately, these results should only be considered as suggestive, as the numerous multiparasitisms by protozoans and other helminths associated with *Fasciola* infection (Esteban et al., 2002; Gonzalez et al., 2011) do not allow for significant clinical studies of children only infected by *F. hepatica* in these rural Andean areas of Peru.

In analyses of patient series, headache was noted in 17.5% of 14 liver fluke-infected children and in 27.6% of 60 adults in Lima, Peru (Blancas et al., 2004), and in 11 out of 28 fascioliasis patients in Turkey, of whom 5 presenting no eggs in stools (Karahocagil et al., 2011). Headache has also been usually found in patients analysed in France (Dauchy et al., 2007; Dejean, 1960), Switzerland (Schussele and Laperrouza, 1971a,b), Spain (Aguirre Errasti et al., 1981), and Argentina (Mera y Sierra et al., 2011).

Cephaleas, when specified, were mostly frontal (Arlet et al., 1966; Berenger, 1984; Bereni and Duboureau, 1963; Leng-Levy et al., 1965; Stemmermann, 1953), sometimes frontotemporal (Ayadi et al., 1991), or occipital (Brouet et al., 1951, Dejean, 1960). In one patient, they were both frontal and occipital, associated with vertigo (Garde et al., 1961), and in another patient, they were described to be holocranial or generalized (Nuñez Fernández et al., 2001). In other reports, cephaleas were found to mainly appear or increase during night (Coulaud et al., 1970; Saimot et al., 1971) as to give rise to nocturnal vomiting (Berenger, 1984). In several cases, cephaleas were noted be persistent (Cames et al., 1947; Desage, 1926; Málaga et al., 2012; Nuñez Fernández et al., 2001; Wang et al., 2007; Ying et al., 2007), intermittent and appearing in episodes or recurrent (Schussele and Laperrouza, 1971a,b; Stemmermann, 1953), intense (Arjona et al., 1995; Brouet et al., 1951; Cames et al., 1947; Dejean, 1960; Dunet, 1924; Guyot, 1962; Mendoza, 1922), or violent (Berenger, 1984; Desage, 1926). When of long duration, severe cephaleas may be of only 1 week (Birjawi et al., 2002), 3 weeks (Brouet et al., 1951), 1 month (Dunet, 1924), 2 months (Ahualli and Arias, 1961), 5 months (Domart et al., 1967), 1 year (Ragab and Farag, 1978), or even 15 years (Cames et al., 1947).

In many patients, headache was reported to appear during the invasive phase, sometimes very early or be even the first symptom (Aliaga et al., 1984; Andresen et al., 2000; Anton Aranada et al., 1985; Arlet et al., 1966; Berenger, 1984; Bereni and Duboureau, 1963; Bernheim et al., 1958; Brouet et al., 1951; Coulaud et al., 1970; Giffoniello et al., 1983; Guyot, 1962; MacLean and Graeme-Cook, 2002; Nuñez Fernández et al., 2001; Schussele and Laperrouza, 1971a,b; Tezer et al., 2013; Ying et al., 2007). However, cephaleas have also been many times reported in

patients diagnosed during the biliary phase (Ahualli and Arias, 1961; Berenger, 1984; Birjawi et al., 2002; Cames et al., 1947; Carena et al., 1972; Domart et al., 1967; Dunet, 1924; Garde et al., 1961; Málaga et al., 2012; Mendoza, 1922; Patrick and Isaac-Renton, 1992; Pesse and Atias, 1956; Ragab and Farag, 1978; Sonzini Astudillo et al., 1973; Strada, 1961; Torrealba, 1922), sometimes even in the very advanced chronic stage, after many years postinfection or a long time after from the first typical symptoms, as 8 years (Bello, 1916; Stemmermann, 1953), 10 years (Kristoferitsch et al., 1982), 11 years (Desage, 1926), 12 years (Del Valle and Donovan, 1928), or even 15 years (Padilla Antoni et al., 1970). Whether on the invasive phase or in the biliary phase, when the patient was treated with a specific fasciolicide drug such as emetine or dehydroemetine, cephaleas regressed rapidly, thus proving to be related to *Fasciola* infection (Berenger, 1984; Guyot, 1962).

Dizziness also appears in many of such studies. This symptom appeared indeed statistically associated with liver fluke infection in 49.2% schoolchildren (Marcos et al., 2006a; Raymundo et al., 2002) and also in surveys of total population, although it was considered to be not likely caused by fascioliasis (Marcos et al., 2005). Unfortunately, the aforementioned problem of the numerous multiparasitisms associated with *Fasciola* infection (Esteban et al., 2002; Gonzalez et al., 2011) again poses doubts on the statistically significant relationship of dizziness with fascioliasis in these human hyperendemic rural Andean areas of Peru. Dizziness was also noted in 8 out of 18 patients in Egypt (Ragab and Farag, 1978).

Interestingly, headache is frequently detected in patients coinfected with other infectious agents, such as hepatitis C virus (Wahib et al., 2006), brucellosis (El-Metwally et al., 2011; Mohammad et al., 2011), and toxocariasis (Tezer et al., 2013).

Headache followed by dizziness and also nausea and urticaria are also the side effects most frequently highlighted after fascioliasis treatment. Such adverse events are usually directly proportional to the intensity of infection and can be classified as systemic or mechanical. These symptoms are considered systemic events when caused by biological substances released by the dying worms (Villegas et al., 2012). In fascioliasis, such side effects have been described after treatment with different drugs, such as Glucantime (Brouet et al., 1951); Entobex®, with important frontal cephalalgias reappearing after four cures with that drug (case 1 of Berenger, 1984); praziquantel, including both headache and dizziness in 46.15% of 26 schoolchildren treated (Yassien et al., 1996); chloroquine, including additionally migraines and marked

tremors (Facey and Marsden, 1960); metronidazole (Mansour-Ghanaei et al., 2003), artemether (Keiser et al., 2011); and triclabendazole (Aksoy et al., 2005; Keiser et al., 2011; Tezer et al., 2013; Villegas et al., 2012).

Migraines have been described in fascioliasis patients more sporadically (Berenger, 1984; Boissiere et al., 1961), similarly as vertigo (Boissiere et al., 1961; Garde et al., 1961; Mendoza, 1922; Schussele and Laperrouza, 1971a,b). A delusional state does neither appear to be frequent (Dunet, 1924; case 2 of Berenger, 1984), as is the case of temporary sleepiness (Bernheim et al., 1958), although repeated changes from insomnia to sleepiness and back to insomnia, also related to important agitation mainly at the moment to seat at table, have been reported from a patient (Dejean, 1960). An extreme case of daily sleepiness, including frequent episodes of continuous 22 h sleep per day, accompanied by an unusual irritability, has been reported (Aimard et al., 1984).

5.2. Major manifestations

Three main clinical pictures may be distinguished in the fascioliasis patients showing major manifestations: (i) genuine neurological cases in which a neurological syndrome dominates, (ii) meningeal cases in which meningitis symptoms are predominating, and (iii) psychiatric or neuropsychic cases in which mental disorders stand out.

5.3. Cases with genuine neurological manifestations

In fascioliasis patients presenting with neurological manifestations but in whom no ectopic fluke directly affecting the central nervous system is detected, genuine neurological symptoms and signs prove to be the most usual and also the most impressive, sometimes surprising by their evolution and easily giving rise to misleading diagnosis.

These neurological manifestations should be differentiated from those of meningeal origin and those of psychiatric or neuropsychic characteristics. However, the overlap of genuine neurological manifestations with meningeal and psychiatric manifestations within the clinical picture of the same patient may become of confusing interpretation. Indeed, given symptoms may have different underlying causes, whether a cerebral–cerebellar or a meningeal origin.

Genuine neurological symptoms and signs found in these fascioliasis patients are listed in the following. Such major neurological manifestations are shown whether in the invasive or in the biliary phase, sometimes of

sudden appearance, and even as first symptoms, although usually previous discrete symptoms (e.g. fever, asthenia, and digestive manifestations) are found. In several cases, symptoms appeared relatively shortly after the suspected metacercarial ingestion, and in other cases, after a delay of several months. In other cases, manifestations extended along both phases. In a few cases, they have even been reported to only appear after a long-term infection of several years, and in other cases reported long time ago, the same clinical picture was noted to reappear after many years:

(A) *Paresis*: Conditions of weakness of voluntary movement, partial loss of voluntary movement, or impaired movement appear to be the most frequent manifestations in neurological patients infected by *Fasciola*. Most cases reported included impressive limb involvement and only a few have referred to facial affection.

In cases with facial involvement, paresis has been described from the lower left part (Aguirre Errasti et al., 1981) and as right facial hemiparesis asymmetrically attracting the mouth leftwards and with repercussion on speech (case 1 of Berenger, 1984).

In cases of limb affection, conditions of monoparesis, paraparesis, hemiparesis, and even tetraparesis or quadriparesis have been reported. In patients showing upper limb paresis, the most usual conditions seem to be right arm monoparesis (Campo et al., 1984a; Coulaud et al., 1970), accompanied by the sensation of dead hand and fingertip paraesthesia (case 2 of Berenger, 1984) or including weakness in the flexion of the right wrist and fingers (Park and Sohn, 2010). Left upper limb monoparesis has also been reported (Aguirre Errasti et al., 1981).

Paresis of the left lower limb was reported, whether predominant (Domart et al., 1971), accompanying paresis of the right upper limb (Gil et al., 1970; Lefevre et al., 1970), or of both upper limbs in a curious case of triparesis (Kristoferitsch et al., 1982). Paresis of both lower limbs has also been mentioned (Llanos et al., 2006), in a case as a spastic paraparesis with exaggerated tendon reflexes and muscle hypertonia in both legs (Caturelli et al., 2008).

Hemiparesis when present was interestingly always affecting the left side (Aubertin et al., 1966; Ayadi et al., 1991; Cattan et al., 1953; Domart et al., 1967; Frances et al., 1994; Pelletier et al., 1995), sometimes accompanied by problems in respective hand (Domart et al., 1967) or bilateral disturbances of proprioceptive sensitivity (Frances et al., 1994; Pelletier et al., 1995). The surprising fast appearance of

the hemiparesis in the patients was emphasized in given patients (Cattan et al., 1953; Frances et al., 1994; Pelletier et al., 1995). A spasmodic quadriparesis with initial lower limb buckling later extending to pronounced motor deficit and hyperreflexia in the four limbs has also been reported (Bothier et al., 1968).

Positivity in both the Barré test for the upper limbs and the Mingazzini test for the lower limbs has been noted in fascioliasis neurological patients (Aubertin et al., 1966; Gil et al., 1970; Lefevre et al., 1970).

(B) *Plegia*: Paralysis with facial involvement has only sporadically been noted in fascioliasis patients, such as the one described affecting the left facial side in a 16-year-old boy (Garde et al., 1961). There are, however, several reports on limb paralysis, although in given patients, the term of plegia was noted more in the sense of worsening of the motor deficit up to impede standing up than referring to a total paralysis.

A sudden appearing of short monoplegia affecting the lower left limb, preceded by strong pain immobilizing the right shoulder, and afterwards extending to a paraplegia, was reported from a woman (Domart et al., 1971).

In cases in which hemiplegia was noted, it was always affecting the left side (Cattan et al., 1953; Domart et al., 1971; case 1 of Berenger, 1984), except in one patient in whom it was right (Ragab and Farag, 1978) and another in whom it was not specified (case of Dr. Lesca in Guyot, 1962).

(C) *Walking problems and movement disorders*: Many different conditions including dyskinesia, ataxia, dysmetria, spasticity, apraxia, astasia, and abasia have been reported from fascioliasis patients presenting with neurological disorders.

Dyskinesia of the face, upper limbs, and lower limbs leading to obliged rest (Mendoza, 1922), slight tremor in left lower limb (case 2 of Berenger, 1984), or affecting the trunk (Coumbaras, 1966) have been reported. Other symptoms reported include movement difficulties in the fingers of the right hand (Cattan et al., 1953), gestures with very pronounced slowness (Gil et al., 1970; Lefevre et al., 1970), a context of motor slowdown (Oujamaa et al., 2003), and abnormal movements of the right upper limb (case 1 of Berenger, 1984).

Impressive manifestations include disturbances and difficulties of walking capacity (Cattan et al, 1953; Dejean, 1960; Paul, 1927),

insecure walking (Kristoferitsch et al., 1982), walking with right deviation (Coulaud et al., 1970; Saimot et al., 1971), and spastic walking (Bothier et al., 1968), sometimes noted to be pronounced, of sudden appearance, and related to a frontal ataxia (Gil et al., 1970; Lefevre et al., 1970). A lack of movement coordination of the type of dysmetria was reported (Bothier et al., 1968). In extreme cases, the problem reached the impossibility for walking (case 1 of Berenger, 1984), accompanied by the impossibility to stand up in a combined condition of astasia and abasia (Aubertin et al., 1966; Lesecq et al., 1972; case 2 of Berenger, 1984).

Another curious manifestation is a motor incoordination condition of the impossibility to remain seated (Domart et al., 1967), similarly as the apraxic condition of a female patient who began to get dressed and to undress with some difficulty, to get into the bed, or to sit down in a seat (Linares et al., 2006).

(D) *Paraesthesia*: Such a condition has been described in several fascioliasis patients, affecting different parts of the body.

At facia level, it has been reported from the right side (Arias et al., 1986) and also affecting half of the tongue although without side specification (Aguirre Errasti et al., 1981). In another patient, paraesthesia was noted in both hands, accompanying an abnormal aspect of the first phalanges of the fingers (Coulaud et al., 1970; Saimot et al., 1971).

Lower limb paraesthesia was reported from a 50-year-old man (Llanos et al., 2006). Paraesthesia and also hypoesthesia affecting the left upper and lower limbs, neck, and trunk were showed by a female patient (Auer et al., 1982; Kristoferitsch et al., 1982). On the contrary, a painful hyperaesthesia (allodynia) on the sole of the right foot was reported in another case (Lesecq et al., 1972).

(E) *Areflexia, hyperreflexia, and clonus*: Areflexia in the four limbs has been reported in the same patient (Aimard et al., 1984). In other cases, there was an asymmetry with evident drop of the left lower limb osteotendinous reflexia (Coulaud et al., 1970; Saimot et al., 1971), an abolishment of the right Achilles' tendon reflex (Lesecq et al., 1972), or an abolishment of the cutaneous abdominal reflexes (case 2 of Berenger, 1984).

An opposite condition of osteotendinous hyperreflexia has also been reported (Domart et al., 1971), described as polykinetic (case 2 of Berenger, 1984), asymmetrically more increased at left (Domart et al.,

1967) or at both right limbs (Campo et al., 1984a), in both lower limbs (Caturelli et al., 2008), or affecting the four limbs (Bothier et al., 1968).

A clonus, which gets exhausted, was reported from the right Achilles' tendon (Campo et al., 1984a).

(F) *Neurological pains and sciatica*: Pains in different parts of the body have frequently been reported from neurological patients infected by *Fasciola*. In all the reports, the disorders are simply described as pains, without evoking its origin (neuropathic pains and neuralgias), and in several cases, they are referred to as diffuse myalgias (Coulaud et al., 1970; Saimot et al., 1971) or myalgias in the limbs, mainly the lower ones (Málaga et al., 2012), although in the latter cases, neurological pains may have been probably interpreted as muscular.

In fascioliasis patients with neurological disorders, diffuse pain has been reported from the left facial side and throughout the spinal column or rachialgia (Lemoine, 1954), upper part of the neck or cervicalgia (Frances et al., 1994; Pelletier et al., 1995), violent pains on the neck and thighs and also the lumbar region (Dejean, 1960), and as intense as to immobilize the right shoulder (Domart et al., 1971). Pains in the articulations of hands and fingers have also been reported (Coulaud et al., 1970).

There are also reports of pains in the lower limbs (Kristoferitsch et al., 1982), noted as strong (Becquet and Delassus, 1961) or stabbing (Lesecq et al., 1972), and additionally including intolerable pains on the feet, generating insomnia.

An interesting case of a 1-month-long mononeuritis located in the region of the external popliteus sciatic nerve was reported in a 12-year-old girl (Lesecq et al., 1972). The aforementioned strong pains in the lumbar region (Dejean, 1960) and feet (Becquet and Delassus, 1961) were most probably also sciatic manifestations.

(G) *Speech disorders*: Dysphasia was reported from a fascioliasis patient presenting with neurological manifestations (Arias et al., 1986). Dysarthria has been described both in a 8-year-old girl (Mendoza, 1922) and in a 60-year-old man with decreased attentiveness (Park and Sohn, 2010). Another speech disorder such as aphasia, characterized by intracranial hypertension signs, may also appear, as indeed it was reported from an Argentinean patient with verified neurofascioliasis (Correa et al., 1969; Ruggieri et al., 1967). Intracranial hypertension related to the affection of half side of the tongue has also been noted (Aguirre Errasti et al., 1981).

(H) *Loss of senses*: Both sense disorders of anosmia and ageusia were reported from the same fascioliasis patient infected by *F. hepatica* (Becquet and Delassus, 1961).

(I) *Convulsions, epilepsy, and coma*: Generalized convulsions have been reported from several fascioliasis patients suffering from neurological disorders (Aguilar et al., 1967, 1968; Aguirre Errasti et al., 1981; Ragab and Farag, 1978). Uncontrolled abnormal movements and convulsions were described in a girl (Mendoza, 1922). Several convulsive crises were reported from a boy who showed a worrying clinical aspect and obtundation without meningeal clinical manifestations (Garde et al., 1961). Short convulsive episodes of only a few seconds were shown on the left half-body side by another boy (Ayadi et al., 1991). The ages of all the aforementioned cases should be emphasized: 4 years (Aguilar et al., 1967, 1968; Aguirre Errasti et al., 1981), 8 years (Mendoza, 1922), 10 years (Ragab and Farag, 1978), 12 years (Ayadi et al., 1991), and 16 years (Garde et al., 1961).

Convulsions were, however, also reported from older subjects, as in a 28-year-old female in whom a night convulsive episode led to a 24-h-long ungainliness condition (Domart et al., 1967). Convulsions were noted to be rare among a total of 277 fascioliasis patients (Blancas et al., 2004).

The manifestations were, however, described as repeated epileptic seizures in another male patient (case of Dr. Valverde in Kouri et al., 1938).

An unconsciousness state of coma has been also reported in a patient suffering from fine tremor (Coumbaras, 1966). The coma was described as apparently epileptic due to having appeared after a convulsive crisis in a patient suffering from Jacksonian seizures (Garde et al., 1961). A coma with awaking due to pain and afterwards another deep coma episode was shown by a 67-year-old man who finally died (case 1 of Berenger, 1984).

(J) *Confusion, disorientation, and amnesia*: Besides less serious mental disorders such as those reported in several patients of the type of obtundation (Garde et al., 1961; Gil et al., 1970; Lefevre et al., 1970; case 1 of Berenger, 1984) or progressive mental torpor (Campo et al., 1984a), other fascioliasis cases have been described presenting with confusion, disorientation, and amnesia.

Confusion associated with disturbances of vigilance was noted from a male patient (Aimard et al., 1984). A 1-month state of delirium was

reported from a patient (Dunet, 1924). A state of pronounced confusion and delirium was shown by a female patient infected by *F. gigantica* (Paraf et al., 1967). Disorientation was reported in other cases, whether among a pronounced condition change in the patient (Lemoine, 1954) or as temporospatial disorientation (Gil et al., 1970; Lefevre et al., 1970). Both disorientation and confusion have been sometimes reported from the same patient (Ait Ali et al., 2002).

An extreme condition such as amnesia has rarely been noted (Lemoine, 1954), the disturbances specified to be of the type of an anterograde amnesia in a case of coinfection with *Toxocara* (Oujamaa et al., 2003).

(K) *Babinski's sign*: This characteristic of pyramidal insufficiency has been repeatedly reported from fascioliasis patients suffering from neurological manifestations (Aubertin et al., 1966; Cattan et al., 1953; Domart et al., 1967) and specified to be unilateral, whether at the right (Bothier et al., 1968), left (Gil et al., 1970; Lefevre et al., 1970), or bilateral (Domart et al., 1971; cases 1 and 2 of Berenger, 1984).

(L) *Hoffmann's sign*: This sign have been detected two times in fascioliasis neurological patients. In one case, this sign appeared unilaterally at the right (Coulaud et al., 1970; Saimot et al., 1971), whereas it was bilateral in another case (case 2 of Berenger, 1984).

(M) *Kernig's sign*: This manifestation indicating subarachnoid haemorrhage or meningitis has been described in a 4-year-old boy who finally died (Paul, 1927). It was noted to be discrete in a 33-year-old man (Leng-Levy et al., 1965) but well evident in a 12-year-old girl (Lesecq et al., 1972).

(N) *Rare manifestations*: Although only sporadically reported in fascioliasis patients with neurological disorders, manifestations such as pathological obesity and hydrocephalus merit consideration.

The involvement of the hypothalamus and thyroid gland seems to be sporadic in fascioliasis patients. A rare case of a female child 11 years of age who, after successful emetine hydrochloride treatment, became ravenous for food and developed pathological obesity (Ragab and Farag, 1978) was suggested to have been probably due to a hypothalamic lesion caused by the fluke (Chen and Mott, 1990). This case remembers another neurological case of a 63-year-old woman in whom alterations in the right lobe of the thyroid gland were found with scintigraphy (Gil et al., 1970; Lefevre et al., 1970). As known, hypothalamic and thyroid functions are related, as the hormonal output from the thyroid is regulated by the thyroid-stimulating hormone

produced by the anterior pituitary, which itself is indeed regulated by thyrotropin-releasing hormone produced by the hypothalamus.

Hydrocephalus accompanying macrocephaly was noted in a case of a very precociously infected 1-year-old child in Mexico (Sanchez Vega et al., 2001). A certain degree of hydrocephalus was also detected at arteriography in a 16-year-old male who presented with a sudden and rapid evolution of unilateral neurological symptoms and electroencephalography (EEG) alterations. This patient is, however, more a meningeal case, as indicated by the finding of a 60% eosinophilia at lumbar puncture (Garde et al., 1961).

5.4. Meningeal cases

In several fascioliasis patients presenting with puzzling polymorphic clinical pictures, meningeal manifestations appear associated with other neurological manifestations although a meningeal syndrome is predominant (e.g. Dunet, 1924; Garde et al., 1961; case 1 of Berenger, 1984). In other patients, however, meningeal manifestations may appear isolaterally, without neurological disorders (e.g. Bernheim et al., 1958; case 2 of Dejean, 1960; case 2 of Guyot, 1962; Leng-Levy et al., 1965). Major meningeal manifestations may appear throughout both the invasive and the biliary phases.

Initial minor symptoms as headache and neck stiffness become associated with confusion or altered consciousness and vomiting, normal or elevated temperature, sometimes irritability, and rarely with photophobia. The meningeal affection may be confirmed by the presence of eosinophilia in a muddy cerebrospinal fluid obtained by lumbar puncture. High albumin concentration and hypercytosis are additional elements of value. In several patients lacking a meningeal syndrome (e.g. Aimard et al., 1984; Aubertin et al., 1966; Bothier et al., 1968; Cattan et al., 1953; Coulaud et al., 1970; Domart et al., 1967, 1971; Gil et al., 1970; Lefevre et al., 1970; Lesecq et al., 1972), the cephalorachidian fluid was normal.

Moreover, the maintenance of meningeal manifestations after a therapy nonspecific for fascioliasis, such as the resistance of the meningeal symptomatology to antibiotics, should draw the attention (Bernheim et al., 1958; Domart et al., 1967).

It classically appears as a meningitis with pale eosinophilic liquid (22–60%) (Garde et al., 1961; Leng-Levy et al., 1965; Lunedei and Roselli del Turco, 1934; case 1 of Berenger, 1984), with predominance of polynucleates and lymphocytes (Bernheim et al., 1958) or of polynucleates (case 2 of Guyot,

1962). The cervicospinal fluid protein concentration is in average very high, of 0.96 g/l, with extremes of 0.30 (case 2 of Dejean, 1960) and 1.36 (case 1 of Berenger, 1984), indicating an inflammatory process of the meninges and of normal gamma globulins. In given cases, a clinical meningeal syndrome with normal cephalorachidian fluid was reported (Coulaud et al., 1970; Domart et al., 1967).

A first fascioliasis case presenting with a meningitis syndrome was reported early in the past century (Dunet, 1924). Meningeal manifestations including intense cephaleas, high fever, constipation, and a delirious condition kept for more than 1 month were shown by a 27-year-old female with muddy cerebrospinal fluid and hepatic disorders evolving after 3.5 years and in whom three large fasciolas were found in her common bile duct. A meningoencephalitic case was later reported, but without eosinophilia having been verified in the cerebrospinal fluid (Leger, 1959 in Berenger, 1984).

An interesting case of eosinophilic meningitis was described in a 14-year-old girl. The persistence of the meningitis and eosinophilia in both the blood and cerebrospinal fluid and the absence of any hepatic disorder and fluke eggs in stools and duodenal aspirate suggested a process caused by an ectopic fluke directly contacting with the meninges during the invasive phase (Bernheim et al., 1958). The patient fully recovered after emetine treatment and the ectopic fluke cause remained as the most probable hypothesis.

Symptoms of an acute meningitis with violent neck pain, fever, and vomiting accompanying neurological manifestations were also described in a 54-year-old male, who was cured with emetine (case 2 of Dejean, 1960).

An ectopic fluke location within a vessel neighbouring the meninges was also evoked to explain the sudden beginning and rapid evolution of manifestations including a meningeal reaction and unilaterality of the neurological signs and of the EEG alterations shown by a 16-year-old male. A 60% eosinophilia was found at lumbar puncture. Interestingly, a certain degree of hydrocephalus was detected at arteriography (Garde et al., 1961).

Eosinophilic meningoencephalitis was the final summarized diagnosis for a 37-year-old woman with frequent migraines, violent cephalalgias, biliary vomits, and sleepiness, but without fever. The repetition of these symptoms was interpreted as consequences a meningeal haemorrhage. The meningeal irritation was detected by EEG. Recovery was obtained with emetine treatment (case 2 of Guyot, 1962).

A meningeal syndrome including frontal cephaleas, photophobia, vomiting, constipation, light Kernig's sign, and atypical nervous

manifestations, together with 30% eosinophilia in the cerebrospinal fluid obtained by lumbar puncture and the Loeffler images at radiography, was reported from a 33-year-old man. Two emetine courses were needed to appropriately treat this eosinophilic meningitis picture up to recovery (Leng-Levy et al., 1965). The total absence of genuine neurological manifestations in that case remembers another aforementioned patient report (Bernheim et al., 1958).

A worrying clinical picture of eosinophilic meningitis with altered consciousness and photophobia although apyretic, accompanied by encephalitic affection with multifocal neurological manifestations and cerebellar syndrome, led to a patient's death despite many fasciolicide treatments. The cerebrospinal fluid eosinophilia, positive specific serology, and meningeal haemorrhage suggested a probable direct intracranial fluke involvement (case 1 of Berenger, 1984), although the presence of mucopurulent secretions in the pulmonary artery and atheromatous deposits in the left coronary artery and heart found at autopsy do not rule out an indirect impact. Unfortunately, no intracranial anatomic analysis could be performed.

In a 26-year-old Spanish male patient, the disease caused by an ectopic fluke was not only meningoencephalic and ocular but also pulmonary, throughout a process that was followed in both invasive and biliary phases (Arias et al., 1986). This case shows many aspects in common with the aforementioned meningeal cases. The patient presented with permanent headache, neck back stiffness, 70% eosinophilia in the cerebrospinal fluid and only 16% eosinophilia in the blood, and Loeffler manifestations, with normal results in hepatic tests (although with fever, jaundice, hepatomegaly, and splenomegaly) and no eggs in stools, in the first analyses corresponding to the invasive phase. Dehydroemetine treatment was applied after diagnosis based on appearance of eggs in stools. The patient fully recovered from eosinophilic meningitis, dysphasia and soft palate paralysis, but right eye blindness persisted as unique sequela.

Other typical meningeal symptoms such as alterations of the consciousness, of the type of vigilance disorders (Aimard et al., 1984) or temporospatial disorientation (Gil et al., 1970; Lefevre et al., 1970) and confusion (Ait Ali et al., 2002), and unusual irritability (case 4 of Guyot, 1962; Aimard et al., 1984) have also been reported from fascioliasis patients.

Sporadic cases reported liver fluke-infected subjects suffering from clinical pictures that may be suggested to have been serious long-term consequences of meningitis not treated in due time. One such patient was a Cuban male suffering from repeated epileptic episodes that disappeared after

emetine treatment (Kouri et al., 1938). Another is a case of a very precociously infected 1-year-old child showing hydrocephalus and macrocephaly in Mexico (Sanchez Vega et al., 2001).

5.5. Psychiatric or neuropsychic cases

Similarly as in the aforementioned meningeal cases, within fascioliasis patients presenting with psychiatric manifestations, several have been reported in whom mental disorders appeared isolated (Girard et al., 1959; Kouri et al., 1938; case 13 of Guyot, 1962; three cases of Blancas et al., 2004), whereas in other patients, such disorders were accompanied by other manifestations, whether genuine neurological (Aimard et al., 1984; Domart et al., 1967; Gil et al., 1970; Lefevre et al., 1970; Paraf et al., 1967), only meningeal (case 4 of Guyot, 1962), or only ocular (case 12 of Guyot, 1962).

Psychiatric or neuropsychic cases appear to be rare when compared to neurological and meningeal cases. The psychiatric manifestations described in fascioliasis patients are so surprising that overlooking of a relationship between such manifestations and *Fasciola* infection may be considered as easily understandable. These manifestations in fascioliasis patients include patients with depression or irritability, other patients with exaltation and overexcitement, and other cyclothymic patients showing a bipolar disorder of alternating periods of depression with periods of euphoria. The cause–effect relationship with fascioliasis may possibly be the consequence of a frontal syndrome, as suggested by a patient in whom mental disorders were linked to an intermittent bilateral grasping sign (Gil et al., 1970; Lefevre et al., 1970).

Two facts may be emphasized. In all the cases, mental disorders appeared together with fascioliasis and disappeared with the cleaning of *Fasciola* infection by fasciolicide treatment. The psychiatric manifestations may thus appear during both invasive and biliary phases. In the only exception, a female patient showed a relapse of her mental disease during an hepatic fascioliasis, this relapse being more severe and lengthy (case 12 of Guyot, 1962). Hence, the question arises on whether hepatic fascioliasis may also be able to favour the appearance of mental disorders in subjects having such a neuropsychic tendency.

Psychiatric manifestations reported from fascioliasis patients included fleeting mental disorders (Girard et al., 1959), certain irritability and an instability (case 4 of Guyot, 1962), and psychiatric disorders of the type of depression in three patients among a total of 277 fascioliasis patients examined

(Blancas et al., 2004). A 4-month-long acute case of melancholia was reported from a woman who died after a retropharyngeal abscess and a high degree of biliary cirrhosis (Biggart, 1937). In another case, 1-year-long important mental disorders in a female patient without previous psychiatric record only disappeared after emetine treatment (case 13 of Guyot, 1962). Other manifestations such as disturbances of character were reported accompanying a large neurological syndrome (Domart et al., 1967).

Mental disorders including laughing for no reason and tendency to tell banal jokes, accompanying a confusional condition, temporospatial disorientation, and very slow gestures, were reported from a 63-year-old woman (Gil et al., 1970; Lefevre et al., 1970). Similar behavioural changes including laughing for no reason, tendency to tell false stories, and lack of interest for any activity were described in another 58-year-old man (Aimard et al., 1984).

Highly annoying intense mental disorders, comprising phobias and pronounced fear, obsessive terror, and intense anguish, were reported by a female patient fully cured with emetine (Kouri et al., 1938). A sudden appearance of an anxiety attack and nervousness with feeling of imminent death later worsened with fever and mystic deliria in a Cameroonian woman. After corticosteroid therapy, the patient surprisingly became lucid when pyretic and deliria returned when apyretic. After 3 weeks' transfer to a Paris hospital, this patient showed a wide confusional syndrome and delirium attacks. The intensity and long-term extent of the mental disorders caused by *F. gigantica* in this patient are worth mentioning (Paraf et al., 1967).

The case of a cyclothymic 32-year-old woman showing alternating depression/euphoria episodes throughout a 3-year period should be emphasized. This patient showed a pronounced relapse of the mental disorders 3 years later, worsening and extending a few months thereafter. Improvement was obtained by emetine treatment, although it needed several months. However, the patient again relapsed around 2 years later, when *Fasciola* eggs were still found in her stools (case 12 of Guyot, 1962).

5.6. Brain examination techniques and neuroimaging

Examinations by EEG and different neuroimaging techniques, when applied, did not show any specific character either, similarly as clinical manifestations. Thus, they appear to be of debatable usefulness for the diagnosis of fascioliasis in patients showing major neurological manifestations. Only in direct intracranial affection by an ectopic fluke may they become decisive, as illustrated by a case in which EEG and angiography were both indicating an

expanded brain lesion where two abscesses containing *Fasciola* eggs were later found in surgery (Correa et al., 1969; Ruggieri et al., 1967). Other patients in whom brain and meningeal alterations were observed with these techniques and a fasciolid fluke was finally recovered from the eye (Cheng et al., 2007; Cho et al., 1994; Wang et al., 2007; Ying et al., 2007; Zhou et al., 2008) may be added here.

In patients presenting with major neurological manifestations caused by *Fasciola* infection, the findings are summarized in the following:

(A) *EEG*: It has been applied to several of these cases. With a few exceptions in which it was normal (Domart et al., 1971; Lesecq et al., 1972; case 3 of Campo et al., 1984a,b), EEG showed whether a generalized hypovoltage (Cattan et al., 1953; Coulaud et al, 1970; Domart et al., 1971) or a global slowdown from more pronounced in certain regions (Aimard et al., 1984; Ayadi et al., 1991; cases 1 and 2 of Berenger, 1984; Gil et al., 1970; case 2 of Guyot, 1962; Kristoferitsch et al., 1982; Lefevre et al., 1970; Oujama et al., 2003) to a localized delta slowdown (Aubertin et al, 1966; Garde et al., 1961), similarly as in the aforementioned verified intracranial case (Correa et al., 1969; Ruggieri et al., 1967), sometimes with phases alternating between local and bilateral in the same patient (Aimard et al., 1984). Unfortunately, these results were not always corresponding to the clinical signs showed by the patient.

(B) *CT scan*: It has shown to be normal in several cases (Aimard et al., 1984; case 3 of Campo et al., 1984a,b; Arias et al., 1986). On the contrary, it showed small hypodense central areas bilaterally and also in the left frontal region (Kristoferitsch et al., 1982). Similarly, hypodensity was also revealed in the right subcortical frontal lobe by brain CT scan (Linares et al., 2006). CT was used to confirm a meningeal haemorrhage with the presence of blood in the lateral ventricles and cortical folds (case 1 of Berenger, 1984). In another case, it showed, after injection of contrast agent, a dense temporary left linear image without clear relationship with the clinical picture and that was not refound 6 months later although a moderated cerebral atrophy meanwhile appeared (case 2 of Berenger, 1984). Head CT scan also helped in detecting localized brain haematomas or haemorrhages in other fascioliasis patients (Figs. 2.3 and 2.4) (Cho et al., 1994; Ying et al., 2007; Zhou et al., 2008).

(C) *Cerebral scintigraphy*: This technique has been applied to only a few neurological fascioliasis patients. It was normal in two cases (Coulaud et al., 1970; case 1 of Berenger, 1984). However, it showed a frontal area of

increased uptake in a patient, which appeared very different from the image provided by parasitic cysts or a tumour neoformations. Such blurred and thin hyperfixation area was anyway not refound on a second examination 2 months later. Interestingly, scintigraphy showed alterations of the right lobe of the thyroid gland in the same patient (Gil et al., 1970; Lefevre et al., 1970).

(D) *Magnetic resonance (MR)*: Despite having only been used in a few cases, cerebral MRI has always shown signal anomalies in patients presenting with major neurological disorders. This technique showed multiple nodular cortico-subcortical hypersignal (T2) areas highlighted by contrast in an antiphospholipid syndrome case due to fascioliasis (Fig. 2.5) (Pelletier et al., 1995). MR imaging showed diffuse bilateral signal anomalies, with hypersignal in cortico-subcortical and periventricular regions on fluid-attenuated inversion recovery (FLAIR) images and the presence of haemorrhages indicating acute disseminated encephalitis or diffuse vascular lesions with vasculitis confirmed by biopsy, evoking arteritis of the central nervous system (Oujamaa et al., 2003). Head MR imaging revealed a large subdural haematoma in the left frontotemporoparietooccipital lobe in one case (Zhou et al., 2008). Diffusion-weighted MR imaging showed bilateral multiple

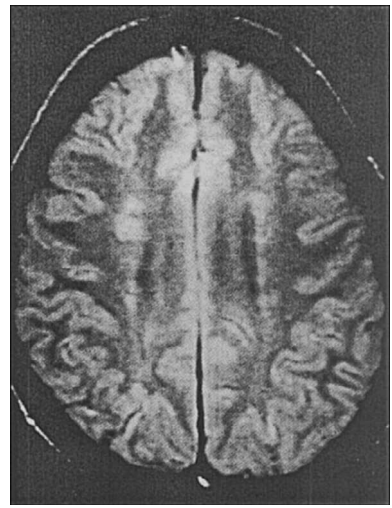

Figure 2.5 Cerebral magnetic resonance (MR) imaging showing cortico-subcortical nodular areas in T2 hypersignal highlighted by contrast in a 40-year-old woman hospitalized due to a tetrapyramidal syndrome. *From Pelletier et al. (1995); Copyright © 1995 Elsevier Masson SAS. All rights reserved.*

high signal intensities in the cortical and subcortical areas of another patient; affected brain areas appeared hyperintense on FLAIR images (Fig. 2.6) (Park and Sohn, 2010).

(E) *Arteriography or angiography*: It was normal in one case, although the aspect of the anterior cerebral artery suggested a certain hydrocephalus degree (Garde et al., 1961). It was also normal in another patient in whom a linear image appeared at CT scan (case 2 of Berenger, 1984). This technique allowed for the detection of an "allergic" vascular process (Coulaud et al., 1970; Saimot et al., 1971). It showed an aspect of parietal malformation in another patient in whom two cystic forms were found at surgery (Correa et al., 1969; Ruggieri et al., 1967) and an obstruction of the middle cerebral artery in another case (Giroud et al., 1979), whereas there was no cardiovascular risk factor and a spasm

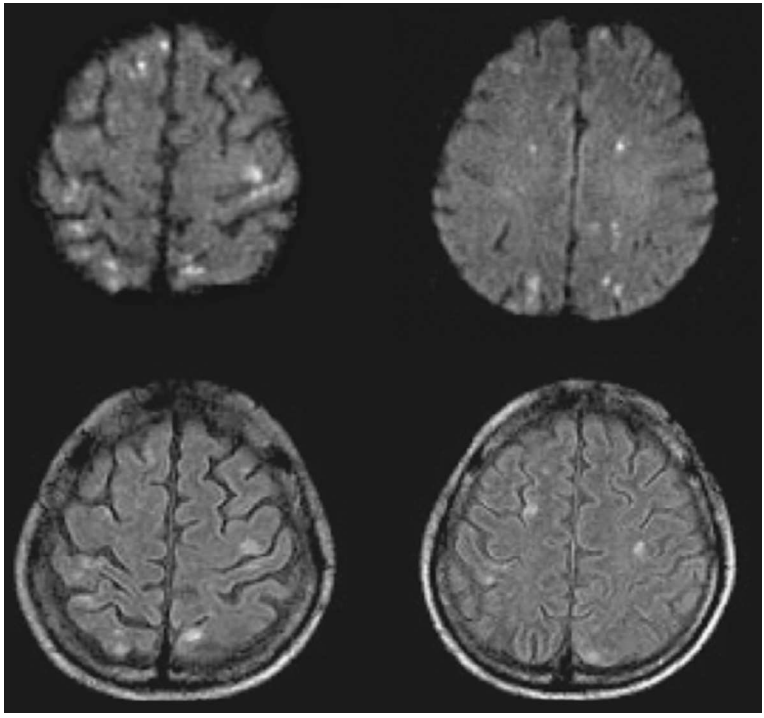

Figure 2.6 Cerebral lesions caused at distance by hepatic fascioliasis: diffusion-weighted magnetic resonance (MR) imaging showed bilateral multiple high signal intensities in the cortical and subcortical areas of a 60-year-old male patient from Korea. Affected brain areas appear hyperintense on fluid-attenuated inversion recovery (FLAIR) images. *Modified from Park and Sohn (2010); © by Korean Stroke Society.*

of the middle cerebral artery in another case in whom a meningeal haemorrhage without visible vascular malformation occurred (case 1 of Berenger, 1984). Interestingly, head DSA revealed similar intracranial aneurysms in the right middle cerebral artery and the left vertebral artery in two different 8- and 10-year-old children (Ying et al., 2007; Zhou et al., 2008).

(F) *Other additional techniques*: Other observations included a normal pneumoencephalography in a patient (Coulaud et al., 1970), a normal myelogram in one case of sciatica (Lesecq et al., 1972), and an electromyography showing signs of peripheral neurogenic affection with slowdown of the motor conduction speed in a patient in whom an areflexia of the four limbs occurred (Aimard et al., 1984). A cervical Doppler ultrasonography helped in identifying a thrombosis located in the left subclavian and axillary veins (Linares et al., 2006).

Although neuroimaging techniques have so far shown the absence of specific characteristics common to neurological patients, the combination of EEG with brain imaging techniques may pronouncedly help in the diagnosis and management of fascioliasis patients with neurological manifestations. CT is helpful in detecting lesions. MR imaging is much more sensitive for accurate localization, characterization of the lesions, delineation of associated parenchymal changes, determination of the activity of some lesions, and monitoring of the patient after therapy. Combining these techniques with diffusion MR, perfusion MR, or MR spectroscopy helps with the differentiation from abscesses and malignancy of other aetiologies. A thorough understanding of the imaging patterns allows to narrow down the options for differential diagnosis and to facilitate the timely implementation of appropriate therapies (Abdel Razek et al., 2011). The application of imaging techniques to ascertain hepatic infection is of additional value, although in fascioliasis, the need for a personal expertise to reach correct interpretations of liver images has already been highlighted, mainly in long-term findings (Kabaalioglu et al., 2007).

6. OCULAR FASCIOLIASIS

6.1. Distribution and frequency

The analysis of the geographic distribution of the fascioliasis case reports including ocular manifestations indicates that eye involvement may be considered rare in this disease. This does not mean, however, that patients showing ocular manifestations caused by fasciolids have been reported from all

continents, except Oceania (Tables 2.1 and 2.2). The country distribution of these cases and the flukes recovered from a few patients indicate that *F. hepatica* is the causal agent of these affections, although nothing suggests that *F. gigantica* or hybrid forms may not be able to also give rise to ocular involvement.

The detection of fascioliasis patients showing ocular manifestations was very sporadic until the 1930s, no case was reported during the following two decades, and reporting restarted from the 1960s (Fig. 2.1). The pronounced difference between the presence of ocular affection in males (67.8%) with regard to in females (32.1%) poses a question mark (Table 2.3). All age groups seem to be susceptible for eye involvement by the liver fluke (Fig. 2.2). The possibility for ocular affection early in life (Table 2.4) represents a factor of negative impact in children during their school period.

The higher number of ocular reports in France (Table 2.1) is due to the fact that many patients presenting with major neurological manifestations do sometimes show additional ocular manifestations. The absence of ocular fascioliasis reports in human fascioliasis hyperendemic areas in countries such as Bolivia, Egypt, and Vietnam or its sporadic appearance in Peru (only one report) (Table 2.2) is surprising and suggests that eye involvement by the liver fluke in these areas may remain overlooked or misdiagnosed.

6.2. Ophthalmofascioliasis and indirect ocular affection

Similarly as with patients presenting with neurological manifestations, cases showing eye involvement may also be distinguished into two main types according to the location of the fluke parasites:

– *Ophthalmofascioliasis*: Cases in which the ocular manifestations are due to the direct effects of a migrating juvenile fluke present in the eye, orbit, or related abducens nerve VI and muscles or to lesions in them suggesting a migrating fluke as the cause. This type of ocular affection has always been described as a unilateral condition. According to the few number of reports on cases of this type, it appears that this modality may be rare. Such an ectopic ocular parasitization is not easy to diagnose, except in patients in whom the fluke becomes externally visible at a given moment or it spontaneously comes out through the orbit. If the fluke is not observed nor spontaneously or surgically obtained, such an orbit infection may be confused with other types of ocular disturbances or remain without diagnosis in remote areas of poor countries where the necessary equipments for appropriate ocular analyses are lacking in the rural health centres.

Therefore, the frequency of such ocular migrating flukes may be higher than the one suggested by the scarce number of published reports. Although patients have been reported who did not manifest any significant symptomatology other than the one related to the affected eye, this type of ocular affection has usually been detected in patients presenting with neurological symptoms suggesting an intracranial migration of the fluke previous to reaching the orbit.

– *Fascioliasis with indirect ocular affection*: Most of the case reports referring to ocular manifestations in fascioliasis patients concern neurological cases in which accompanying ocular manifestations were described. In such cases, eye involvement uses to be unilateral, but cases with bilateral affection have also been reported. Clinical pictures appear to vary pronouncedly, from mild symptoms up to transitory amaurosis or even permanent blindness. These manifestations are not overlooked in patients hospitalized due to their neurological manifestations, but may be so when mild or when even impressive symptoms are misdiagnosed in areas, in both remote rural and urban, where physicians are not aware about the capacity of *Fasciola* to cause such ocular (and neurological) manifestations.

The term of ophthalmofascioliasis should be restricted to patients in whom eye involvement by a migrating fluke has been verified. All other patients showing ocular manifestations caused by fasciolids located in the liver or other organs should be referred to fascioliasis with ocular implications, although sometimes, they may clinically appear more impressive and severe than those in ophthalmofascioliasis.

6.3. The first reports of a human ocular infection

The first two records of trematode findings in a human eye were made almost two centuries ago and *F. hepatica* was suggested to be involved in both. Unfortunately, these two old cases were not analysed in recent reviews on ocular helminthiases (Otranto and Eberhard, 2011; Sabrosa et al., 2010), despite having been much discussed in both ophthalmologic and helminthological literatures during many decades and still continuing to be chiefly of historical interest.

In the first case, eight small specimens, of only 0.3 mm length, were discovered in the upper layers of the lens substance of an old woman who had been extirpated for cataract (von Nordmann, 1832). These specimens were later studied and appeared to have only one apical sucker and were therefore included within monostomes (name for collective group of forms with only

one sucker) as *Monostomum lentis* (Gescheidt in Gescheidt and von Ammon, 1833a). The possible overlook of the presence of an acetabulum due to the small size of the worms recovered led to suggest that the parasites found could be young forms of *Dicrocoelium lanceolatum* (syn. *D. dendritium*), the smaller or lanceolate sheep-living fluke, or of *F. hepatica* (Leuckart, 1864). A dicrocoeliid has never been found in the human eye so far (Otranto and Eberhard, 2011), but two cases of infection by *D. lanceolatum* indicate that this species, although apparently only exceptionally, is able to reach the central nervous system of the patient (Siguier et al., 1952) and also gives rise to ocular manifestations (exophthalmos) accompanying neurological disorders (Coudert and Garin, 1959; Guyot, 1962). Anyway, the relatively high number of worms does not fit well in accepting an infection by *D. dendriticum* or *F. hepatica*. Present knowledge suggests that these eight small worms were most probably protoescoleces of a taeniid cestode individualized after surgical rupture of the thin larval stage wall, the so-called apical sucker (see figures of worms made by von Ammon in Wood, 1918) in fact being not more than the orifice externally resulting from the invagination of the protoscolex. Their size of around 300 μm agrees perfectly. *Echinococcus granulosus*, widely distributed throughout Europe time ago, could be involved as a consequence of blood-borne dissemination of the oncospheres. Ocular localization by the larval form of *E. granulosus* accounts for 1–2% of all reports. Other taeniids present in Europe whose larval forms have been found in human eye are *Echinococcus multilocularis* (although this species gives rise to acephalic larval stages in humans), budding cysticerci of *Taenia crassiceps*, and *Coenurus cerebralis* of *Multiceps multiceps* (Otranto and Eberhard, 2011). Thus, Küchenmeister's (1855) idea that this monostone was perhaps a young *Cysticercus cellulosae* was not far wrong there, despite not at all convincing in that time (Cobbold, 1864).

In the other case, four specimens were removed from between the opaque lens and the capsule of the eye of a 5-month-old child after death from infantile atrophy whilst under care in Dresden. This child presented congenital lenticular cataract with partial opacity of the capsule (von Ammon in Gescheidt and von Ammon, 1833b). These parasites were around 0.50–1 mm long and 0.14–0.30 mm wide, with circular terminal mouth, and acetabulum one-third smaller than the oral sucker. Some years later, an illustration was provided (Cobold, 1879; von Ammon, 1838; Wood, 1918). This distome (name for collective group of forms with one apical sucker and a ventral acetabulum) was initially named *Distomum oculi humani* and later *Distomum ophthalmobium* von Diesing, 1850 (see Braun,

1908; Cobold, 1879). It is clear that the parasite represents a larval immature distome. On the account of the abundance of certain trematodes in the region where this case was found, the authors were inclined to also regard the species as a juvenile of *D. lanceolatum* or of *F. hepatica* (Leuckart, 1863). Other authors also supported that *F. hepatica* was the causal agent involved (Castellani and Chalmers, 1919).

It was emphasized that although the worm discovered apparently had intestinal caeca without bilateral branches, this does not necessarily oppose its belonging to *F. hepatica*, as in this species, the intestinal caeca are originally unbranched, and they only develop lateral ramifications later, between the 12th and the 22nd day of infection (Braun, 1908). Nevertheless, in all *Fasciola* juveniles recovered from the human eye since today, bilateral caecal branching has appeared always very evident (Cho et al., 1994; Dalimi and Jabarvand, 2005; Zhou et al., 2008).

It is also hard to see how infection could have taken place in so young a child. It was suggested that it wandered in from the mother before the child was born; others considered it more probable that infection took place through the use of drinking water in which were by chance free infecting metacercariae (Wood, 1918). An argument supporting the former hypothesis is that prenatal *F. hepatica* infection is well known to take place in different mammal species such as sheep, cattle, and buffaloes (El-Azazy, 1988; Enigk and Düwel, 1959; Ishikawa et al., 1986; Rees et al., 1975; Vermeer et al., 1993). A priori, nothing seems to impede this possibility in humans (Mas-Coma et al., 1999a). Additionally, *F. hepatica* infection at such an early age has been already described, as it was the case of a lactating 1-year-old male (Sanchez Vega et al., 2001).

However, the fact that four specimens were simultaneously present in the eye poses a problem, because such an ocular *F. hepatica* multi-infection has been never found. This, together with the absence of bilateral caecal ramifications, casts serious doubts on the ascription of such small ocular worms to *F. hepatica*. Present knowledge indicates that this old case was most probably due to a multi-infection by mesocercariae of a species of the genus *Alaria*. *Alaria* species are three-host life cycle trematodes that live as adults in the intestine of definitive carnivore hosts, such as the dog. Eggs are passed in faeces and hatch in water, releasing miracidia that penetrate freshwater snails (first intermediate host) and develop into cercariogenous sporocysts. Cercariae released from snails actively penetrate the second intermediate host (tadpoles) becoming infective mesocercariae in about 2 weeks. In the tadpole or in the frogs (following the metamorphosis), mesocercariae accumulate and

may be ingested by a number of paratenic hosts (e.g. other frogs, snakes, small mammals, and wild boar) or directly by the definitive host (Möhl et al., 2009). Cases of human intraocular infection with *Alaria* mesocercariae have been recorded in patients who had ingested undercooked contaminated frog legs (McDonald et al., 1994) in Asia, the United States, and Canada (Otranto and Eberhard, 2011).

Many aspects support that the four worms found in the fatal case of the 5-month-old child in Dresden in 1883 were in fact *Alaria* mesocercariae: (i) the size of the worms and their sucker ratio agree with those of *Alaria* mesocercariae (Freeman et al., 1976; Odening, 1963; Pearson, 1956); (ii) fatal human cases due to infection by high *Alaria* mesocercarial multiinfection have been recorded (Fernandes et al., 1976; Freeman et al., 1976); (iii) transmammary transmission of mesocercariae of *Alaria* have been experimentally demonstrated in primates (Shoop et al., 1990); and (iv) *Alaria* spp. appears to be relatively frequent in game meat in Germany (Möhl et al., 2009; Riehm et al., 2011). Thus, despite the assumption that no human larval alariasis case has been reported in Germany so far (Möhl et al., 2009), it may be concluded that the *D. ophthalmobium* case was most probably due to mesocercarial alariasis after eventual transmammary transmission.

The third human ocular infection case ascribed to *F. hepatica* was reported only a century later (Lunedei and Roselli del Turco, 1934). An Italian, 35-year-old male patient had been diagnosed to be infected by *F. hepatica* according to egg finding in stools and duodenal aspirate. At a given day, he presented pronounced painful at the right side of the shoulder block followed 1 week later by stiffness of the back of the neck, strong frontal pain, violent cephalalgias, and hemiparalysis of the left body and right face, accompanied by ophthalmologic symptoms such as strabism, diplopy, and bilateral reduction of vision, within a condition suggesting VI cranial nerve paralysis. These conditions were kept for 10 months until a day in which a kind of small oval-shaped vesicle of a size of around 4.10 mm, of greenish colour, motionless, and showing a small protuberance at its upper extremity, was observed down in a vitreous body of particulate matter due to the presence of exudate, in his right eye. All was indicating that the ocular form in question was a worm in an ocular ectopic location (Lunedei and Roselli del Turco, 1934). Six months and a week later, after four treatment courses with emetine, the aforementioned form previously observed in the vitreous body was absent and the exudate had disapeared almost completely. The shape and size of the ocular form observed, the many serious manifestations suggesting a direct affection of the central nervous system, the

ophthalmologic symptoms, and the disappearance of the ocular form after emetine treatment indicate that an *F. hepatica* worm were involved in the eye.

The aforementioned Italian patient may thus be considered the first true reported case of ocular fascioliasis in man. That case shows many similarities with the two cases of children recently described in China (Ying et al., 2007; Zhou et al., 2008).

6.4. Manifestations in ophthalmofascioliasis

Additional reports on ectopic *Fasciola* worms infecting the human eye concern only the past 25 years, including a total of six cases: the first in Spain (Arias et al., 1986), the following in Korea (Cho et al., 1994), one in Iran (Dalimi and Jabarvand, 2005), another in Uzbekistan (Cheng et al., 2007), and finally two in China (Ying et al. 2007; Zhou et al., 2008).

Among these reports, those from Iran and Uzbekistan are surprising because of dealing with patients who did not manifest any significant symptomatology other than the one related to the affected eye.

In the case of Iran (Dalimi and Jabarvand, 2005), a 44-year-old woman from the city of Anzali presented with a red, painful left eye for 10 days. The affected eye showed perception of light with relative afferent pupillary defect and endophthalmitis. The anterior chamber was shallow with mixed hyphaema and inflammatory cells. There were 360° of posterior synaechiae and iris bombe. Intraocular pressure was 17 mmHg whereas only 14 mmHg in the right normal eye. There was some cataract but the fundal view was obscured by dense vitritis and vitreous haemorrhage. A small, flat, moving parasite was removed from the anterior chamber at surgery, during which patches of ischaemia were seen in a retina that appeared flat on ophthalmoscopy. Examination showed 3+ conjunctival hyperaemia, 2+ corneal oedema, and deep anterior chamber with 2+ flare. Applanation intraocular pressure was 16 mmHg. Subconjunctival injection of betamethasone 2 mg and gentamicin 40 mg was given. On the first day after surgery, best corrected visual acuity was 6/60. After 3 weeks, the eye was found to be quiet. After 6 months follow-up, the visual acuity decreased to 6/120 owing to corneal oedema and deep vascularization. Based on morphology, an immature *F. hepatica* was identified. The white blood cells were raised to 14,300/μl (normal range, 5000–10,000/μl), but there was no eosinophilia and liver function tests and liver ultrasound were normal. Serology using indirect fluorescent antibody test was negative and no eggs were detected

in stool analyses repeated five times. The route of entry of the worm up to the anterior chamber of the eye could not be identified, but a possible route via the central retinal artery into the vitreous was suggested.

The case of Uzbekistan concerned a 6-year-old boy with facial swelling and monocular blindness (Cheng et al., 2007). Clinical findings and radiological investigation suggested sinusitis, and the patient underwent a sinus drainage procedure. Following surgery, the patient was found to have proptosis, chemosis, dilated episcleral vessels, complete paralysis of all extraocular movements, no perception of light in the left eye, a dispersed hyphaema, and a cataractous lens. A presumptive diagnosis of traumatic posterior orbital arteriovenous fistula was made. Four days later, a leaf-shaped, undulating "stingray"-like organism with cephalic cone was briefly visible in the anterior chamber of the left eye (Fig. 2.7). He was empirically treated with oral mebendazole. On the following day, the optic disc was noted to be markedly swollen on ultrasound examination, but there were no signs of the parasite within the globe. CT of the brain demonstrated a left frontal lesion, and ultrasound of the liver revealed two cystic lesions. A complete paralysis of extraocular movements was likely the result of retro-orbital tension on the nerves and muscles, attributable to inflammation and/or a possible post-surgical arteriovenous fistula.

The two aforementioned cases are important because they suggest that, in endemic areas, fascioliasis with ocular involvement should be considered, as in cases of uveitis. Patients may have no other systemic involvement, with only subacute infection and even negative coprology and only low serological positivity, if any.

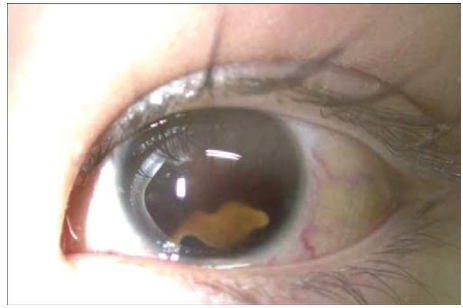

Figure 2.7 Left eye of a 6-year-old male patient from Uzbekistan, showing leaf-shaped, undulating "stingray"-like organism with cephalic cone, consistent with *Fasciola hepatica* infection. *From Cheng et al. (2007); © by Oxford University Press on behalf of the Infectious Diseases Society of America.*

The remaining four ocular cases share an evident affection of both the central nervous system and eye in the same patient (Arias et al., 1986; Cho et al., 1994; Ying et al. 2007; Zhou et al., 2008). Besides the respective neurological pictures already analysed in the text earlier, the patients presented with the following ophthalmologic manifestations.

In the case of the 26-year-old Spanish male patient, the disease caused by the ectopic fluke was not only ocular and meningoencephalic but also pulmonary, throughout a process that was followed in both invasive and biliary phases (Arias et al., 1986). Right retro-ocular pain and eyelid oedema appeared unexpectedly 3 months after typical hepatic fascioliasis symptoms and 2 months after the Loeffler syndrome, beginning of neurological manifestations, 70% eosinophilia in the cerebrospinal fluid, and only 16% eosinophilia in the blood, with normal results in hepatic tests and no eggs in stools. Ocular disorders increased progressively up to evident proptosis and conjunctival chemosis. Pain and exophthalmos improved during the 10 following days, but loss of vision in the right eye remained. Upon examination, right eye amaurosis appeared accompanied by scarce exophthalmos, eosinophilia in both cerebrospinal fluid and blood, and hepatosplenomegaly had disappeared, but there was the presence of numerous eggs in stools. After a 2-week treatment with dehydroemetine, right eye amaurosis was still present. A subsequent 2-year follow-up showed that the patient fully recovered, except right eye blindness that persisted as unique sequela. Although a fasciolid fluke was never recovered, it was concluded that the ocular problem was due to ectopic *Fasciola* infection in the invasive phase, an aetiology also indicated by the retro-ocular pain and initial high eosinophilia in the cerebrospinal fluid and by the soft palate paralysis additionally detected indicating a mononeuritis probably due to direct compressive pathology. Although an immunologic pathogenicity origin of the amaurosis could not be ruled out, the lack of response of eye blindness to the dehydroemetine treatment reinforces the ectopic origin of the ocular problem.

The case of Korea concerned a 28-year-old male who had been ill with headache and motor weakness for a month and developed sudden pain and blindness of the right eye (Cho et al., 1994). This eye was protruded, the lens was displaced to the posterior chamber, and the posterior border of the right eyeball was thickened (Fig. 2.8). Corneal oedema and conjunctival injection worsened 1 day after. During ophthalmoscopy, a worm was recognized penetrating the iris, occupying the anterior chamber for a brief period, returning back behind the iris, and leaving corneal oedema with hyphaema and extensive retinal bleeding. The enucleated eye revealed areas of a focal

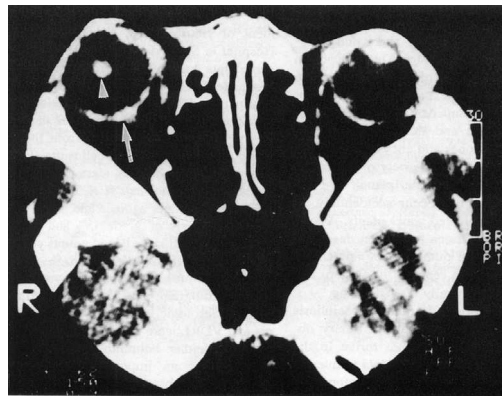

Figure 2.8 Orbital computed tomography showing the protrusion of the right eye, posterior displacement of the lens (arrowhead), and thickening of the posterior border of the right eyeball (arrow) in a patient in whom a migrating fluke was observed in the eye. *From Cho et al. (1994); © by American Society of Tropical Medicine and Hygiene.*

degeneration of sclera and intraocular haemorrhage. An abrupt tissue defect and few inflammatory reactions in the uvea suggested very recent migration of a moving worm. The flatworm detected in the anterior chamber was identified to be a juvenile *Fasciola*. After surgery, neurological symptoms disappeared gradually within 6 months, the patient showing no clinical problems in a 3-year follow-up.

In China, an 8-year-old boy suffered, as the results of worm migration, significant cerebral and ophthalmic tissue damage before the diagnosis could be made (Ying et al., 2007). On the 19th day after hospital admission due to many neurological disorders, the patient had swelling of the right eyelid accompanied with conjunctiva oedema and mild protrusion of the eyeball. Examination of the eyes revealed blurred margins of the bilateral optic disc, optic disc, and surrounding area oedema of the right eye. On the 26th day, the patient experienced an itchy and aching sensation on his right lower eyelid, and a red dot-like moving object was found underneath the mucosa of his right lower eyelid. Subsequently, the moving object penetrated through and gradually emerged out of the mucosa. Whilst half of the object was still inside the tissue of the right eye, a 1 cm long trematode moved out from the tissue of the eye completely when pricking the object slightly with a cotton swab. The patient was treated with 300 mg praziquantel, three times a day for 5 days, and was followed up for 9 months without any further signs or symptoms. The parasite was identified as a juvenile *F. hepatica* by morphology.

Also in China, a 10-year-old boy suffered from intermittent headache, vomiting, repeated fever, ophthalmalgia, eyelid swelling, conjunctival chemosis, and vision loss of the right eye in the 4 months previous to hospital admission (Zhou et al., 2008). On admission, there was complaint of ophthalmalgia, the patient presenting with eyelid swelling, conjunctival chemosis, and vision loss of the right eye during the past 4 months. Examination showed bilateral papilloedema and narrowness of the right rima oculus, with the right pupil 4 mm and the left pupil 3 mm in diameter, but no neurological symptom. A blood test revealed leucocytosis (5.19×10^9/l) and slight eosinophilia (6.7%), but liver function and blood coagulation were normal. Besides mild liver enlargement, CT scan showed different cerebral haematomas and haemorrhages during subsequent days, and aneurysms were observed on angiography. On the 17th hospital day, the patient developed progressive pain in the right eye with swelling eyelids, exophthalmos, conjunctival congestion and swelling, and fever. Subsequently, the patient had paralysis of the right abducens nerve. On the 20th hospital day, head MR imaging revealed a large new subdural haematoma in the left frontotemporoparietooccipital lobe. Three days later, a small leaf-shaped parasite was recovered when moving out of the swollen conjunctiva of the right eye. According to morphology and positivity of serological tests, the parasite was identified as an immature *F. hepatica* worm (Fig. 2.9A), despite the absence of eggs in stools. Three days after praziquantel treatment, temperature returned to normal, and the eyelid swelling and conjunctival congestion gradually relieved. One month later, headache and malaise in the right eye disappeared. Three months later, clinical symptoms and signs disappeared. Total leucocyte and eosinophil counts returned to normal. Serological test and ELISA test were negative. More than 1 year after treatment, haematomas had disappeared but one of the aneurysms remained unchanged. This case shows that ectopic fascioliasis in the eye and brain may be associated with multiple brain haemorrhages and haematomas and that an intracranial infection by *Fasciola* may be indicated by a fever of unknown origin, eosinophilia, and iterative intracranial haemorrhages (Zhou et al., 2008).

6.5. Ocular disorders in indirect affection

Fascioliasis patients presenting neurological manifestations may also simultaneously show different ophthalmologic problems. In other helminthiases, eye affection is usually due to an ocular location of the parasite, different

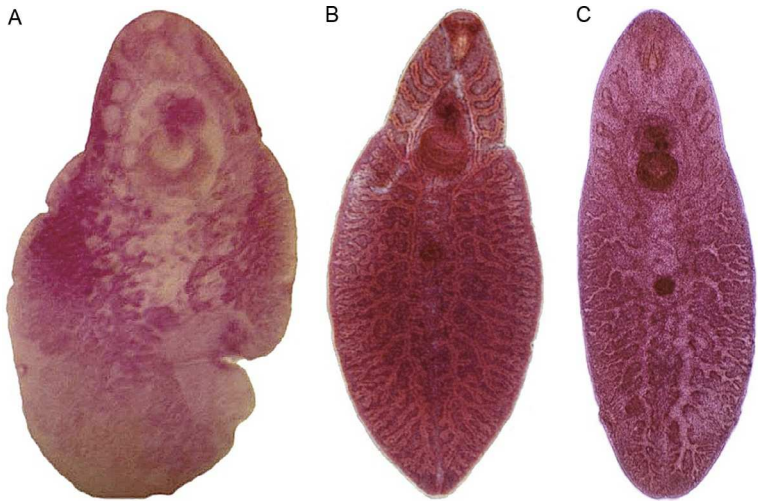

Figure 2.9 Comparison of immature forms of *Fasciola hepatica*: (A) 4.4 mm long specimen obtained after having moved out of the swollen conjunctiva of the right eye of a 10-year-old boy from China; (B) 3.9 mm long specimen found migrating inside the abdominal cavity of an experimentally infected mouse; (C) 3.2 mm long specimen found in the liver parenchyma of an experimental laboratory rat. Note the widely ramified caeca bilaterally. *Photograph A from Zhou et al. (2008); © by Elsevier Ltd.*

symptoms depend on the eye region involved (a few even showing characteristic lesions, such as in onchocerciasis or river blindness), and in given diseases, swelling of the eyelids occurs without the eye itself being attacked by the parasites, as in trichinelliasis or ascariasis (Grüntzig, 1988).

However, ocular disorders in fascioliasis do not appear to be specific but the capacity for ophthalmologic affection by fluke infection at distance should be highlighted. Different visual disturbances and ocular manifestations have been mentioned, several of which sometimes in the same patient (manifestations found in the patient of Arias et al., 1986, here included because the ectopic fluke was not found and hence ophthalmofascioliasis was not definitely proved).

Many of them may be considered mild manifestations:

- Blurred vision (Domart et al., 1967)
- Sudden bilateral reduction of the visual acuity (Oujamaa et al., 2003)
- Eye-roll sign (Coumbaras, 1966)
- Retro-ocular pain and unilateral exophthalmos (Arias et al., 1986)
- Unilateral haemorrhage in the lamina cribrosa (Málaga et al., 2012)

Other disorders become worrying, such as the following:

- Transitory or intermittent diplopia (Domart et al., 1971; Garde et al., 1961; case 1 of Berenger, 1984; Málaga et al., 2012)
- Unilateral concentric narrowing of the field of vision (case 1 of Berenger, 1984)
- Disorders of the visual accommodation (Domart et al., 1967)
- Nystagmus, from simple (Bothier et al., 1968; Kristoferitsch et al., 1982; case 1 of Berenger, 1984; Ayadi et al., 1991) to extremely intense (Garde et al., 1961)

And finally, others become serious problems:

- Visual hallucinations (Aimard et al., 1984)
- Visual deformation of objects and superimposition of geometric images, as signs of occipital affection (Coulaud et al., 1970; Saimot et al., 1971)
- Loss of bilateral vision (Llanos et al., 2006), which may become almost complete vision loss (Aguilar et al., 1967, 1968)
- Amaurosis, from transient (Ayadi et al., 1991) to permanent (Arias et al., 1986; Becquet and Delassus, 1961) and noted to be due to ophthalmoneuritis (Becquet and Delassus, 1961)

Ocular manifestations have been found in both the invasive phase (Domart et al., 1967; case 1 of Berenger, 1984; Arias et al., 1986; Ayadi et al., 1991; Llanos et al., 2006; Oujama et al., 2003) and the biliary phase of the disease (Aguilar et al., 1967, 1968; Arias et al., 1986; Bothier et al., 1968; Domart et al., 1971; Garde et al., 1961), sometimes even appearing in the long-term chronic phase after many years (Becquet and Delassus, 1961; Kristoferitsch et al., 1982; Málaga et al., 2012). In a patient, ocular manifestations were detected still in the invasive phase but showed long-term persistence in the biliary phase (Coulaud et al., 1970; Saimot et al., 1971).

7. AFFECTION OF RELATED OR CLOSE ORGANS

Clinical and parasitological findings in several fascioliasis patients reported are of interest to understand the physiopathogenic mechanisms involved in the neurological and/or ocular manifestations. Only information from cases and findings giving light for that purpose is analysed in the following.

7.1. Dorsal spine

There is only one case concerning an ectopic immature *Fasciola* in the dorsal spine of a patient presenting neurological symptoms.

A 30-year-old woman in India presented with complaints of gradual onset bilateral lower extremity weakness and numbness. On examination, she was conscious and well oriented, her cranial nerves were intact, and her fundus examination was within the normal limit. She had sensory–motor spastic paraplegia at T6 and below, with bladder and bowel involvement. She had no cerebellar and meningeal signs. Examination of other systems was normal. Neuroimaging revealed an epidural mass lesion isointense on T1-weighted images and hyperintense on T2-weighted images, extending from the T4–T7 vertebra with epidural cord compression. At removal of the epidural mass during surgery and T4–T7 laminectomy, a live, intact, mobile, leaflike, flat, pink coloured immature *Fasciola* measuring $2.7 \times 0.5 \times 0.2$ cm was extracted from deep inside the epidural granulation tissue. Postoperatively, the patient improved neurologically. Sensory loss disappeared and bladder and bowel control was regained, and recovery of motor and sensory loss was complete after 3 months. Interestingly, neither fluke eggs were found in serial stool examination on three consecutive days, nor evidence of liver or any other abdominal organ involvement was found with sonograph (Vatsal et al., 2006). This worm probably gained access to the portal venous system, subsequently to the epidural venous plexus, and finally, it migrated from the venous blood to the epidural space. After that case, one unavoidably wonders whether similar neurological cases could be related to an overlooked ectopic *Fasciola* located in the dorsal spine.

7.2. Pulmonary manifestations

Pulmonary manifestations seem to be rare in fascioliasis (only a 3.0% among a total of around 1000 case reports in France; 4.2% among 72 cases in Spain). These manifestations include, as main disorders, pulmonary infiltrates of the Loeffler type and pleural haemorrhage, usually scarce, at the right side and poor in eosinophiles, lasting between 3 days and 2 months. Spontaneous pneumothorax, haemoptysis, and pyopneumothorax appear only sporadically (Aliaga et al., 1984).

In patients with neurological and/or ocular affection due to fascioliasis, pulmonary manifestations do not always appear but their frequency appears to be higher (21.0% out of 38 patients with complete clinical analyses in France and Spain) than in nonneurological patients. Several of the aforementioned patients showing neurological and/or ocular affection and also pulmonary manifestations were diagnosed in the invasion phase (Aimard et al., 1984; Aliaga et al., 1984; Cattan et al, 1953; case 1 of Berenger,

1984). Others were detected during the biliary phase (Becquet and Delassus, 1961; Garde et al., 1961; Leng-Levy et al., 1965), and two patients were diagnosed in the invasion phase but their disease was followed into the biliary phase (Arias et al., 1986; Schussele and Laperrouza, 1971a,b).

The pulmonary manifestations in neurological patients do not seem to be different from those described in patients affected by fascioliasis without neurological disorders. The findings in two neurologically affected patients corresponded to pyopneumothorax, one case alive (Schussele and Laperrouza, 1971a,b) and another verified postmortem, death suggested to be caused by bronchopneumonia with Mendelson's syndrome (case 1 of Berenger, 1984).

7.3. Heart and vessel affection

In several patients suffering from neurological and ocular manifestations due to *Fasciola* infection, concomitant cardiac disorders were found. Such cases with cardiac complications included both patients diagnosed during the invasive phase (Arlet et al., 1966; case 1 of Berenger, 1984; Frances et al., 1994; Pelletier et al., 1995) and others in the biliary phase (Domart et al., 1971), including cases with manifestations in the invasive phase but persisting in the biliary phase (Coulaud et al., 1970; Saimot et al., 1971) and also in long-term chronic phase after many years (Kristoferitsch et al., 1982). The persistence of myocardial disorders, almost analogous to those observed at the beginning of the disease including neurological manifestations, and their disappearance a few weeks after fasciolicide treatment demonstrate the link between the two types of manifestations (Coulaud et al., 1970; Saimot et al., 1971).

The importance of heart affection in patients with neurological fascioliasis does concern not only the potential implications of cardiac disorders but also the necessity to take it into account for the treatment, as indeed the drugs most successfully used in neurological cases were emetine and dehydroemetine, which are well known for their cardiac toxicity.

The aspect worth emphasizing is, however, the coexistence of cerebral and cardiac thrombotic symptoms related to an antiphospholipid syndrome in the most recent dual case report (Frances et al., 1994; Pelletier et al., 1995).

With regard to circulatory vessels, a case of vascular cerebral lesion concerning the obstruction of the right middle cerebral artery was observed by arteriography in a subject lacking classical thrombotic-embolism affections

(Giroud et al., 1979). An axillary–subclavian deep venous thrombosis, clearly distended superficial veins, pain and swelling of the left arm, and an acute cerebrovascular stroke were associated with *F. hepatica* infection in an apraxic old woman (Linares et al., 2006).

7.4. Findings in blood vessels

The capacity of fasciolids to enter the circulatory system is known since long ago. Already three centuries ago, two young *Fasciola* specimens were obtained from the tibial vein spontaneously broken in a 17-year-old child in Leipzig (Treutler, 1793 in Bürgi, 1936). In autopsies performed many years later, six *F. hepatica* specimens were found inside the portal vein in a 40-year-old man in Rennes (Duval, 1842 in Senevet and Champagne, 1929), and an alive specimen of the same fasciolid species was found inside a vein in a man from Malta (Vital, 1845 in Senevet and Champagne, 1929).

In animals, the eggs of *F. hepatica* have been found inside the blood vessels, both arteries and veins, of the liver of cattle in a frequency, suggesting this location to be more usual than previously thought (Marcos et al., 2006b).

The pronounced transversal enrolment capacity of these thin flatworms enables them to survive and probably progress within the blood vessels of relatively small diameter. Although fasciolids are flukes that tend to cross-breed (Mas-Coma et al, 2009a), their hermaphroditic condition allows them also for selfing, which would explain the finding of eggs inside the blood vessels.

7.5. Skin and dermatologic reactions

In fascioliasis, urticaria, with dermatographia, is considered a distinctive feature in the early stage of the fluke invasion. Pruritus may also appear in the biliary phase. Such dermatologic manifestations are usually described within the clinical pictures of patients suffering from fascioliasis with neurological consequences.

With regard to fascioliasis patients presenting with neurological affection, the combination of urticarial eruptions or rash, Quincke's oedema, and the Loeffler infiltrate has been many times evoked to support an immunoallergic origin of the neurological manifestations (Aimard et al., 1984; Aubertin et al., 1966; Berenger, 1984; Bothorel, 1953; Guyot, 1962; Mignot et al., 1971). Cutaneous manifestations have been described in neurological patients both in the invasive (Bernheim et al., 1958;

Boissiere et al., 1961; Coulaud et al., 1970; Domart et al., 1967; Frances et al., 1994; Pelletier et al., 1995; Saimot et al., 1971) and in the biliary phases (Auer et al., 1982; Becquet and Delassus, 1961; Brouet et al., 1951; Domart et al., 1971; Kristoferitsch et al., 1982). Such skin affections included simple urticaria (Aimard et al., 1984; Boissiere et al., 1961), sometimes acute or very pruriginous (Auer et al., 1982; Coulaud et al., 1970; Domart et al., 1967; Kristoferitsch et al., 1982; Saimot et al., 1971), with unique or multiple oedematous nodules in different parts of the body (Becquet and Delassus, 1961). Generalized urticaria, sometimes erythematous and maculous, was noted in several of these neurological patients, usually fleeting or brief (2–3 days up to 3 weeks) (Becquet and Delassus, 1961; Bernheim et al., 1958; Brouet et al., 1951; Domart et al., 1971). In another patient, a curious cutaneous symptom such as livedo, first in the lower limbs and later expanding to the abdomen, back, and upper limbs, was described (Frances et al., 1994; Pelletier et al., 1995).

7.6. Ectopic mature flukes and upper body locations

Although it was believed that ectopic flukes would be not able to mature and produce eggs (Chen and Mott, 1990), today, it should be admitted this may occur but not usually. Adult fasciolas are able to deposit eggs in inflamed liver parenchyma (Acosta-Ferreira et al., 1979; Goodman et al., 1973; Hardman et al., 1970; Jones et al., 1977). *Fasciola* eggs were recovered from the aspirate of an hepatic abscess (Perry et al., 1972). Hence, these flukes could presumably do the same in other well vascularized tissues. Egg granulomas have been described several times, including human pancreas (Chitchng et al., 1982), caecum (Park et al., 1984), peritoneum, and intestinal wall (Mohammadi-Ghalehbin et al., 2012). Several case reports refer to fasciolas in organs different from the liver and that should have been gravid based on observation of tissue-embedded eggs from different locations, such as the right iliac fossa (Park et al, 1984), pelvis (Yazici et al. 2005), breast (Naresh et al., 2006), colon (Makay et al., 2007), and abdomen (Ongoren et al., 2009).

However, ectopic mature gravid fasciolas have been recovered only sporadically. Adult flukes and eggs of *F. hepatica* were found on a pulmonary surgical sample in France (Couraud et al., 1975). A 2 cm long *F. gigantica* specimen noted to be mature was found in an abdominal mass in Egypt (Ragab and Farag, 1978). Another 16 mm long specimen of *F. gigantica*, with intrauterine eggs of 140 μm by 75 μm, was obtained on incision of an

anterior swelling of a 2-month duration in the upper third of the sternum of a 17-year-old male in Uganda (Ongom, 1980).

In a surprising and interesting case of lymphatic invasion in a 46-year-old female in Australia, a mature *F. hepatica* surrounded by scattered clusters of eggs was observed in the sections of an enlarged lymph node, of approximately 4 cm diameter in size, from the left posterior triangle of the neck. Apart from the subcutaneous lump, which she had first noticed 10 days previously, she had been quite well. Nothing of significance was found on examination. Sections from the resected lymph node revealed severe eosinophilic and granulomatous lymphadenitis, with foci of vasculitis and necrosis. The fluke may have entered in the usual manner and then reached the lymph node perhaps via blood or lymphatic channels (Prociv et al., 1992). This Australian case remembers the first manifestation showed by a 36-year-old man in France, including a 6-month-long cervical adenopathy involving 3–4 indolent lymph nodes along the sternocleidomastoid muscle and an additional one in the axillary region but without blood eosinophilia, who later developed a wide neurological syndrome (Cattan et al., 1953).

A case very similar to the aforementioned Australian case was the one of a 58-year-old man, who used to live in a livestock-raising area in the Andean region of Peru and presented with an asymptomatic 5 cm left anterior cervical tumour that had been present for over a year. The findings of CT scan suggested an inflammatory lesion. Histological analysis revealed chronic inflammation and granuloma with giant cells surrounding *F. hepatica* eggs. A *Fasciola*-specific antigen (Fas2) was detected in the tissue by immunohistochemistry. Serology for *F. hepatica* was positive, but stool examinations were negative, and eosinophil count and abdominal ultrasonography were normal. A month later, the tumour recurred, and a mature *Fasciola* adult was found during excision. The patient received triclabendazole, and after 6 months, serology was negative (Marcos et al., 2009b).

The latter two findings (Marcos et al., 2009b; Prociv et al., 1992), together with that of the recovery of *F. hepatica* specimen after incision of a subcutaneous abscess in the shoulder of a 23-year-old female in France (Malherbe, 1898 in Senevet and Champagne, 1929), have the additional interest of demonstrating that ectopic flukes may reach locations in the upper body very far from the normal fasciolid microhabitat of the liver. Other three findings proving that ectopic flukes may reach the head are (i) the obtaining of an *F. hepatica* specimen from a walnut-sized cutaneous abscess behind the ear of a 38-year-old man in the United Kingdom (Fox, 1857 in Senevet and Champagne, 1929), (ii) an *F. gigantica* specimen

recovered from the external ear of a patient, and (iii) another *F. gigantica* fluke sneezed out by another patient who presented with a migrating mass in the neck that was associated with paralysis of the recurrent laryngeal nerve, both latter cases in Hawaii (Stemmermann, 1953).

The aforementioned ectopic findings of gravid and immature flukes explain the detection of *Fasciola* eggs in the brain of an Argentinean patient, in whom the fluke was unfortunately not found (Correa et al., 1969; Ruggieri et al., 1967).

8. POLYMORPHISMS, MULTIFOCALITY, MANIFESTATION CHANGES, AND SYNDROMES

The clinical complexity of the patients here in question relies on the puzzling polymorphisms resulting from the combination of symptoms, signs, and syndromes of different types, sometimes with multifocal traits:

- Genuine neurological
- Meningeal
- Psychiatric or neuropsychic
- Ocular

The aforementioned diversity of clinical pictures is additionally accompanied by a series of symptoms that are classically or rarely caused by fascioliasis:

- Hepatic
- Cutaneous
- Pulmonary
- Cardiac and circulatory

In the follow-up of an extreme case throughout the invasive and biliary phases of fascioliasis, manifestations of all the aforementioned types may be found. However, the usual situation is a combination of neurological and meningeal together with hepatic and cutaneous manifestations. Ocular involvement is added more frequently than psychiatric or neuropsychic manifestations. Pulmonary symptoms may be detected in the invasive phase during juvenile intraorganic migration. Heart and vessel involvements are the less frequently reported, which in the case of vessels may probably be an underestimation due to lack of detection because of not having been looked for.

Major neurological, meningeal, psychiatric, and ocular manifestations rarely appear isolated. Different combinations of them are the usual condition within the manifestations of the same patient. This gives rise to very diverse clinical pictures between different patients, although similarities

between a few patients may be observed when not entering into details. Moreover, (i) the different complex associations between them; (ii) the transitory trait sometimes shown to be several of the manifestations; (iii) their changes including appearance, spontaneous disappearance, and reappearance along with the evolution of the disease in a patient; and (iv) different successions from one another according to the different patients add to the surprising and disconcerting complexity of these fascioliasis patients. If not treated against fascioliasis or not completely cleaned from flukes, the length of such disorders may take several months or similar clinical pictures reappear after years.

Additionally, these neurological, meningeal, psychiatric, and ocular symptoms, signs, and syndromes are not pathognomonic. The large diversity and the lack of specificity are the two main outstanding features of these manifestations. Shared aspects by these different semiologies have been mentioned in several cases and include a preceding urticaria, a hyperthermia, the rapid onset, and a drop of the general health status. Unfortunately, exceptions are numerous including patients without cutaneous reactions, patients without fever, patients showing a gradual progressive worsening of the disorders, and even cases of ocular fascioliasis without any other manifestation.

Additional to the cases of neurofascioliasis and ophthalmofascioliasis previously summarized, several complex cases of indirect neurological affection of the central nervous system are appropriate examples to illustrate the surprising and disconcertating evolution of the clinical pictures and impressive evolutionary changes of symptoms and signs appearing and disappearing along the disease process a fascioliasis patient may show (Aguirre Errasti et al., 1981; Aimard et al., 1984; Aubertin et al., 1966; Becquet and Delassus, 1961; Berenger, 1984; Bothier et al., 1968; Cattan et al., 1953; Coulaud et al., 1970; Domart et al., 1967, 1971; Garde et al. 1961; Girard et al., 1959; Guyot, 1962; Kristoferitsch et al., 1982; Lefevre et al., 1970; Leng-Levy et al., 1965; Lesecq et al., 1972; Paraf et al., 1967; Pelletier et al., 1995).

An enumeration of the diversity of main syndromes that may appear in such fascioliasis patients is included in the following. It should be considered that these different syndromes may appear accompanied by aberrant signs such as generalized convulsions, epileptic seizures, sense disorders, and other disconcerting manifestations as the psychiatric ones.

(A) *Pyramidal syndromes or upper motor neuron lesions*: Comprising symptoms caused by partial or complete damage of the pyramidal tract and suggested by progressive muscle weakness, movement slowness,

spasticity, increased deep tendon reflexes, and Babinski's sign. Such pyramidal insufficiencies in the fascioliasis patients have been reported many times and noted to manifest focality through unilateral or bilateral motor deficit abnormalities including monoparesis, hemiparesis, quadriparesis, monoplegia, and hemiplegia (see numerous references in Section 5.3).

(B) *Extrapyramidal syndromes*: Related to various movement disorders and appear only sporadically in *Fasciola*-infected subjects in whom it has been reported to be unilateral and accompanying neurological polymorphic pyramidal manifestations (Coulaud et al., 1970; Saimot et al., 1971).

(C) *Brainstem syndromes*: Including cerebellar syndrome that presents with symptoms of an inability to coordinate balance, gait, extremity, and eye movements. In the fascioliasis patients, the cerebellar syndrome has been reported to be either static (case 1 of Berenger, 1984), kinetic (Bothier et al., 1968; Garde et al., 1961) or nonestablished (case 4 of Guyot, 1962). Related manifestations include nystagmus and dysmetria (Bothier et al., 1968; Garde et al., 1961; case 1 of Berenger, 1984) and vertigo (Domart et al., 1967; Garde et al., 1961). A Millard–Gubler syndrome has also been noted in a neurofascioliasis case (Lunedei and Roselli del Turco, 1934).

(D) *Cranial nerve syndromes*: Involvement of the VI abducens cranial nerve (Coulaud et al., 1970; Saimot et al., 1971) including palsy (Zhou et al., 2008). Paralysis of the VII cranial nerve showing facial affection (Arias et al., 1986; Garde et al., 1961) and with additional repercussion on half tongue (Aguirre Errasti et al., 1981; Lunedei and Roselli del Turco, 1934).

(E) *Encephalic syndromes*: Such as delirium, hallucinations, confusion, behaviour disorders, altered intellectual functioning, and attentiveness disturbances (Dunet, 1924; case 2 of Guyot, 1962; Aimard et al., 1984; Berenger, 1984; Gil et al., 1970; Lefevre et al., 1970).

(F) *Meningeal syndromes*: Presenting with cephalalgias, vomiting, normal or elevated temperature, usually with transitory neck stiffness, and classically appearing as an acute meningitis with eosinophilic cerebrospinal fluid (Dunet, 1924; Garde et al., 1961; case 2 of Dejean, 1960; Bernheim et al., 1958; Leng-Levy et al., 1965; case 2 of Guyot, 1962; case 1 of Berenger, 1984). This meningeal syndrome may appear isolaterally (Leng-Levy et al., 1965; case 2 of Dejean, 1960; case 2 of Guyot, 1962; Berheim et al., 1958) or associated with focal

neurological manifestations (Dunet, 1924; Garde et al., 1961; case 1 of Berenger, 1984). The persistence of the meningeal manifestations after nonspecific therapy is worth noting.

(G) *Bone-marrow syndromes*: Neurological abnormalities appeared related to bone-marrow involvement in several cases (Bothier et al., 1968; Murase et al., 1998; Vatsal et al., 2006). Bone-marrow damage may also be inferred from a clonus reported from the right Achilles' tendon (Campo et al., 1984a).

(H) *Peripheral syndromes*: Mononeuritic sciatica (Dejean, 1960; Lesecq et al., 1972), sensitive polyneuritis of the lower limbs and optical retrobulbar neuritis (Becquet and Delassus, 1961), and osteotendinous areflexia with peripheral neurogenic affection at electromyography in a patient with polyradiculoneuritis and encephalopathy (Aimard et al., 1984).

9. PATHOGENIC AND PHYSIOLOGICAL MECHANISMS

Until the 1990s, two physiopathologic hypotheses were noted to explain the neurological manifestations of *Fasciola* infection: (i) an ectopic localization of the parasite and (ii) an immunoallergic process, including meningovasculitis due to an eventual transitory migration of a fluke or fluke eggs and a seric allergic mechanism related to parasites staying in biliary ducts and inducing antigenic reactions at distance.

Today, findings in recent case reports and results of experimental infection in laboratory animal models are sufficient to establish that two physiopathogenic mechanisms underlie the neurological and ocular manifestations of fascioliasis, the second one showing a large physiological complexity.

9.1. Ectopic flukes as causal agents

Many reports demonstrate that intracranial invasion by ectopic juvenile, immature fasciolids may occur in the invasive phase (Arias et al., 1986; Cho et al., 1994; Wang et al., 2007; Ying et al., 2007; Zhou et al., 2008) and also spread into the biliary phase (Arias et al., 1986) or occur in the biliary chronic phase when reinfection occurs (Lunedei and Roselli del Turco, 1934). The fatal case of the female patient of Argentina (Correa et al., 1969; Ruggieri et al., 1967) does not allow to conclude whether it was caused by an ectopic fluke from a reinfection that was able to mature and produce eggs intracranially or by a mature gravid fluke that reentered intraorganic

migration after a long-term biliary phase, although the latter possibility is hard to believe.

The fact that all these neurofascioliasis or intracranial fascioliasis cases seemed to be caused by only one ectopic *Fasciola* specimen is worth emphasizing.

Different patients (Lunedei and Roselli del Turco, 1934; Ying et al., 2007; Zhou et al., 2008) in whom an ectopic migrant worm was involving first an intracranial location and afterwards an ocular location are proves of the capacity of sporadic fluke juveniles to migrate upwards and reach the central nervous system. The recovery of immature fasciolids of similar characteristics from the eye of other patients (Cheng et al., 2007; Cho et al., 1994; Dalimi and Jabarvand, 2005) confirms such a capacity.

In fact, this capacity of *Fasciola* is not surprising, taking into account that ectopic cases involving many numerous extrahepatic locations have been reported and that in other trematodiases, cerebral localizations have also been reported, such as in several intestinal (Africa et al., 1936) and mainly pulmonary (Shih et al., 1958) distomatoses. In paragonimiasis, migration is known to occur by blood way or by migration through conjunctive tissues of the neck, along vessels and nervous stems. In *Fasciola*, findings in blood vessels indicate that young flukes are able to enter the bloodstream and pass to distant parts of the body.

Hence, the pathological changes in the nervous system will be influenced by the route of entry and the size and mobility of the parasite. These changes may thus be expected to fall into three categories:

- Haemorrhagic changes may be caused by parasites in the arterial circulation or to the laceration of the blood vessels as the parasite moves through the tissues.
- Degenerative changes may include disruption of nervous tissues, swelling of axis cylinders, and degeneration of neurons. Degenerative changes in the vicinity of the parasite should be expected to appear only if the parasite has become quiescent. On the contrary, relatively normal tissue may surround parasites in movement, whereas extensive damage may be found in other parts of the central nervous system.
- Proliferative changes may be diffuse, including perivascular hyperplasia of the reticulum, or focal, usually consisting of granulomatous aggregations in the vicinity of the parasite, sometimes comprising cellular reaction of mostly glial proliferation.

Several observations made in neurological fascioliasis patients may be explained in this way, such as the finding of an area of increased uptake

in brain scan (Gil et al., 1970; Lefevre et al., 1970) suggesting the presence of an inflammatory granuloma in contact with the parasite and the detection of a linear image by means of CT in another patient (case 2 of Berenger, 1984). Similarly, the obstruction of the middle cerebral artery, found in a patient without cardiovascular risk (Giroud et al., 1979), may be interpreted as being due to a parasitic embolism.

The repeated five-episode intracerebral haemorrhages and haematomas detected in a 10-year-old Chinese boy with neurological manifestations were explained by three probable physiopathologic mechanisms: (i) the worm migrated into the brain parenchyma and/or directly penetrated through the vessel wall into the brain parenchyma, its finding when moving through the swelling conjunctiva demonstrating the strong penetration capacity of such a relatively big *Fasciola* juvenile (of around 4.4 mm length according to the illustration provided—see Fig. 2.9A) through the tissues; (ii) inflammatory changes and vessel damages were associated with fascioliasis; in the central nervous system infections, direct inflammation can induce cerebral blood vessel wall destruction (the adventitia, tunica media, and tunica intima of arteries and/or arterioles become oedematous and infiltrated with the inflammatory cells, resulting in panarteritis, and focal arteritis leads to a severely weakened and necrotic vessel wall); and (iii) involved vessels may become occluded by thrombosis or the parasite, resulting in focal vessel wall destruction (Zhou et al., 2008).

The lesions produced in the nervous tissue are more likely to be symptomatic than aberrant migrations in other tissues. It is also common to find lesions typical of those produced by migratory parasites without being able to locate the parasite. The clinical signs associated with parasites in the nervous system depend on the neuroanatomical structure affected, but as *Fasciola* has no specific selectivity for any part of the nervous system, the worms can produce any clinical sign referable to that area. Lesions in the nervous tissue may explain why sequelae have been described to persist in several neurological fascioliasis patients even after treatment.

The Argentinean case always used in the past as an argument to support a possible cerebral location of a migrant worm (Correa et al., 1969; Ruggieri et al., 1967) is now the only case still posing doubts of interpretation. The question refers on how to understand the detection of numerous *Fasciola* eggs in the human brain where no worm was found in the surgical intervention. Two explanations may be evoked for such a finding:

– When considering that ectopic flukes in humans are almost always immature worms, one may conclude that eggs have been accumulated in a

cerebral place after having been laid by a mature fluke in an hepatic blood vessel and passively transported by the bloodstream from the liver along the circulatory system up to the central nervous system. However, this hypothesis hardly explains the concentration of so numerous eggs surrounded by a lymphocytic infiltrate reaction in only one place of the brain by simple progressive transport accumulation.

– The egg concentration suggests their shedding through the genital pore of a mature fluke adult in one lay. Cases of infection by ectopic mature *F. hepatica* in upper body locations indicate that *Fasciola* may reach and produce eggs in locations close to the central nervous system, whether (i) in the rare probability of having begun its extrahepatic migration after becoming gravid in the liver or (ii) most probably when a migrant worm initiates the extrahepatic migration as a juvenile and matures during the migration or once in its ectopic location.

The question why, after host infection, a juvenile of *Fasciola* becomes an ectopic migrant even able to reach the central nervous system (and/or the eye), instead of succeeding in reaching its appropriate final hepatic microhabitat, remains a matter of speculation. Although reinfection of the same host individual is known to occur in subjects inhabiting human fascioliasis hyperendemic areas (Valero et al., 2003), it has been argued that previous specific immunologic sensitization may give rise to a certain difficulty barrier for the correct intraorganic migration route to be followed by reinfecting new flukes. Although such a possibility applies well to the Italian patient studied by Lunedei and Roselli del Turco (1934), as these authors already emphasized, it cannot be argued for those patients in whom the migrant fluke seemed to be the only infecting worm (Dalimi and Jabarvand, 2005; Vatsal et al., 2006; Ying et al., 2007).

9.2. Physiopathogenic processes indirectly affecting the central nervous system

Many observations made in neurological patients affected by fascioliasis support the existence of mechanisms acting at distance:

– The fact that, in patients presenting neurological manifestations, flukes are most usually found in their specific hepatic microhabitat and, consequently, liver fluke eggs are found in the patient faeces.
– In neurological fascioliasis patients, there is a frequent association with cutaneous manifestations of allergic type, such as urticaria, Quincke's disease, and dermatographia (Aimard et al., 1984; Bernheim et al., 1958; Domart et al., 1967, 1971).

- In neurological fascioliasis patients, the appearance of an allergic pulmonary picture such as the Loeffler syndrome has been noted several times (Garde et al., 1961).
- The neurological disorders appearing sometimes at the invasion period of the infection, their polymorphism, and their character are sometimes multifocal.
- The regression of the neurological symptoms sometimes spontaneous and without sequelae.

Other arguments sometimes noted to support an allergy mechanism cannot however be retained. The efficacy of corticotherapy cannot be evoked because of the spontaneous recoveries and its incapacity to prevent patient's worsenings (Bothier et al., 1968; case 2 of Berenger, 1984). The existence of a blood hypereosinophilia and sometimes also a rachidian hypereosinophilia does not appear to be a formal argument, as indeed a hypereosinophilia may be the reaction to a larval migration, as in the case of the eosinophilic meningoencephalitis by *Angiostrongylus cantonensis* or a meningeal haemorrhage by ruptured aneurysm. The following aspects should be considered:

(A) *Intensity of infection and host sensitivity*: A priori, one would expect such impressive neurological manifestations to be related with high infection burdens. However, the numbers of fasciolid specimens recovered from patients do not suggest a direct relationship between burden and symptomatology. Although the number of patients in whom the burden could be established is obviously reduced, data appear to speak for itself.

In different patients with minor neurological symptoms, only one *Fasciola* was found by surgery (Del Valle and Donovan, 1928) or endoscopically (Birjawi et al., 2002; Giffoniello et al., 1983). A finding of 25 flukes by endoscopy appears to be an exception (Coral et al., 2007).

Only three fasciolid specimens were found in a patient showing major neurological symptoms (Dunet, 1924), and a sole fluke was causing the neurological disorders in an ectopic case in dorsal spinal cord (Vatsal et al., 2006). In fatal neurological cases, the fluke number found at autopsy was also low in almost all cases, such as 6 (Frank, 1823 in Bürgi, 1836), only 1 (Biermer, 1863 in Bürgi, 1836), 2 (Blanchod, 1909 in Bürgi, 1836), 12 (Biggart, 1937), 2 (Aguilar et al., 1967, 1968), and 11 (Pan and Huang, 1954). The only exception among fatal cases is a patient in whom 26 flukes were found (Humble und Lush, 1881 in Bürgi, 1836).

This speaks about the high pathogenicity of a sole fasciolid specimen and/or suggests differences in host sensitivity. The latter should

be related to the question of why neurological manifestations are caused in certain individuals and not in others, even despite massive infections.

(B) *Infection phase, fluke location, and the role of abscesses*: Reports on major neurological manifestations refer to patients in the invasive phase, symptoms sometimes appearing suddenly and very early, and in the biliary phase. This means that the induction of neurological disorders may be caused by juvenile flukes migrating through different tissues and organs and by mature flukes in the liver.

A first question mark refers to the pathogenicity of the migrant juvenile flukes. Does an infecting small form of about 250 μm have the capacity to induce such impressive manifestations or are there many infecting flukes needed (possibly only few reaching the final biliary microhabitat and the many other dying in their endeavour), and how do they produce these neurological reactions in such a short time after the infection. These facts suggest the involvement of immunoallergic and toxic processes that may later worsen with the damage to the liver.

The mechanisms underlying all these processes are still far from well elucidated, but recent knowledge indicates the involvement of the following:

- Inflammation, vasculitis, arteritis, and thrombosis
- Local and systemic responses
- Circulating immune complexes and complement system activation
- Autoimmune responses
- Hypersensitivities, type III hypersensitivity, and Arthus reaction
- Impairment of the blood–brain barrier
- Hepatic encephalopathy and relationships with ammonia, sodium, manganese, and others
- Hypereosinophilia-induced encephalopathy
- Excretory/secretory products of *Fasciola* and their metabolic end products
- Proteases and cathepsins
- Proinflammatory cytokines and oxidative/nitrative stress
- Neurotoxins
- Substances derived from the damage, malfunction, and failure of the liver
- Coinfections and sepsis
- Associated conditions such as antiphospholipid syndrome and intravascular lymphomatosis

The large complexity and interrelationships of these immunoallergic and physiopathogenic processes and their involvement in human fascioliasis merit an exhaustive analysis, which will be made in an additional article.

A second question is posed by patients in whom such neurological manifestations appear after many years of infection, that is, in the late chronic phase, such as in a fatal case in Argentina (Correa et al., 1969; Ruggieri et al., 1967) and in another patient from Chile who was diagnosed after 5 years living in Austria (Auer et al., 1982; Kristoferitsch et al., 1982). In the former, a reinfection could not be ruled out, but in the Austrian case, it was.

An explanation to the second question may be based on observations such as those showing the presence of an immature fluke in between mature gravid flukes among fasciolids arrived simultaneously to the biliary ducts (Neghme and Ossandon, 1943), the finding of living flukes locked in abscesses in the liver parenchyma for a long time, months, or even years (Mohr et al., 1951), and the detection of multiple liver abscesses in a patient (Teichmann et al., 2000). Aberrant specimens that do not reach the biliary ducts may survive as immature flukes in other organs for long periods. This could be related to a high antigen contact responsible for allergic pathomechanisms also in the post-invasive phase. Additionally, the breakage of such abscesses may release large amounts of antigens producing subsequent allergic reactions.

(C) *Experimental studies*: The confirmation of the existence of mechanisms acting at distance has recently been experimentally obtained. A study in the laboratory rat model demonstrated that (i) *F. hepatica* infection in the liver was able to impact on remote tissues, (ii) an altered neural nucleotide signature was one of the strongest metabolic responses in infected rats, and (iii) the perturbed immunologic function suggested by the altered nucleotide levels in the brains of *F. hepatica*-infected animals proved the neural effects to be specific for *Fasciola* when compared to other trematodes (Saric et al., 2010). Significant perturbations of the nucleotide balance in the brain, together with an increase of an important direct negative regulator of inflammatory cytokines in macrophages such as plasma IL-13, were found. This suggested a shift towards modulation of immune reactions by secretion of nucleotide-degrading enzymes to minimize inflammatory response, which may favour the coexistence of the parasite in the host. The pattern of differentiating metabolites in the neurochemical profiles was composed of

a significant increase in the relative levels of inosine, tyrosine, and phenylalanine. Conversely, the relative tissue concentrations of GPC, succinate, inosine mono-, di-, and triphosphate; adenosine; and adenosine mono-, di-, and triphosphate were lower in the brains of infected animals.

Minimization of such an intense immune reaction, and hence prolongation of the period in which the parasite can remain undetected by the host at earlier stages of infection, is clearly beneficial for the survival of the worms (Saric et al., 2010). Moreover, the inflammatory response manifested not only at the level of the metabolite signature but also at the level of structural damage. Additionally, hepatic dysfunction in *F. hepatica* infection is known to result in increased circulating toxins, such as ammonia, thiols, and phenols. It was shown that hepatic failure induced an increased passive permeability of the blood–brain barrier for several substances, among which phenylalanine and tyrosine were found to increase up to 30% in the host brain.

The question of why the percentage of fascioliasis patients showing neurological manifestations is relatively low, whereas *Fasciola* in the liver has been proved to be able to induce modifications in the host brain is still pending. It may be argued that results of *Fasciola* infection studies on rats may not be extrapolated to human beings. However, the laboratory rat model has shown to be useful for this purpose in other aspects (Gironés et al., 2007; Valero et al., 2002, 2003, 2007, 2008).

10. DIAGNOSIS OF NEUROLOGICAL AND OPHTHALMOLOGIC FASCIOLIASIS

10.1. Clinical and paraclinical diagnosis

Major neurological manifestations usually show such puzzling polymorphisms, comprising extremely varied clinical pictures where irritative, deficitary, and meningeal signs appear intricated, that they may easily lead to confusion with other diseases such as cerebral tumours, multiple sclerosis, lesions of the brainstem, or cerebromeningeal haemorrhages. Complementary examinations, EEG, and arteriography are not conclusive; they may show either focal signs guiding towards a tumour formation or diffuse fleeting signs. Characteristics that attract attention include the existence of diffuse lesions, the surprising evolution with sometimes unexpected spontaneous regression of the disorders or on the contrary their resistance to symptomatological

treatments, and clinical polymorphisms with sometimes association of pulmonary, cutaneous, digestive, and also cardiac manifestations.

The long time needed until the correct diagnosis in patients showing such disconcerting clinical pictures was reached was emphasized in several cases: 4 months (Ragab and Farag, 1978), not longer than 4 months only thanks to finding a similar literature report by chance (Coulaud et al., 1970; Saimot et al., 1971), 11 months until correct diagnosis, and even a total of 33 months to reach final cure (case 12 of Guyot, 1962). The consequences of the complexity of such confusing and changing neurological polymorphisms have been highlighted, including long hospitalization periods, surgical interventions, and many treatment courses with different drugs for the same patient (Paraf et al., 1967).

There are, however, aspects for presumption of *Fasciola* infection. Eosinophilia, in both the blood and cerebrospinal fluid, and typical source of infection appearing in anamnesis may greatly help in guiding towards the correct diagnosis.

Eosinophilia is a usually constant biological sign, mainly during the acute phase, which, when relying on it, allows for a correctly channelled aetiological diagnosis of the aforementioned neurological manifestations. Blood eosinophilia is usually high and constantly found (except for rare cases without eosinophilia). Rachidian eosinophilia, if present and detected, is an additional factor of value.

The source of infection can usually be found easily in the anamnesis and greatly helps in considering a *Fasciola* infection to be in the background of the neurological disorders detected in the patient. Aspects to be considered include ingestion of wild watercress and/or any other freshwater vegetable species potentially carrying infective liver fluke metacercariae, fascioliasis diagnosis in family members or persons having participated in the same suspicious meal, and patient living or having visited a human fascioliasis endemic area or living in an area where livestock is known to be infected by the liver fluke.

Finally, the discovery of eosinophilia and the interest of the source of infection are the data leading to the performance of the appropriate biological examinations, including the search for the presence of eggs/coproantigens in stools and/or serological tests that assure the correct diagnosis.

Elements indicating liver affection, such as hepatomegaly, pain in right upper quadrant, and abnormal hepatic function tests (although these tests have also been found to be normal even in several patients with major neurological manifestations—e.g. Bernheim et al., 1958; Coulaud et al., 1970;

Saimot et al., 1971), are of additional support when found. There is the need to know how to adequately place all the neuromeningeal, respiratory, and cutaneous elements within the anamnestic, haematologic, parasitological, and serological context, in the way to identify them.

Other interesting guiding results found in the analyses of patients with major neurological manifestations include an increased erythrocyte sedimentation rate (usually present), high to very high levels of serum IgE (Berenger, 1984; Cames et al., 1947; Park and Sohn, 2010), high prothrombin rate (Domart et al., 1971; Gil et al., 1970; Lefevre et al., 1970), and shortening of the partial thromboplastin time (Linares et al., 2006). Indicators that should be emphasized due to their potential special significance are a high circulating immune complexes rate (Aimard et al., 1984; Auer et al., 1982; Kristoferitsch et al., 1982), elevated level of C-reactive protein (Murase et al., 1998; Paraf et al., 1967; Park and Sohn, 2010), and positivity to antiphospholipid antibodies with anticardiolipin antibody IgG (Frances et al., 1994; Linares et al., 2006; Oujama et al., 2003; Pelletier et al., 1995). Subungual splinter haemorrhages, a nonspecific sign that has been related to infective endocarditis, inflammation in blood vessels all around the body (systemic vasculitis), and also the antiphospholipid syndrome, have been reported from neurological patients showing cardiac affection (Auer et al., 1982; Coulaud et al., 1970; Frances et al., 1994; Kristoferitsch et al., 1982; Pelletier et al., 1995; Saimot et al., 1971), although also in subjects in whom heart involvement was not noted (Lemoine, 1954).

In cases of patients only showing minor neurological manifestations, whether in human endemic areas or not, reaching fascioliasis suspicion may need to rely on other symptoms and/or signs characteristic of the acute and chronic phases of this disease. These combined characteristics may thereafter lead to the analysis of eosinophilia and questioning about potential infection sources during anamnesis.

In patients with ophthalmologic manifestations, the diagnosis may be simple if the fluke is directly observed in the eye. If such an observation is not made, accompanying neurological disorders and typical symptoms of acute and/or chronic phases rapidly channel towards fascioliasis suspicion and subsequently to the corresponding analysis for eosinophilia and questioning about the source of infection.

Once *Fasciola* infection is diagnosed, the more or less rapid, total disappearance or pronounced regression of neurological and ophthalmologic disorders and the cure obtained after a successful specific fascioliasis treatment

become the final confirmation or an additional argument of the correct diagnosis.

10.2. Eosinophilia in the blood and cerebrospinal fluid

In patients presenting with neurological manifestations, blood eosinophilia is almost always present, in both the invasion and biliary phases, and thus becomes a key parameter to look for already from the beginning. It is usually accompanied by a hyperleucocytosis (polynucleosis of 80% or even 90% of the elements). Blood eosinophilia in hepatic fascioliasis is known to differ not only between individuals but also throughout the disease evolution, according to the traditional curve including an initial quick rise up to a peak around the fourth month from infection to decrease progressively afterwards until stabilization at low level even during years. Factors potentially modifying that curve include fluke burden, coinfections, allergic phenomena, and reinfections. Thus, hypereosinophilia should not be considered a specific reaction, as a low eosinophilic rate does not allow to rule out fascioliasis.

Very high blood eosinophilia has been reported from neurological patients, up to levels of 71% (Boissiere et al., 1961), 75% (Ayadi et al., 1991), or even 80% (Ragab and Farag, 1978). However, it should be considered that blood eosinophilia may vary pronouncedly throughout the infection, as observed in several cases (from no eosinophilia during the first symptoms up to between 33% and 56% later by Cattan et al., 1953, and between 1% and 55% by Aimard et al., 1984).

One of the misleading situations is that in which eosinophilia is not present. This is rare in fascioliasis infection but may be relatively frequent in subjects in the biliary phase in human fascioliasis hyperendemic areas, as suggested by the 61.9% of the 134 infected subjects showing no eosinophilia in Peru (Vera, 1989, in Blancas et al., 2004). The absence of eosinophilia has been sporadically reported in patients with minor neurological symptoms in the biliary phase (Birjawi et al., 2002; Patrick and Isaac-Renton, 1992) and in a few showing major neurological manifestations (Gil et al., 1970; Girard et al., 1959; Lefevre et al., 1970).

The other important misleading situations are found in fascioliasis patients with coinfections with other helminths, which may also be responsible for blood eosinophilia. Coinfections by *Fasciola* and other helminth species are indeed a very frequent situation in human fascioliasis endemic areas, mainly in children (Esteban et al., 1997a,b, 1999, 2002, 2003; Gonzalez et al., 2011; Zumaquero-Ríos et al., 2013). The problem posed

by such coinfected patients when hospitalized due to their neurological manifestations in developing countries where helminth infections are common in rural areas becomes evident. A 27-year-old woman living in Yaoundé, Cameroon, and admitted in a local urban hospital, who was coinfected by *Taenia saginata*, *Trichuris trichiura*, *Strongyloides stercoralis*, and *Onchocerca volvulus*, could not be correctly diagnosed as a subject whose persistent intense neuropsychic manifestations were caused by *F. gigantica* until four and a half months later, after having been transferred to France and a long stay in an hospital in Paris. In that patient, the varying blood eosinophilia (5%, 18%, 33%, and 2% depending on hospitalization day) became misleading instead of helpful (Paraf et al., 1967).

Additionally to blood eosinophilia, in several fascioliasis patients presenting with neurological and/or ocular manifestations, eosinophilia was also found in the cerebrospinal fluid (Aguirre Errasti et al., 1981; Ayadi et al., 1991; Bernheim et al., 1958; Cames et al., 1947; Campo et al., 1984a,b; Cattan et al., 1953; Coulaud et al., 1970; Gil et al., 1970; Girard et al., 1959; Lefevre et al., 1970; Leng-Levy et al., 1965; Oujamaa et al., 2003; Saimot et al., 1971; Schussele and Laperrouza, 1971a,b). Usually, the presence of eosinophilia in the cerebrospinal fluid indicates meningeal affection, whether isolated or accompanying neurological disorders; on the contrary, patients with only genuine neurological manifestations usually show a normal cerebrospinal fluid.

A cerebrospinal fluid eosinophilic pleocytosis should be considered an important clinical observation because of the small range of aetiological possibilities (mainly helminths included *Fasciola*, rarely fungal and bacterial infections, and also myiases) (Kuberski, 1979). The levels of eosinophilia in the cerebrospinal fluid of neurological patients have usually been noted to be not very high (between 20% and 41%), although exceptions of cases showing a very high one have also been reported: 60% (Garde et al., 1961), 79% (Aguirre Errasti et al., 1981), 82% (Lunedei and Roselli del Turco, 1934), and 90% (Bernheim et al., 1958).

A few cases with neurological manifestations should be emphasized due to the fact of presenting eosinophilia in the cerebrospinal fluid but none in blood (Gil et al., 1970; Girard et al., 1959; Lefevre et al., 1970). This indicates that there is no parallelism between eosinophilia in the blood and in the cerebrospinal fluid. The absence of blood eosinophilia may imply wasting of long time (Girard et al., 1959). When both eosinophilias are present in the same neurological patient, it is usually higher in the blood than in the cerebrospinal fluid, but here, also exceptions of patients in whom the

eosinophilia in the cerebrospinal fluid was higher have also been reported (Aguirre Errasti et al., 1981; Leng-Levy et al., 1965). All in all, the experience accumulated suggests the convenience to look for a possible eosinophilia in the cerebrospinal fluid in patients presenting with neurological and/or ocular disorders. Its presence may initially be considered an indicator of potential neurofascioliasis or intracranial infection by ectopic flukes and secondarily of fascioliasis with neurological and/or ocular involvement.

10.3. Differential diagnosis from other parasitic infections

The finding of eosinophilia not only in the blood but also in rachidian fluid leads to the diagnosis of an infection by metazoan parasites, not only by helminths but also arthropods. There are many metazoan parasites that may affect the central nervous system (Abdel Razek et al., 2011; Bia and Barry, 1986; Brown and Voge, 1982; Chacko, 2010; Cook and Zunmia, 2009; Graeff-Teixeira et al., 2009; Hughes and Biggs, 2002; Kristensson et al., 2002; Lowichik and Ruff, 1995a,b; Lowichik and Siegel, 1995; Lv et al., 2010; Vercruysse et al., 1988) and/or the eye (François et al., 1985; Grüntzig, 1988; Otranto and Eberhard, 2011; Sabrosa et al., 2010).

Moreover, the eosinophilia in the cerebrospinal fluid, when detected, may help in ruling out many neurological disorders of different aetiology.

10.4. Helminthiases

Regarding helminthiases, there are many helminth species known to give rise to neurological and ophthalmologic symptoms, more or less frequently depending on the parasite species involved. Knowledge about the geographic distribution and previous human casuistry by the helminth species in the area in question may help in differentiating from *Fasciola* infection.

Among trematodiases, paragonimiasis should be highlighted as it appears to be the most frequently affecting the central nervous system (Kusner and King, 1993; Oh, 1968a; Shih et al., 1958) and also the eye (Oh, 1968b; Wang et al., 1984) and should therefore be taken into account in certain areas of Asia, Africa, and Latin America where an overlap with human fascioliasis is known. Heterophyiasis is of importance because of its high pathogenicity in neurogenic cases although human infection by *Heterophyes* appears very rarely (Africa et al., 1936; Collomb and Bert, 1957, 1959; Collomb et al., 1960). Dicrocoeliasis has only been sporadically diagnosed in humans and a cerebral affection by *Dicrocoelium* has only been reported once (Siguier et al., 1952). Finally, the infection of the central nervous

system by species of *Schistosoma* seems to be a neglected and an under-recognized complication of schistosomiasis as a neurological consequence of the immune reaction around the fluke eggs in the central nervous system, which may provoke severe disability (Carod-Artal, 2010).

Ocular infection by *Fasciola* should be differentiated from that caused by mesocercariae of species of *Alaria* usually infecting canids and that by adult stages of *Philophthalmus* species that are bird eye trematodes. Human infections by trematode species of the genus *Alaria* have been reported in North America and Asia, including fatal human mesocercarial multi-infection cases (Fernandes et al., 1976; Freeman et al., 1976; McDonald et al., 1994; Shea et al., 1973). Ocular philophthalmiasis in humans has been reported in Europe (former Yugoslavia, now Serbia), Israel, Asia (Thailand, Sri Lanka, and Japan), and the Americas (i.e. Mexico and the United States) (Gold et al., 1993; Kalthoff et al., 1981; Lamothe-Argumedo et al., 2003; Markovic, 1939; Rajapakse et al., 2009; Waikagul et al., 2006). Contrary to *Alaria* infection, philophthalmiasis is not known to cause serious illness in humans, although granulomatous anterior uveitis reported as an outbreak in numerous children from southern India has also been tentatively attributed to *Philophthalmus* (Rathinam et al., 2001, 2002). Morphological characteristics of the *Alaria* mesocercariae (Odening, 1963) and *Philophthalmus* adult digeneans (e.g. Lamothe-Argumedo et al., 2003; Markovic, 1939) should be considered for its differentiation regarding juveniles of *Fasciola*, the ramified caeca of the latter being the first differential and easily visible feature to be looked for.

With regard to cestodiases affecting the central nervous system, neurocysticercosis by *Taenia solium* is the most important, mainly in overlapping areas with fascioliasis in Latin America and may be in Africa and Asia. Relatively high numbers of patients with ocular cysticercosis, at a constant frequency throughout the past two decades, have been reported in the area of Hyderabad, India (Madigubba et al., 2007). Hydatidosis and sparganosis appear to be of secondary importance with regard to the differential diagnosis with fascioliasis. Sparganosis by species of *Spirometra* and larval stages of taeniid species such as *T. crassiceps*, *E. granulosus*, *E. multilocularis*, and *E. oligharthrus* and *M. multiceps* are cestodes able to affect the human eye (Otranto and Eberhard, 2011) and should therefore be considered in the differential diagnosis with *Fasciola*.

Affection of the central nervous system is also known in many nematodiases, such as trichinelliasis or trichinosis, eosinophilic meningoencephalitis or angiostrongyliasis, and strongyloidiasis or anguillulosis, among many others caused by filarids and larva migrans by different nematode

species. Among the latter, toxocariasis stands out (Dousset et al., 2003), even including neurological cases of coinfection with fascioliasis (Oujamaa et al., 2003). Neurological abnormalities were also observed in patients coinfected by *Fasciola* and *Anisakis* (Murase et al., 1998).

The aforementioned coinfected patients suffering from neurological manifestations highlight the diagnostic problems that may be encountered in human endemic areas of developing countries, where *Fasciola* coinfections with other helminthiases appear to be very frequent. Indeed, the parasitological spectrum of protozoan and helminthic species found in the inhabitants of the human fascioliasis endemic areas, the multiparasitisms, and the associations between liver fluke infection and infection by other pathogenous parasites all appear to be similar in the different human endemic zones (Esteban et al., 1997a,b, 1999, 2002, 2003). These synergistic associations of fascioliasis with other pathogens are believed to be at the base of the high morbidity and mortality rates of Aymara children inhabiting the northern Bolivian altiplano (Mas-Coma et al., 1995).

The presence of eosinophilia may, however, help in patients from human endemic areas in whom coinfection is with pathogenic microorganisms, such as viruses as HIV virus (Wahib et al., 2006) or bacteria as *Brucella* (El-Metwally et al., 2011; Mohammad et al., 2011).

Many nematode species are known to be able to infect the human eye. Among them are strongyloides (*A. cantonensis*), ascarids (*Toxocara canis*, *Toxocara cati*, and *Baylisascaris procyonis*), spirurines (*Gnathostoma* spp.), thelaziids (*Thelazia* spp.), and many onchocercid filariids (*Dirofilaria* spp., *Acanthocheilonema* spp., *Onchocerca* spp., and *Loaina* spp.) (Otranto and Eberhard, 2011). Their cylindrical roundworm form and external cuticle allow for an easy differentiation from the plathelminth form and external tegument of *Fasciola*.

10.5. Myiases

Concerning arthropods, neurological and ocular manifestations of fascioliasis may give rise to confusion mainly with brain myiasis and ophthalmomyiasis. Myiases are infestations by dipterous larvae (maggots) that feed on dead or living vertebrate tissues for a variable period. They induce specific and nonspecific immune responses of the hosts with practical implications in the diagnosis (Otranto, 2001). Although serological tests are available, myiasis diagnosis is basically made by finding and carrying out a characteristic morphological identification of the larvae of the Diptera.

Brain myiasis in humans appears to be rare. Only very few cases of brain myiasis in humans have been reported worldwide (Terterov et al., 2010), including fatal cases in intracerebral myiasis. Infestations of the nose and ears are extremely dangerous because they provide the larvae with access to brain tissue. Aural myiasis causing meningitis and brain infestations have been reported (Yuca et al., 2005). In cases of brain penetration by the larvae, the fatality rate has been mentioned to be as high as 8% (Noutsis and Millikan, 1994). Severe complications may also be related to the involvement of the skull base (Arbit et al., 1986; Caça et al., 2003; Ciftcioglu et al., 1997; Werminghaus et al., 2008).

Aural myiasis occurs frequently in children and less frequently in adults, especially when mentally retarded (Baynder et al., 2010). Larval stages of *Calliphora* sp., *Lucilia sericata*, and *Musca domestica* have been found to be involved with myiasis of the nose, paranasal sinuses, pharynx, and ears (Kaczmarczyk et al., 2011). *Sarcophaga* has also been listed as causing mystic scalp and skull infection (Arbit et al., 1986). Fatal scalp myiasis caused by *Cochliomyia hominivorax* having reached the brain cavity has been reported (Oliva et al., 2007).

Larvae of species of *Hypoderma* appear to be of particular importance in intracerebral myiasis, mainly in children (Doby and Beaucournu, 1970; Kalelioglu et al., 1989; Lewicka-Urbanska, 1955; Pouilaude et al., 1980). *Hypoderma* larvae can penetrate into the brain, causing cerebral haematoma and clinical signs. The severity of hypodermiasis relies on the fact that intraocular and nervous locations accompanied by paraplegia have also been reported. The migration of the larval stages of *Hypoderma* may take place through the central nervaxis, involving a neurological syndrome with rachidian hypereosinophilia of several weeks, until the appearance of larvae under the skin and a reincrease of blood eosinophilia. Finally, eosinophils of the cephalorachidian fluid may thus participate in a paraneoplastic syndrome. *Hypoderma lineatum* gives rise to linear dermatitis chiefly at the level of the head, for example, behind the ear, whereas *Hypoderma bovis* gives rise to mobile ambulatory tumours with gelatinous oedema and finally giving rise to a pseudofuruncular lesion containing the larval stage (Doby and Beaucournu, 1970). The differential diagnosis between fascioliasis and hypodermiasis may sometimes pose serious difficulties, mainly in cases of myiasis with deep tissue infections or abortive forms and in overlapping endemic areas such as western France. Computerized tomography shows the haematoma, and carotid angiography shows the absence of a vascular malformation, but these examinations do not allow aetiological diagnosis

of myiasis. Diagnosis is indicated by seroimmunological examinations and/or by the discovery of the larva during operation (Pouilaude et al., 1980). In those cases, only the use of a *Fasciola*-specific serological test may confirm the fascioliasis diagnosis of the patient.

Unlike the rarity of brain myiases, there are numerous cases of eye myiasis reported from many regions, including both developed and developing countries. Ocular involvement or ophthalmomyiasis is seen to occur in about 5% of all cases of myiasis. Most commonly, larvae attack the external surface of the eyes or ocular adnexa, for example, the lids, conjunctiva, or lacrimal ducts (external ophthalmomyiasis). External ophthalmomyiasis is often a benign self-limiting disease that may usually be remedied without complications. However, outbreaks of human external ophthalmomyiasis have also been reported (Dunbar et al., 2008).

In uncommon circumstances, the maggots may penetrate into the eyeball itself (internal ophthalmomyiasis) or may involve the orbit (orbital myiasis), which may result in serious damage and often lead to severe loss of vision, blindness, or even loss of the eye, mainly in cases of larvae with burrowing habits that can give rise to very destructive forms of ophthalmomyiasis, especially in debilitated patients (Chodosh and Clarridge, 1992; Khurana et al., 2010; Verstrynge and Foets, 2004). Ophthalmomyiasis interna may be further subdivided into anterior and posterior based on the presence of the larva in the anterior or posterior segment of the eye, respectively. However, posterior migration of an anterior larva has also been reported (Miratashi et al., 1997; Sharifipour and Feghhi, 2008). The unpredictable behaviour of the larva inside the eye results in difficulty in making treatment decisions.

Fly species of the genera *Calliphora*, *Lucilia*, *Sarcophaga*, *Gasterophilus*, *Hypoderma*, *Musca*, *Callitroga*, *Cuterebra*, *Dermatobia*, *Chrysomya*, *Wohlfahrtia*, and *Oedemagena* are known to cause internal ophthalmomyiasis in humans (Lagace-Wiens et al., 2008; Syrdalen and Stenkula, 1987; Thakur et al., 2009; Verstrynge and Foets, 2004). Diagnosis is made by surgery and identification of the dipteran larva involved.

10.6. Fascioliasis diagnosis

There are several types of techniques for human fascioliasis diagnosis, although some suggestive clinical presentation aspects may be useful. Direct parasitological techniques, indirect immunologic tests, and other noninvasive diagnostic techniques are presently used for human fascioliasis diagnosis.

Besides the detection of eggs in a coprological analysis or in a duodenal aspirate, adults and eggs may be also found elsewhere by means of other invasive techniques: obtaining duodenal fluid, duodenal, and biliary aspirates; surgery (laparotomy, cholecystectomy, and sphincterotomy); and histological examination of the liver and/or other organ biopsy materials. Serological, intradermal, and stool antigen detection tests have been developed. Immunologic techniques present the advantages of being applicable during all periods of the disease, but fundamentally during the invasive or acute phase, and to the other situations in which coprological techniques may present problems. However, immunologic techniques offer other types of problems related mainly to sensibility and specificity. Different serological tests have been used for human diagnosis. Almost all of these techniques concern the detection of circulating antibodies and only very few have been designed to detect circulating antigens and immune complexes. Also, several serological techniques have recently proved to be useful for monitoring posttreatment evolution. Noninvasive diagnostic techniques of complementarily use for human diagnosis include radiology, radioisotope scanning, ultrasound, CT, and MR (Esteban et al., 1998; Hillyer, 1999; Mas-Coma et al., 1999a, 2005).

Although almost all human fascioliasis patients showing neurological manifestations concerned *F. hepatica*, one such case proved to be caused by *F. gigantica* (Paraf et al., 1967). Thus, differentiation between the two fasciolids may be of interest. With regard to this purpose, it should be highlighted that the differential diagnosis between *F. hepatica* and *F. gigantica* in humans cannot be achieved by clinical, pathological, coprological, or immunologic methods. This is a problem in overlap areas of both species as this differential diagnosis is crucial owing to their different pathological, transmission, and epidemiological characteristics. To distinguish between *F. hepatica* and *F. gigantica*, a simple and rapid PCR-restriction fragment length polymorphism (RFLP) assay, based on the 28S rRNA gene obtained from populations of South America, Europe, and Africa, has been described. This assay provides unambiguous results (Marcilla et al., 2002). A similar PCR-RFLP assay was later developed to differentiate between Chinese liver flukes (Huang et al., 2004). Nevertheless, in China and in many other areas of Asia and Africa, the overlap of both fasciolids has given rise to hybrid intermediate forms whose extraordinary genetic variability makes characterization impossible unless DNA sequencing is applied (Mas-Coma et al., 2009a).

The aetiological diagnosis of patients with neurological symptoms has traditionally been performed relying on (i) the identification of flukes and/or

fluke eggs of a *Fasciola* species after their recovery by surgery, (ii) fasciolid egg finding in patient faecal samples, and/or (iii) specific serological tests.

10.7. Fluke and/or fluke egg recovery by surgery

Although there may be cases in which diagnosis may be achieved by means of surgical intervention, as it was in the discovery of flukes in the choledoc of one patient (Dunet, 1924), such a situation may only happen rarely. Fluke recovery in neurological patients usually happens in intraocular cases.

Cases of eye affection by migrating flukes permit their visualization (Fig. 2.7) (Cheng et al., 2007) and subsequent surgical intervention or enucleation for its extraction. In several of these reports, a description and/or figure of the fluke obtained is included to demonstrate its belonging to *Fasciola* (e.g. Zhou et al., 2008), which is mainly facilitated by the presence of highly ramified bilateral intestinal caeca (Fig. 2.9).

In these cases, a juvenile immature *Fasciola* worm was encountered. The size (length/width) of the worm obtained was of 5.20/2.88 mm, in a 28-year-old man from Korea who also was positive in an IgG ELISA serological test (Cho et al., 1994). The worm was 4.26/2.04 mm in a 44-year-old woman from Iran in whom no eggs were found in stools and a serological test (indirect fluorescent antibody test) was also negative (Dalimi and Jabarvand, 2005). The fluke obtained was 8/4 mm in a 8-year-old boy from China in whom fasciolid eggs were not found in faeces (Ying et al., 2005).

Although still far from the normal size of a mature liver specimen of *F. hepatica* or *F. gigantica*, the relatively large size of these immature worms (4.26–8.00/2.04–4.00 mm) when compared to the small size of the early migrating *Fasciola* juveniles in the abdominal cavity (250 μm in length) indicates that these ectopic worms had fed on host's substances and had grown during their migration from the moment of the infection. This pronounced size difference also suggests that the time elapsed between the patient's infection and the worm extraction was more than a few days. Indeed, a 10-day hospitalization stay was noted for the aforementioned Korean patient (Cho et al., 1994) and a similar 10-day period was noted to be the duration of the painful eye symptomatology in the Iranian patient (Dalimi and Jabarvand, 2005), which agrees with the similar yet relatively small size of their respective worms. The case of the larger worm of the Chinese patient also agrees with the longer 26-day hospitalization stay noted for that case (Ying et al., 2005).

Intracranially, *Fasciola* eggs have been found only once, during the microscopic observation of material from the left parietal brain lobe initially considered to be two hydatid cysts infecting a 44-year-old Argentinean woman. Eggs of *F. hepatica* appeared surrounded by fibrotic granulation tissue with lymphocytic infiltration within a necrosis (Correa et al., 1969; Ruggieri et al., 1967).

10.8. Analyses with faecal samples

In cases reported with neurological symptoms, two situations have been described: patients in whom *Fasciola* eggs were found in stools and patients in whom eggs were not present in their faecal samples.

Case reports including neurological manifestations in the hepatic phase (fascioliasis chronic phase) are consequently patients in whom *Fasciola* eggs may be detectable in faeces in a coprological analysis. Indeed, many of the patients showing neurological manifestations were noted to shed eggs in their faeces (Berenger, 1984), which suggests (i) a usually late diagnosis of these neurological patients and (ii) a possible massive infection (Domart et al., 1967).

Techniques ranging from a simple direct smear to different concentration methods may be used. Egg concentration has been achieved by flotation and sedimentation techniques. The sedimentation techniques appear to be more accurate and sensitive than flotation techniques, as most of the hyperosmotic solutions distort the eggs (Esteban et al., 1998; Mas-Coma et al., 1999a).

If eggs are found, their size and shape allow for their specific determination. *Fasciola* eggs should not be confused with those of other trematodes existing in overlap areas and presenting similar egg shape and size (*Paragonimus* spp., *Fasciolopsis buski*, and *Gastrodiscoides hominis*) (Mas-Coma, 2013).

Although almost all neurological fascioliasis patients concerned *F. hepatica*, one was proved to be caused by *F. gigantica* acquired in Cameroon (Paraf et al., 1967). The absence of shoulders in a 2.7 cm long worm found in a 30-year-old woman presenting with neurological manifestations in India suggests that it was a young intermediate fasciolid closer to *F. gigantica* despite having been identified as *F. hepatica* (Vatsal et al., 2006). Thus, causal fasciolid species differentiation may be needed. A recent study was undertaken to validate the identification of *Fasciola* species based on the shape and size of eggs shed by humans. These egg length/width results obtained should

be a useful tool for clinicians (Table 2.5) especially since the application of the classic egg size range in human samples according to traditional literature (books and monographs related to medical parasitology and/or tropical medicine and in guides for clinicians and diagnosis analysts) may lead to erroneous conclusions (Valero et al., 2009a). When measuring the eggs shed by patients, the geographic origin of the patient or the place where the patient was probably infected should be taken into account. Thus, three main world regions may a priori be distinguished (Table 2.5):

- Areas where *F. gigantica* is absent (as in the Americas and Europe).
- Areas where both fasciolid species are present (as in parts of Africa and Asia); the size of eggs shed by humans may be intermediate between the above-mentioned data for *F. hepatica* and *F. gigantica* in humans and such situations may be interpreted as infections by intermediate or hybrid forms.
- Areas where *F. hepatica* is absent (as in parts of Africa).

Future studies in other areas may perhaps widen these ranges for fasciolid eggs shed in human stools, but the exhaustive study performed suggests that only very slight differences will be found, if any (Valero et al., 2009a). An egg length of 140–197 μm was reported for the *F. gigantica* infecting the aforementioned neurological patient from Cameroon (Paraf et al., 1967), which perfectly fit the egg size range for this fasciolid species in areas where *F. hepatica* is absent (Table 2.5).

Egg shedding allows for a quantitative coprological analysis. This is very important in fascioliasis, as the threshold of 400 eggs per gram (epg) of faeces shall be taken into account to establish the treatment dose for the patient. Such a threshold was established to avoid the risk of colics in massively infected subjects and in whom epg counts should be higher (WHO, 2007). In patients shedding more than 400 epg, a repeated, timely spaciated mid-dose instead of a complete one dose of triclabendazole is recommended. Similar dose precaution should be taken when treating the patient with another drug.

In fact, all coprological techniques may be used for egg count if started from a known stool volume. Such techniques have already been used quantitatively in human fascioliasis. Anyway, the cellophane faecal thick-smear technique (Kato or Kato-Katz) appears to be the most appropriate, taking into account the advantages of being rapid and its very low cost, sensitivity, and reproducibility (Esteban et al., 1998; Mas-Coma et al., 1999a). The low sensitivity of this quantitative technique may be solved by applying it with more than one smear for patient or by using it only for quantification, after

Table 2.5 Size (length/width) of eggs of *Fasciola hepatica* and *F. gigantica* in different world regions according to the absence or existence of overlap of the two fasciolid species (intermediate hybrid forms have egg size ranges different from pure species)

Endemic areas	Geographic distribution	*Fasciola hepatica*		*Fasciola gigantica*	
		In humans	In animals	In humans	In animals
Areas where *F. gigantica* is absent	The Americas and Europe	100.6–162.2/ 65.9–104.6 μm	73.8–156.8/ 58.1–98.1 μm	–	–
Areas where both fasciolid species are present	Parts of Africa and Asia	106.5–171.5/ 63.9–95.4 μm	120.6–163.9/ 69.2–93.8 μm	150.9–182.2/ 85.1–106.2 μm	130.3–182.8/ 74.0–123.6 μm
Areas where *F. hepatica* is absent*	Parts of Africa	–	–	137.2–191.1/ 73.5–120.0 μm*	129.6–204.5/ 61.6–112.5 μm

All data from Valero et al. (2009a,b), except values for pure *F. gigantica* infecting humans in areas where *F. hepatica* is lacking (*)

egg detection with a qualitative technique. Another quantitative technique called FLOTAC has also recently proved to be useful for liver fluke egg quantification (Duthaler et al., 2010) and its somewhat more infrastructure demanding performance makes it ideal for hospital work.

In several neurological fascioliasis patients, however, egg finding in faeces was negative even when coprological techniques were repeatedly applied (Aubertin et al., 1966; Coulaud et al., 1970; Lefevre et al., 1970; Lesecq et al., 1972; case 2 of Berenger, 1984). This may be due to one of the following situations: (i) the patient having been diagnosed when still in the acute phase; (ii) the patient harbouring but a very few flukes in the liver shedding eggs in very small quantities and irregularly throughout in such a way that they may be overlooked; and (iii) the patient harbouring liver flukes not producing eggs (Esteban et al., 1998; Mas-Coma et al., 1999a). In these cases, two solutions are recommended: whether apply a *Fasciola*-specific coproantigen test or apply a specific serological test.

At present, coproantigen tests have proved to be very useful for human diagnosis of infection by both *F. hepatica* and *F. gigantica* (Ubeira et al., 2009; Valero et al., 2009b). Moreover, a coproantigen test, when positive, assures the presence of the flukes in the liver, that is, when negative only ectopic forms may be responsible for positivity of the specific serological tests. Additionally, a coproantigen test is more useful for a posttreatment efficacy follow-up, because negativization of a coproantigen test occurs very rapidly, opposite to serological tests due to the long-lasting post-treatment negativization of antibodies. Thus, it has proved to be useful for control in human hyperendemic areas (Valero et al., 2012a). Additionally, a new preservative/diluent, CoproGuard, developed for preservation of *Fasciola* coproantigens, has demonstrated to enhance coproantigen extraction without affecting the detection limit of the assay, the antigenicity of *Fasciola* coproantigens in faecal samples being retained throughout long periods. Thus, the combination of CoproGuard with a coproantigen test becomes a very useful tool for the diagnosis of human fascioliasis (Ubeira et al., 2009).

10.9. Analyses with blood samples

In patients with neurological manifestations who do not shed eggs in their faeces, a serological test may be used for fascioliasis diagnosis. Many different serological tests have been used for human fascioliasis diagnosis since long ago (Esteban et al., 1998; Hillyer, 1999; Mas-Coma et al., 1999a). However,

in the past two decades, efforts have been concentrated in obtaining purified excretory/secretory antigens and/or recombinant molecules to improve the efficiency of serological tests for this disease, owing to the problems of the parasitological diagnosis because of (i) the delay in its usefulness in the acute phase (coprological examination positive only after 3–4 months post-infection); (ii) intermittent egg output dynamics, very low, or even the absence of egg shedding in cases of only one or a few fluke adults and old, chronic infections; (iii) "false" fascioliasis related to eggs in transit after ingestion of infected liver from domestic animals; and (iv) flukes unable to attain maturity in human subjects in nonhuman endemic areas (Mas-Coma et al., 2007).

Ectopic *Fasciola* infections may be added to this list of situations in which a serological test becomes highly recommended. This also refers to patients with neurological symptoms in whom a migrant fluke causing the disorders is initially suspected or liver fascioliasis may be indirectly causing the neurological picture despite the absence of egg shedding in stools. No eggs were noted to be found in patients who were precociously diagnosed and treated (Aubertin et al., 1966; Bernheim et al., 1958). In such cases in which *Fasciola* eggs are not found in patient's faeces, serological tests furnish the most useful tool because of the earliness of their positivity and their high specificity. They may additionally allow for the follow-up of the posttreatment infection evolution, although the relative long period of several months needed for negativization should be considered.

Cysteine proteinases are secreted by the adult and juvenile forms (Dalton et al., 2003; Law et al., 2003) and are highly antigenic in both animals (Cornelissen et al., 2001; Neyra et al., 2002) and humans (Cordova et al., 1997). Several cysteine proteinases offer highly sensitive and specific markers for human fascioliasis serodiagnosis for *F. hepatica* (Cordova et al., 1997, 1999; Espinoza et al., 2007; O'Neill et al., 1998, 1999; Rokni et al., 2002; Sampaio-Silva et al., 1996) and for *F. gigantica* infection (Ikeda, 1998; Intapan et al., 1998, 2004; Maleewong et al., 1999; Tantrawatpan et al., 2005). *F. hepatica* recombinant cysteine proteinases produced in yeast (O'Neill et al., 1999) or in *Escherichia coli* (Carnevale et al., 2001) have been used in ELISA methods for human infection diagnosis, offering results similar to native antigens. Individual evaluation of several of these serological tests with samples from human endemic areas allows for the detection of the advantages and problems each one may pose for one or another diagnostic purpose, infrastructure availability, and epidemiological situation (Espinoza et al., 2007; Valero et al., 2012b).

Among them, a quick field method for the detection of anticathepsin L antibodies in blood samples (Strauss et al., 1999) is worth mentioning because of its simplicity, even despite its somewhat lower sensitivity. Similarly, a new lateral flow test (SeroFluke™) constructed with a recombinant cathepsin L1 from *F. hepatica* (Martinez-Sernandez et al., 2011) should also be highlighted. The latter test is a rapid, simple, and inexpensive immunochromatographic diagnostic method that appears to perform better than other more complex indirect methods, providing similar specificity and higher sensitivity, has the advantage of being applicable to both serum and whole blood samples in the acute and chronic phases, and can be used for individual patient diagnosis in hospitals and for human fascioliasis endemic areas where passive case finding (e.g. Vietnam) and selective treatment (e.g. Egypt) are the control strategies followed within the worldwide initiative of WHO against this disease (WHO, 2008).

11. NEUROLOGICAL AND OPHTHALMOLOGIC FASCIOLIASIS TREATMENT

11.1. Treatment of patients with neurological manifestations

In patients with minor neurological symptoms, nothing should a priori differentiate their treatment from that recommended for other patients showing no neurological manifestations. With regard to patients suffering from major neurological symptoms, except the patients in whom a spontaneous cure occurred without treatment or those cases in which the diagnosis was made during a surgical intervention, all the patients received a specific fasciolicide treatment.

The neurological evolution has been reported to be in the way for cure in given cases, several times in a spontaneous way (Cattan et al., 1953; Dunet, 1924; cases 4 and 12 of Guyot, 1962; Aimard et al., 1984; Aubertin et al., 1966; Ayadi et al., 1991; Domart et al., 1967; Leng-Levy et al., 1965). A transitory spontaneous improvement has been described in several patients (Arias et al., 1986; Bothier et al., 1968; Lunedei and Roselli del Turco, 1934). In another patient, the spontaneous regression of neurological manifestations occurred before any treatment and was followed by a symptom reaccentuation 1 month after the first fasciolicide treatment and by a clear improvement after the second treatment (Coulaud et al., 1970; Saimot et al., 1971).

However, in other cases, patients with major neurological manifestations recovered only after fasciolicide treatment (Bernheim et al., 1958; two cases

of Kouri et al., 1938; Lemoine, 1954; case 2 of Dejean, 1960; Garde et al., 1961; Aguirre Errasti et al., 1981; Auer et al., 1982; Domart et al., 1971; Gil et al., 1970; cases 2 and 3 of Guyot, 1962; Kristoferitsch et al., 1982; Lefevre et al., 1970; Lesecq et al., 1972; Paraf et al., 1967; case 1 of Berenger, 1984; Ait Ali et al., 2002; Ayadi et al., 1991; case 8 of Campo et al., 1984a; Frances et al., 1994; Linares et al., 2006; Llanos et al., 2006; Málaga et al., 2012; Oujamaa et al., 2003; Park and Sohn, 2010; Pelletier et al., 1995).

Whereas an anti-*Fasciola* treatment keeps essential, anti-inflammatory therapeutics with corticosteroids have also been usually applied as adjuvants to the antiparasitic treatment of neurological patients (Domart et al., 1967, 1971; Oujamaa et al., 2003; Paraf et al., 1967; Park and Sohn, 2010) and also patients suffering from concomitant minor neurological symptoms and heart affection (Arlet et al., 1966). A systematic use of such an accompanying anti-inflammatory treatment appears logical, given the allergic part of the aforementioned suspected physiopathologic process. Thus, corticotherapy (usually by prednisone) allowed for a regression of clinical and biological symptoms, such as disappearance of eosinophilia in 3 days and sedimentation time returning to normal values (Coulaud et al., 1970; Saimot et al., 1971). An anti-inflammatory treatment was applied in one patient with tetracosactide (Synacthene®) before the fascioliasis diagnostic was reached and a pronounced improvement of the patient was obtained (Berenger, 1984). Curative doses of anticoagulant by K antivitamin and antiplatelet drug (lysine acetylsalicylate) were administered to a 40-year-old woman showing thrombotic symptoms (cutaneous, cerebral, and cardiac) associated with hepatic fascioliasis and with the development of an antiphospholipid syndrome (Frances et al., 1994; Pelletier et al., 1995). Antiepileptic drugs have also been sporadically applied with satisfactory results on the neurological and ocular manifestations (Oujamaa et al., 2003).

11.2. Antiparasitic drugs used in neurological patients

For the treatment of human fascioliasis, drug preferences have been changing throughout time. Dehydroemetine (2-dehydroemetine) was considered the therapy of choice a few decades ago, including the 1950–1980 period in which most of the neurological fascioliasis case reports were published. Its dosage included 1–2 mg/kg/day in treatments of 10–20 days separated by 15 days, administered by subcutaneous way in two doses (or *per os*). Its additional high efficacy against fasciolid juveniles during the invasion period should be highlighted and was crucial for (i) a treatment giving rise to cure

without sequelae and (ii) for patients suffering from neurological manifestations that in many cases appear whether as the first detectable symptoms or at the beginning of the clinical picture. Most of the patients showing major neurological manifestations were treated with emetine (Domart et al., 1971; case 2 of Dejean, 1960; Garde et al., 1961; Bernheim et al., 1958; cases 2 and 3 of Guyot, 1962; Lefevre et al., 1970; Lesecq et al., 1972; case 1 of Berenger, 1984). The outcome of a treatment with dehydroemetine was usually a very evident symptomatologic and biological improvement of the neurological patient, although patients in whom dehydroemetine failed and moreover was badly tolerated have also been reported (Schussele and Laperrouza, 1971a,b).

However, emetine posed several problems, such as its extremely painful administration sometimes reported; the need to frequently apply two or three treatment courses (Leng-Levy et al., 1965; Paraf et al., 1967), sometimes four (Dejean, 1960), or even the need of subsequent treatments with other drugs after or in between the two or three emetine courses (Berenger, 1984; Guyot, 1962); its long treatment course; and mainly the variety of toxic manifestations, involving the heart, liver, and digestive tract. Its cardiac toxicity may even impose an electrocardiographic follow-up.

As an alternative to avoid emetine toxicity, a few patients showing neurological manifestations were treated with Entobex® (4,7-phenantroline-5,6-quinone = phanquinone), administered *per os* at a dose of 8–10 pills of 50 mg per day during 10 days (Bothier et al., 1968; Saimot et al., 1970), sometimes after emetine treatment or in between different emetine courses (Berenger, 1984; Domart et al., 1971; Guyot, 1962). This drug was known because of its efficacy at the biliary invasion phase, its rapid biliary elimination, and its well tolerability, although its failure in given patients was noted already from the beginning (Coudert and Garin, 1959). Entobex® treatment may give rise to nauseas and vomiting, which are controlled by parasympathicolytics. Its application in neurological patients was useless in several cases (Becquet and Delassus, 1961; Bothier et al., 1968; Schussele and Laperrouza, 1971a,b).

The aforementioned problems caused by emetine treatment led Bithionol® [2,2-thiobis-(4,6-dichlorophenol)] to take this place of drug of choice for human fascioliasis during years. This drug was used at the dosage of 30 mg/kg *per os* during 10 days. Bithionol® furnished excellent results regarding adult liver flukes (Bacq et al., 1991; Bassiouny et al., 1991; Lienert, 1962; Yoshida et al., 1974) and was also successfully used in patients presenting with neurological manifestations (Anton Aranada et al., 1985;

Arjona et al., 1995; Coral et al., 2007). Side effects such as vomiting and epigastric cramps may oblige treatment interruption (Schussele and Laperrouza, 1971a,b).

With regard to praziquantel (Biltricide®), a drug useful and highly effective against all trematodiases, it should be highlighted that *Fasciola* species are a surprising exception. *F. hepatica* and *F. gigantica* are the only flukes not responding to praziquantel treatment. In neurological fascioliasis patients, the uselessness of praziquantel in biological or clinical improvement has also been emphasized in cases of both minor symptoms (Patrick and Isaac-Renton, 1992) and major symptoms (Berenger, 1984), although surprisingly, it was mentioned to be applied with effective response in other such patients (Ait Ali et al., 2002; Ayadi et al., 1991; Nuñez Fernández et al., 2001).

The lack of consensus about the therapy of choice for human fascioliasis finished when it was proved that appropriately dosified triclabendazole, a benzimidazole derivative [6-chloro-5-(2,3-dichlorophenoxy)-2-methylthiobenzimidazole], was highly efficient in humans (Mas-Coma et al., 1999a). Triclabendazole for human use (Egaten®) is at present the drug of choice for human fascioliasis caused by both *F. hepatica* and *F. gigantica* (Keiser et al., 2005; Savioli et al., 1999) and the global human fascioliasis control strategy of the WHO is relying on this drug (WHO, 2007, 2008).

Triclabendazole is active during both the invading phase (acute phase) (Picot et al., 1992) and the chronic phase of fascioliasis (Brennan et al., 2007), which for neurological ectopic *Fasciola* infection appears to be of great value when considering the neurological fascioliasis cases reported in both very early invasion phase (in fact, neurological symptoms being the first to appear) and chronic phase. Several reports already refer to its effectiveness in treating neurological patients (Caturelli et al., 2008; Linares et al., 2006; Llanos et al., 2006; Málaga et al., 2012), including even up to resolution of previous multiple cerebral lesions (Park and Sohn, 2010).

Triclabendazole is better adsorbed if administered after meals (Lecaillon et al., 1998). The recommended dosage is two separate regimens of 10 mg/kg. A cure rate of 79.2% when first used and 100% after a second round of therapy was found in Chile (Apt et al., 1995) and 79.4% and 93.9%, respectively, in Egypt (El-Morshedy et al., 1999). Triclabendazole appears to keep its efficiency in human endemic areas after years (Talaie et al., 2004). However, the side effect of a transient biliary obstruction syndrome linked to the expulsion of the dead flukes, around 3–7 days after the drug administration (Millan et al., 2000), should be considered. Such a

situation seems to be more frequent if triclabendazole is readministered each 48 h or in cases of double dose. Thus, triclabendazole administration should be initially recommended at a dose of 10 mg/kg. Additionally, a total of 8% Cuban patients, with *F. hepatica* infection refractory to previous chemotherapy with other antihelminthics, were still shedding eggs in faeces on day 60 posttherapy with two doses of 10 mg/kg administered after food intake 12 h apart, although all these failure cases responded to a third triclabendazole dose (Millan et al., 2000).

In that sense, the extent of triclabendazole resistance in animals in different countries should be taken into account: first in Australia, later in Europe, and more recently also in South America (see review in Zumaquero-Ríos et al., 2013). During several years, triclabendazole resistance only concerned livestock in animal endemic areas. However, unfortunately resistance to triclabendazole has also been recently detected in the area of Neuquén, Argentina (Olaechea et al., 2011), where human infection has already been reported twice (Mera y Sierra et al., 2011), and more recently even described in infected humans (Ortiz et al., 2013) in a human fascioliasis hyperendemic area such as the Andean valley of Cajamarca, Peru (Gonzalez et al., 2011). Strategies to minimize the development of resistance in animals include the use of synergistic drug combinations (Fairweather and Boray, 1999), although this approach has the risk of building up multiple drug resistance. Additionally, studies suggest that our understanding of the mechanism of resistance to triclabendazole remains far from complete (Brennan et al., 2007; Fairweather, 2005, 2009), so that there is even a knowledge gap regarding its spreading capacity.

Nitazoxanide (2-acetyloxy-*N*-(5-nitro-2-thiazolyl) benzamide; commercialized under different registered marks such as Colufase®, Daxon®, or Paramix®) is a drug with reported efficacy on a broad parasitological spectrum, such as intestinal protozoans and helminths. Nitazoxanide had demonstrated its efficacy against human fascioliasis in a few trials, in Egypt (Kabil et al., 2000; Rossignol et al., 1998) and Peru (Favennec et al., 2003). It appears to be the only alternative drug for human fascioliasis at present, mainly in those countries where triclabendazole is still not registered but nitazoxanide is since several years, as it is the case of Mexico (Zumaquero-Ríos et al., 2013). Its usefulness for the treatment of human cases not responding to triclabendazole (Gargala et al., 2005) is of important additional value, given the spread of the resistance to this drug in liver flukes. Its long treatment course for fascioliasis, including 500 mg each 12 h during 7 days, makes its application somewhat difficult, mainly in remote rural

areas. However, differences in fasciolid susceptibility to nitazoxanide may exist depending on geographic strains. Thus, no response to nitazoxanide treatment was reported in 24 cases of liver fluke infection in Esmeralda, Camagüey, Cuba (Del Risco Barrios et al., 2001), and a triclabendazole-resistant *F. hepatica*-infected patient not responding to nitazoxanide treatment has recently been reported in the Netherlands (Winkelhagen et al., 2012). The usefulness of nitazoxanide for the treatment of fascioliasis patients with neurological affection has not yet been verified.

Other drugs recently proposed as fasciolicides for human treatment proved unfortunately to be of insufficient efficacy, such as Mirazid®, a drug prepared commercially from myrrh (an oleo–gum–resin obtained from thorny trees *Commiphora molmol* and other species of the family Burseraceae) (Botros et al., 2009) and artemether (Keiser et al., 2011).

11.3. Prognosis, sequelae, and fatal cases

The prognosis of fascioliasis depends from the promptness of the treatment. At the phase of hepatic invasion (acute phase), the treatment most usually gives rise to cure without sequelae. However, when treated late, the prognosis becomes subordinated to the importance of the affection of the biliary ducts.

With regard to cure criteria, from the clinical point of view, the improvement of the general condition of the patient with fever disappearance and weight recovery indicates a good prognosis, although one should be cautious as relapses are possible. The normalization of blood eosinophilia as best criterion in all cases, the disappearance of eggs in stools in cases of patients diagnosed in the biliary phase, and the progressive disappearance of hepatic function test abnormalities are helpful indicators of treatment effectivity.

In most of the fascioliasis patients suffering from major neurological and ocular manifestations, reports referred to a complete fascioliasis cure and recovery from the disorders. Anyway, the regression of the neurological manifestations, and also the normalization of EEG, may sometimes be slow (Gil et al., 1970; Lefevre et al, 1970).

However, the seriousness of fascioliasis was emphasized in many cases, with regard to the progressive general deterioration of the patients with slimming, anorexia, and physical and neuropsychic asthenia and the fact that cure does not mean total recovery but that fascioliasis left them as handicapped and frail subjects (Becquet and Delassus, 1961). In several patients presenting

neurological and ocular manifestations, the given sequelae were noted to persist despite treatment:

- Only light improvement of neurological manifestations after symptomatologic treatment, although a specific fasciolicide was not administered (Mendoza, 1922).
- Paraesthesia of upper left limb (Lunedei and Roselli del Turco, 1934).
- Paleness, insomnia, and dyspnoea when hiking (Dejean, 1960).
- Right eye amaurosis linked to ophthalmoneuritis, partial dysgeusia and anosmia, painful lower limbs, and minor functional impotence (Becquet and Delassus, 1961).
- Left facial paralysis of peripheral type and subnormal EEG (Garde et al., 1961).
- Subnormal EEG (Aubertin et al., 1966).
- Left hyperreflexivity (Domart et al., 1967).
- Bilateral pyramidal syndrome, with hyperreflexivity, outline of Babinski's sign, light dissymmetry, and evident spasm signs (Bothier et al., 1968).
- Very long duration of neurological disorders and imperfect regression 8 months after appearance despite two fasciolicide treatments, including persistence of finger articulation pain, phalange aspect modification, electrocardiac signs, and visual disorders linked to the abducens cranial nerve VI (Coulaud et al., 1970; Saimot et al., 1971).
- Right Achilles' tendon reflex abolished (Lesecq et al., 1972).
- Right hemiplegia only markedly improved (Ragab and Farag, 1978).
- Motor deficit in the right upper limb, with hyperreflexivity (Campo et al., 1984a,b).
- Right hemiparesis and bilateral pyramidal syndrome (Berenger, 1984).
- Permanent right eye amaurosis (Arias et al., 1986).
- Cortical and subcortical nodular lesions with MRI hypersignal and lower limb livedo spread throughout abdomen, back, and upper limbs (Frances et al., 1994; Pelletier et al., 1995).

These sequelae were sometimes important (Bothier et al., 1968; case 2 of Berenger, 1984), even making difficult or impeding the professional activity of the patient, as it was the case of a patient keeping nervous sequelae translated into visual disorders and a right upper limb ungainliness (Coulaud et al., 1970; Saimot et al., 1971). It should be stressed that limb sequelae sometimes remain when the picture was markedly close to a pyramidal syndrome of the four limbs. With regard to the neurological sequelae observed in a patient in whom sequelae of the kind of amaurosis and muscular deficiency of lower

limbs were found (Becquet and Delassus, 1961), reserves were initially noted concerning the possible responsibility of the therapeutics used in that case (Domart et al., 1967), although similar sequelae were later found in other patients treated according to standards.

A unfavourable negative evolution towards the death of the patient has anyway been noted in several cases. Six *Fasciola* specimens were found in the autopsy of an 8-year-old girl died showing convulsions in Italy (Frank, 1823 in Bürgi, 1936). A Swiss soldier fallen ill in Sumatra was transferred to Switzerland where he died presenting with delirium; one *Fasciola* in the main hepatic duct and a widely affected liver were found at autopsy (Biermer, 1863 in Bürgi, 1936). A total of 26 fasciolas were found in the hepatic ducts at the autopsy of a 52-year-old farmer who died presenting with deliriums after only two months of symptoms in UK (Humble und Lush, 1881 in Bürgi, 1936). A 53-year-old man died after 12 years of symptoms, depression, and suicide thoughts accompanying hepatic and digestive system disorders; only two fasciolas were found at autopsy despite the typical dilatation and thickening of biliary ducts and hepatomegaly in Switzerland (Blanchod, 1909 in Bürgi, 1936). A young woman 25 years of age died after 3 years of illness and 1 year after admission showing articular pain and tetanic contractions in France (Mauriac, 1922 in Bürgi, 1936). A 50-year-old man suffering from persistent, violent cephalalgias appeared 11 years postinfection died in Algeria after a symptomatology suggesting 13 years of liver fluke affection (Desage, 1926). A 4-year-old boy, who had problems in walking and right limb, died after a 6-month illness in Austria (Paul, 1927). A 36-year-old female patient reported as an acute case of melancholia died in an hospital of United Kingdom after 5 months of unchanged mental condition until retropharyngeal abscess that proved fatal. A marked degree of biliary cirrhosis and a dozen fasciolid flukes were found in the biliary system at autopsy (Biggart, 1937). At autopsy, a total of 11 adult flukes were found in the duodenum of an hospital patient who died due to cerebral abscess in China (Pan and Huang, 1954). A 4-year-old child presenting almost complete loss of vision and bad general conditions died in Guatemala. Cerebral microgliomatosis, severe cerebral oedema, hepatic fibrosis, and two *F. hepatica* specimens in the liver were found at autopsy (Aguilar et al., 1967, 1968). An Argentinean patient died 2 days after the surgical intervention, autopsy showing numerous *Fasciola* eggs in intracerebral cysts (Correa et al., 1969; Ruggieri et al., 1967). Another French patient was noted to have died due to a complication during the resuscitation process (Berenger, 1984), although the detection of

meningeal haemorrhages remembering other fatal cases due to intracranial trematode infection (Africa et al., 1936) poses doubts about the aforementioned interpretation.

Finally, given the possibility of an immunologic background within these neurological forms, the importance of prophylactic measures to avoid the reinfection of fascioliasis patients seems crucial, mainly concerning the vegetables most incriminated in the transmission of the disease and on all other aforementioned human infection sources (Mas-Coma, 2004), mainly in high infection risk areas known to be human endemic and in areas where fascioliasis widely affects livestock.

11.4. Treatment of patients with ophthalmologic manifestations

Patient's cure has been verified in cases that were solved by surgery without any antiparasitic treatment (Cho et al., 1994; Dalimi and Jabarvand, 2005). In other cases showing neurological manifestations, the cure of the patients in whom the fluke was recovered from the eye has been reported to be obtained by praziquantel treatment (Cheng et al., 2007; Ying et al., 2007; Zhou et al., 2008). However, in the latter cases, patient's recovery was most probably due to the extraction of the only infecting worm than to the drug therapy.

A case of a patient keeping nervous sequelae translated into visual disorders after treatments with dehydroemetine and Entobex® was reported. These important sequelae impeded the professional activity of the patient (Saimot et al., 1970).

12. CONCLUDING REMARKS

Data indicate that fascioliasis patients with neurological and ocular manifestations have been reported from so much countries and continents (Table 2.1) that it would be better to henceforth consider these complications within the general clinical frame of this trematode disease.

Almost all of the reports analysed (Table 2.1) concern patients in whom fascioliasis was diagnosed in a well-equipped hospital, usually of a large city. One unavoidably inquires which may be the situation of fascioliasis affected people in human endemic areas as those known in Latin America, Africa, and Asia (Mas-Coma et al., 1999a, 2009a), where prevalences and intensities reach high levels in humans, mainly children (Esteban et al., 1997a,b, 2002, 2003; Gonzalez et al., 2011; Mas-Coma et al., 1999a). The very low number of reports on neurological cases in countries including human fascioliasis

endemic areas, for instance, Bolivia, Peru, Egypt, or Vietnam, should be highlighted. Reports of patients having been diagnosed in developed countries but infected in developing countries or areas (Auer et al., 1982; Bürgi, 1936; Frances et al., 1994; Kristoferitsch et al., 1982; Paraf et al., 1967; Pelletier et al., 1995) clearly indicate that neurological cases are overlooked in human fascioliasis endemic areas and also in developing countries in general. On the other side, the reference to a case with a cerebral abscess in Australia (Prociv et al., 1992), a country where human fascioliasis cases have always proved to be sporadic (Mas-Coma et al., 1999a), makes one think.

In these human endemic areas of developing countries, most of the subjects keep their infection for a long time because they do not attend a health centre for diagnosis. Hence, acute lesions superimposed on chronic disease should be relatively frequent due to the high reinfection risk in these high transmission areas (Valero et al., 2003). With regard to these repetitive reinfections, one wonders on whether a previous sensitization of the subjects may stimulate ectopic migration of new-entering juvenile forms, when not a more pathogenic development of the disease caused by reinfecting flukes and higher burdens, involving effects on the central nervous system. In such remote zones, rural health centres and small hospitals in or near the human endemic areas do not dispose of the appropriate equipments for neurological analyses. Moreover, physicians may not be aware about the potential relationship between liver fluke infection and neurological implications, and such cases may therefore remain misdiagnosed.

In developing countries, moreover, the usual coinfections by *Fasciola* and other helminths in the same patient (Esteban et al., 1997a,b, 2002, 2003; Gonzalez et al., 2011; Mas-Coma, 2004; Mas-Coma et al., 2005; Zumaquero-Ríos et al., 2013) pose a serious additional problem, as blood eosinophilia loses its value as main decisive specific element guiding towards the correct fascioliasis diagnosis. A Cameroonese case is a good example of the long-term problems posed by such a coinfection in a neurological patient (Paraf et al., 1967).

Globally, neurological complications of fascioliasis should be given an importance higher than previously assumed. All data suggest that such complications did not receive the appropriate focus in the past two decades. Their repercussions on the health of the patient are evident. Hence, priority should henceforth be given to their consideration in human endemic areas, and efforts should be implemented to assess their characteristics and frequency.

Their impact should also be considered when estimating the global burden of fascioliasis by calculating the disability-adjusted life years (DALYs) for

this disease or any other estimation way (Payne et al., 2009). Decisive aspects include (i) the presumably higher prevalence of such cases worldwide; (ii) the need for long-term hospitalization, of several months or even years, sometimes with disease processes requiring hospital readmissions of the same patient; (iii) the disconcerting evolution of the disease requesting the intervention of physicians of different specialities and the patient going through different speciality services; (iv) the application of neuroimaging techniques not affordable for rural areas; (v) the need for a transfer from a rural area to a urban hospital or even from one country to another better equipped one and with more expertise; (vi) sometimes the need for different repeated treatment courses with combination of different drugs, when not surgery; (vii) the many sequelae reported, several as important as to impede professional activities or leaving handicapped patients behind; and (viii) the high mortality in nontreated patients and its consequences in human endemic areas of developing countries and the fatal cases reported even in treated ones. Unfortunately, when recently analysing fascioliasis among the estimations of the global burden of food-borne trematodiases (Fürst et al., 2012), neurological, meningeal, psychiatric, and ocular infections were not considered.

All in all, the conclusions obtained about the neurological and ocular pathogenicity and impact of fascioliasis emphasize the importance of the control efforts launched by the WHO in human fascioliasis endemic areas. Adequately scheduled mass treatments in hyperendemic areas of remote poor rural areas lacking medical infrastructures for the care of such patients will contribute to the decrease of these neurological and ocular affections.

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CHAPTER THREE

Measuring Changes in *Plasmodium falciparum* Transmission: Precision, Accuracy and Costs of Metrics

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Contents

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Abstract

As malaria declines in parts of Africa and elsewhere, and as more countries move towards elimination, it is necessary to robustly evaluate the effect of interventions and control programmes on malaria transmission. To help guide the appropriate design of trials to evaluate transmission-reducing interventions, we review 11 metrics of malaria transmission, discussing their accuracy, precision, collection methods and costs and presenting an overall critique. We also review the nonlinear scaling relationships between five metrics of malaria transmission: the entomological inoculation rate, force of infection, sporozoite rate, parasite rate and the basic reproductive number, R_0. Our chapter highlights that while the entomological inoculation rate is widely considered the gold standard metric of malaria transmission and may be necessary for measuring changes in transmission in highly endemic areas, it has limited precision and accuracy and more standardised methods for its collection are required. In areas of low transmission, parasite rate, seroconversion rates and molecular metrics including MOI and mFOI may be most appropriate. When assessing a specific intervention, the most relevant effects will be detected by examining the metrics most directly affected by that intervention. Future work should aim to better quantify the precision and accuracy of malaria metrics and to improve methods for their collection.

1. INTRODUCTION

Changes in malaria transmission must be measured accurately and precisely in order to evaluate the impact and cost-effectiveness of new and existing interventions. As malaria transmission declines across much of sub-Saharan Africa, there has been renewed focus on the need to codify a set of metrics, expectations about likely changes in those metrics across the spectrum of transmission in response to control, defined end points for measuring changes in the intensity of transmission and the associated reductions in malaria burden (Cohen et al., 2010; Corran et al., 2007; Hay et al., 2008; Smith and Hay, 2009; Steketee et al., 2010), with a concurrent increase in funding directed towards improving capacity for monitoring and evaluation (Cibulskis et al., 2007; Nahlen and Low-Beer, 2007).

Malaria transmission, defined herein as the process by which a malaria parasite completes its life cycle, involves parasites being passed from a female anopheline mosquito through the skin, during a blood meal, and via the liver into human blood, and later from the blood back into the mosquito during a subsequent mosquito blood meal, leading to parasite development within a mosquito. The intensity of transmission, described by Macdonald (Macdonald, 1957; Smith et al., 2012), is a general concept describing the

potential frequency of transmission, but it may also be defined as the number of times each day that a parasite infection is initiated in a human or the number of times a pathogen infection is initiated in a mosquito. Transmission intensity varies enormously within malaria-endemic areas and is determined not only by the vectorial capacity of local mosquito populations but also by other factors, including human immunity and the interventions in place (Smith et al., 2010). Transmission is intrinsically 'noisy' due to fluctuations in underlying mosquito populations, temperature-induced changes in mosquito interactions with the parasite, immunologic changes affecting human–parasite interactions and the spatial heterogeneity over which these occur. There is also variation in the efficiency of transmission, the number of uniquely identifiable infections caused by each infectious bite, which is affected by heterogeneous biting, multiple infections and acquired immunity (Smith et al., 2010). Spatiotemporal variability in the quantities of interest raises questions about the precision and accuracy of these metrics that must be understood to interpret these parameters properly and to measure changes over time.

Malaria control interventions slow transmission at specific points during the complex parasite life cycle, and likewise, there are several points during this cycle at which the intensity of transmission may be measured, using various metrics pertaining to the three players: mosquitoes, parasites and humans (Carter and Mendis, 2006; Hay et al., 2008). Each metric represents a quantity that is an important step in the transmission process, as illustrated in Fig. 3.1.

Metrics of malaria transmission change on different temporal scales, reflecting the dynamics of mosquito populations, parasite infections in humans, the kinetics of changing human immunity and human demographics. The metrics are causally interrelated (Fig. 3.1), but based on both *a priori* arguments and *a posteriori* examinations of patterns, some of these relationships are nonlinear when considered across the spectrum of transmission intensity (Smith et al., 2010). These nonlinearities, together with variability in transmission and measurement errors, weaken the associations between those metrics separated by a greater number of steps in the transmission cycle. The most substantiated and relevant effects on transmission are found by examining the metric that is most directly affected by an intervention, for example, the biological efficacy of a transmission-blocking vaccine is best assessed directly by measuring κ (Fig. 3.1). However, when it is not possible to measure an effect directly, the study should follow the chain of causation and examine the nearest attainable downstream metric. Generally, the end points of greatest interest are the direct outcomes of human infections:

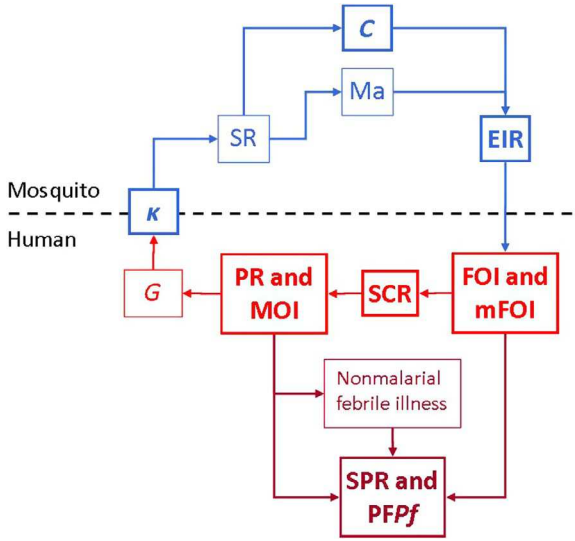

Figure 3.1 Metrics of malaria transmission. Metrics evaluated in this chapter are in bold. Blue indicates entomological metrics; red indicates clinical metrics; dark red indicates asymptomatic and symptomatic infections identified in health facilities. κ: net infectiousness of humans; SR: sporozoite rate; *C*: vectorial capacity; *Ma*: human biting rate; EIR: entomological inoculation rate; FOI: force of infection; mFOI: molecular force of infection; MOI: multiplicity of infection; SCR: seroconversion rate; PR: parasite rate; *G*: gametocyte rate; SPR: slide positivity rate; PF*Pf*: proportion of fevers parasitaemic. Parasite rate (PR) is the proportion of the proportion of people who are infected with parasites and the gametocyte prevalence is the proportion of people carrying gametocytes in their blood. The human biting rate is the number of bites by vector mosquitoes received per human per day, denoted *Ma*, and some portion of mosquitoes biting infectious humans become infected. Since gametocytes must be present for a mosquito to become infected, gametocyte rates give an index of the net infectiousness of the human populations to mosquitoes, which is defined as the probability that a mosquito becomes infected after biting a human, denoted κ. Thereafter, each mosquito gives some number of infectious bites. The average number of human blood meals taken by a mosquito over a lifetime has been called the stability index, *S*, and the proportion of infected mosquitoes that survive long enough to transmit, *P*. The sporozoite rate, SR, in a stable population is related to κ by a formula $SR = (SP\kappa/1 + S\kappa) \approx SP\kappa$. EIR is the expected number of infectious bites per person per day, a product of SR and *Ma* (Onori and Grab, 1980b). Vectorial capacity, *C*, describes the relationship between κ and EIR and reflects the efficiency of the malaria vector, or 'the expected number of humans infected per infected human, per day, assuming perfect transmission efficiency' (Smith and McKenzie, 2004). The *t*-day attack rate, denoted $A(t)$, is the proportion of people who become infected over some interval of time of length *t*. This is the typical metric used to count human infections. The annual force of infection (aFOI) is the number of infections per person per year. In a population with homogenous risk, the attack rate is related to the force of infection by the relationship $A(t) = 1 - e^{-ht}$. Two measures

(Continued)

infection *per se*, clinical malaria, hospitalisation and death. However, the relationships between these clinical metrics and transmission are complex and are among the most difficult to measure (Ghani et al., 2009; Trape and Rogier, 1996).

The future need to approve new interventions and to evaluate existing strategies aimed at reducing transmission highlights the specific requirement for robust methods to measure a change in transmission (invariably a decrease) and the need to account for nonlinear patterns and expectations between metrics when interpreting data from intervention studies. To help guide the appropriate design of future trials seeking to evaluate transmission-reducing interventions, we first critically evaluate the precision, accuracy and costs of the metrics that have been developed to measure the transmission of falciparum malaria. To our knowledge, this is the first comprehensive review of these attributes. Second, we review the nonlinear scaling relationships between five major metrics of malaria transmission: the entomological inoculation rate (EIR), force of infection (FOI), sporozoite rate (SR), parasite rate in humans (PR) and the basic reproductive number, R_0.

2. ACCURACY, PRECISION AND COSTS OF MALARIA METRICS

The suitability of malaria transmission metrics as end points for measuring changes in transmission is determined by costs, precision, accuracy, the need for and availability of experts, the intrinsic variability of the metric across space and time and overall familiarity with the metric because of common use. In this chapter, we review 11 metrics of transmission: (1) net infectiousness of humans, (2) PR in humans, (3) EIR, (4) FOI and molecular force of infection (mFOI), (5) multiplicity of infection (MOI), (6) seroconversion rate (SCR), (7) slide or clinical positivity rate (SPR or CPR), (8) incidence of clinical malaria or annual parasite index (API), (9) proportion of fevers with *P. falciparum* parasitaemia (Pf*Pf*), (10) vectorial capacity and (11) basic reproduction number. In addition to giving a general description

Figure 3.1—Cont'd that are closely related to the AR and the FOI are the clinical attack rate (cAR) and the clinical force of infection (cFOI), which are defined in the same way as their respective clinical measures, but they are accompanied by clinical symptoms. The seroconversion rate describes the rate at which a population develops detectable malaria antibodies in the serum as a result of malaria infection.

of each metric, we also assess the (a) methods for collection and the (b) accuracy, (c) precision and (d) costs of collection and give an overall critique. The findings of the chapter are summarised in Table 3.1.

Accuracy is defined as the closeness of measurements of a quantity to the true value of that quantity, while precision is the degree to which repeat measurements under the same conditions give the same results. When metrics are used to measure changes in some metric, practical consideration must be given to statistical power—the sample sizes required to obtain the required degree of precision. Precision can be improved by increasing sampling effort, and the sample sizes must be sufficiently large to determine whether a change is statistically significant. This remains true even if the parameter is inaccurate. Further thought is required to determine what a change in the value of a metric means. Many factors can affect the accuracy of these metrics independently of their precision, such that the validity of any measured change must be scrutinised as it may or may not reflect a true change. A persistent issue for interpreting a change in the value of a malaria metric is the source of bias and whether that bias affects the estimates of the metric in the same way in, for example, pre- and postintervention estimates. To put it in other terms, a biased metric may be useful if it is consistent, even if it is inaccurate. Because most metrics are intrinsically biased, as we discuss later, it may be appropriate to utilise consistent and precise metrics as a way of measuring the magnitude of change. Both accuracy and precision are therefore critical considerations when choosing outcomes for the evaluation of interventions and when drawing inferences about changes in malaria transmission using a given metric.

2.1. Net infectiousness of humans to mosquitoes (κ)

The most direct assessments of malaria interventions that aim to reduce malaria transmission measure a change in the net infectiousness of humans to mosquitoes, known as κ and defined as the proportion of mosquitoes that become infected after feeding on humans. The net infectiousness of humans to mosquitoes is affected by processes acting in both humans and mosquitoes. In humans, fluctuations in gametocyte density, naturally acquired transmission-blocking immunity and the efficiency with which the gametocytes are taken up by mosquitoes in a blood meal relative to their measured density in blood play a role in determining κ. In mosquitoes, the effective contact rate with humans and factors influencing the susceptibility of mosquitoes to malaria infection determines κ. The susceptibility of anophelines

Table 3.1 Summary of sampling issues, accuracy and precision of major malaria transmission metrics

	Sampling issues	Accuracy	Precision
Net infectiousness of humans to mosquitoes (κ)	• Feeding assays: restriction to patent gametocyte carriers leads to different answers than xenodiagnostic studies • Wild-caught vector infection rates: sampling mosquitoes will be affected by natural variations in mosquito populations	• Poor association with transmission intensity • Mosquito-feeding assays need to take into account the likelihood of being bitten • Skin-feeding assays may result in higher infection rates than membrane-feeding assays[a] • Susceptibility of mosquito colony may differ from wild-caught mosquitoes	• Affected by seasonality, natural variation in mosquito populations and frequency of sampling • Mosquito-feeding assays have unknown precision
Parasite rate in humans (PR)	• Age groups for sampling affect estimates • Seasonal patterns affect outcomes • Convenience sampling leads to selection bias with plausibly more parasite-positive individuals (cluster sampling approach)	• Substantial proportion of infections will be missed by microscopy and RDTs • PR depends on season and age groups	• Standardised sampling approach in combination with high-quality microscopy methodology will allow good precision • Consistency in methodology is required
Entomological inoculation rate (EIR)	• Seasonal variation • Convenience sampling	• Relative contribution of outdoor biting to transmission	• *Ma* difficult to measure precisely due to spatial,

Continued

Table 3.1 Summary of sampling issues, accuracy and precision of major malaria transmission metrics—cont'd

	Sampling issues	Accuracy	Precision
	(selecting high-burden households) may bias estimates	poorly characterised • Variation in procedures to sample mosquitoes • Inconsistencies in protocols for the same procedures • Heterogeneous biting limits accuracy at high transmission intensity	temporal and seasonal variability in vector density • SR affected by initial infectiousness and average age of adult mosquitoes
Force of infection (FOI), molecular force of infection (mFOI)	• Age groups for sampling affect estimates • Seasonal patterns affect outcomes • Convenience sampling leads to selection bias with plausibly more parasite-positive individuals (cluster sampling approach)	• FOI, but probably not mFOI, will saturate at a certain transmission intensity • Strong association between mFOI and seasonality, age and ITN use indicates relatively high accuracy	• Variation in sample quality and extraction efficiency may result in fluctuations between surveys[b] • Fluctuation in parasite densities below detection thresholds limits precision
Multiplicity of infection (MOI)	• Age groups for sampling affect estimates • Seasonal patterns affect outcomes • Convenience sampling leads to selection bias	• Substantial number of clones may be missed by PCR if sampling is restricted to 1 day[c] • Accuracy limited by diversity	• Variation in sample quality/extraction efficiency that may result in fluctuations between surveys[b]

Table 3.1 Summary of sampling issues, accuracy and precision of major malaria transmission metrics—cont'd

	Sampling issues	Accuracy	Precision
	with plausibly more parasite-positive individuals (cluster sampling approach)	of parasite clones	
Seroconversion rate (SCR)	• Age groups for sampling affect estimates • Seasonal patterns affect outcomes • Convenience sampling leads to selection bias with plausibly more parasite-positive individuals (cluster sampling approach)	• Short-lived and long-lived responses will affect accuracy • Limitations in detecting small changes in transmission intensity	• Standardised procedures and positive controls make precision of estimates reasonably high
Clinical surveillance: slide positivity rate (SPR), incidence of clinical malaria and proportion of fevers with *P. falciparum* parasitaemia (PF*Pf*)	• Attendance to health facilities varies • Variations in clinical decision making[d]	• Clinic attendance to health facilities may be suboptimal and vary between times and sites • Saturation of incidence at high transmission intensities • SPR and PF*Pf* affected by incidence of other febrile illness	• Depends on consistency in diagnostic practices and methodology
Vectorial capacity (C) and the basic	• C and R_0 difficult to measure directly	• C and R_0 only as accurate as	• Precision affected by natural

Continued

Table 3.1 Summary of sampling issues, accuracy and precision of major malaria transmission metrics—cont'd

	Sampling issues	Accuracy	Precision
reproduction number (R_0)	• Convenience entomological sampling (selecting high-burden households) may bias estimates of C	their constituent components • Relative contribution of outdoor biting to transmission poorly characterised • Variation in procedures to sample mosquitoes	fluctuations in vector densities, biting patterns and variation in performance of trapping methods • When measuring R_0, heterogeneous biting must be accounted for

[a]Bousema et al. (2012).
[b]Baidjoe et al. (2013).
[c]Koepfli et al. (2011).
[d]Bastiens et al. (2011).

to malaria infection differs between mosquito strains as a result of differences in mosquito innate immunity (Blandin et al., 2004; Molina-Cruz et al., 2012; Smith et al., 2004; Trape et al., 1987) and midgut microbiota (Cirimotich et al., 2011). There may also be differences in malaria susceptibility of different molecular forms of *Anopheles gambiae* and *A. arabiensis*. Until the importance of these phenomena in determining transmission efficiency is established, a general estimate of κ is deemed appropriate.

The infectiousness of individual humans can be measured directly using mosquito-feeding assays, but net infectiousness of a human population must be estimated from field data because it is mediated by the complex biting patterns of mosquitoes on humans. This involves entomological sampling since field measures are based on the prevalence of infections in mosquitoes. κ has been collected relatively infrequently, with approximately 37 estimates between 1955 and 2005 (0.7 estimates per year) (Killeen et al., 2006).

2.1.1 How to collect

The contributions of individual humans to κ are most commonly measured using direct skin-feeding assays (SFAs), using standard membrane-feeding assays (MFAs) or using infection rates in natural vector populations.

SFA: In theory, one of the most reliable ways to measure the individual human's contributions to κ is using a direct SFA, where laboratory-reared mosquitoes are directly fed on infected humans to observe the fraction that become infected (Killeen et al., 2006). Human subjects may be either a randomly selected sample of the whole population (Bonnet et al., 2003; Boudin et al., 1993; Burkot et al., 1988; Graves et al., 1988; Muirhead-Thomson, 1957) or purposely chosen gametocyte carriers (Bousema et al., 2012).

MFA: An alternative and more ethically acceptable method is a MFA with blood from naturally infected humans. MFAs and SFAs can use both oocyst prevalence and density as outcome measures. This is particularly relevant in the evaluation of transmission-blocking interventions where the transmission-blocking effects may be dependent on parasite exposure (Churcher et al., 2012).

Evidence from a recent meta-analysis of 930 transmission experiments from Cameroon, The Gambia, Mali and Senegal indicates strong correlation between estimates from direct SFAs and standard MFAs ($p < 0.0001$), although direct SFAs generally produced higher mosquito infection rates than MFAs (OR 2.39, 95%CI 1.94–2.95) (Bousema et al., 2012). In practice, direct SFAs and standard MFAs can be difficult and lengthy. Since membrane feeds use mosquitoes of a fixed age (normally 3–5 days) that are kept in optimum conditions, survival rates after feeding are artificially increased. The number of mosquitoes used is also often unrealistically high for a natural setting (i.e. it is unlikely that in reality an individual human would receive 50–75 bites per night). This means that estimates of κ derived from infectiousness in MFAs and SFAs, though accurate, may not be representative in the context of natural mosquito exposure. However, even if the estimates of κ are not representative, these assays may be used to give an accurate assessment of a change in κ.

Infection rates in the natural vector population: To determine net κ, infection rates in the natural vector population can be measured. Mosquitoes are caught and examined for parasites, either in the midgut examining for the presence of the oocyst stage or in the salivary glands for detection of sporozoites. κ is then calculated using biodemographic models of the vector population (Burkot et al., 1990; Charlwood et al., 1997; Graves et al., 1990; Saul, 1990). Killeen and colleagues recently added human blood index into the previous model (Charlwood et al., 1997) to allow estimation of κ using oocyst (γ) or sporozoite prevalence (z) (Killeen et al., 2006). A simple, but useful, rule for approximating these rates is $\gamma \approx S\kappa p^{o}$, and $z \approx S\kappa p^{n}$, where S is the expected

number of human bites a mosquito will give over its lifetime, p is the probability of surviving 1 day, o is the day after infection on which oocysts appear, and n is the day on which sporozoites appear (Killeen et al., 2006). This approximation is inaccurate in two ways: first, it assumes that mosquito populations have a stable age distribution, which is violated in populations that are fluctuating, and second, it overestimates by a small amount that is accounted for by reinfection of mosquitoes that were already infected earlier in life, but for realistic values of κ, this effect is very small.

Other methods: If the data for the previously mentioned parameters are unavailable, alternative approaches to calculating κ are to use oocyst infection rates, sporozoite accumulation rates in ageing populations of mosquitoes or age-specific sporozoite prevalence curves (Killeen et al., 2006). κ has also been predicted using stochastic individual-based models, using data from neurosyphilis patients given malariatherapy to predict the probability that a mosquito feeding on an infected patient becomes infected, as a function of recent history of asexual parasite density, together with data on EIR to predict the parasite density distributions for populations exposed since birth to seasonal transmission (Killeen et al., 2006).

2.1.2 Accuracy

Values of κ simulated using EIR and data from neurosyphilis patients given malariatherapy have been shown to correlate well with EIR at relatively low transmission intensities (EIR < 10), yet a paucity of field estimates of κ from areas with EIR < 10 has precluded evaluations of this relationship at low transmission intensities using field data (Killeen et al., 2006). Where EIR > 10, empirical data from 37 sites across Papua New Guinea and Africa indicate little relationship between field estimates of κ and EIR (Killeen et al., 2006). This suggests little association between the infectiousness of humans to mosquitoes and mosquito-to-human transmission intensity at higher transmission intensities. It is unlikely that this is explained by the acquisition of transmission-blocking immunity since there is little evidence that this type of immunity varies with cumulative exposure (Boudin et al., 2004; Killeen et al., 2006; Sauerwein et al., 2011). Therefore, κ is currently not thought to be an accurate assay of malaria transmission intensity, although more data are required to substantiate this. Furthermore, estimates of κ that are based on mosquito-feeding assays need to take into account natural mosquito exposure and possible differences between membrane-feeding and skin-feeding mosquito infection rates to be accurate.

2.1.3 Precision

The limited number of observations makes an assessment of the precision of κ difficult. It will be influenced by seasonality and both the frequency and intensity of mosquito sampling. The number of estimates may increase with the use of more frequent mosquito sampling techniques (Chaki et al., 2012) and that κ may be a potential end point for transmission-blocking interventions.

2.1.4 Costs

The cost of measuring κ depends on the method used. If SFAs or MFAs are used, laboratory rearing of mosquitoes is required, along with associated laboratory equipment for the assay. These assays have proven difficult to establish and only a handful of laboratories are routinely measuring human infectiousness to mosquitoes (Bousema et al., 2012). If κ is indirectly measured using data on sporozoite or oocyst prevalence in mosquitoes, costs may be comparable to and usually associated with an estimation of EIR (see the succeeding text).

2.1.5 Critique

Some transmission-reducing interventions are likely to have a direct effect on κ, such as transmission-blocking vaccines or gametocyte-reducing chemotherapy. However, due to its relatively low precision and accuracy, κ is not considered a robust metric for detecting a change in malaria transmission intensity. SFAs or MFAs will probably play a role in assessing the biological efficacy of a transmission-blocking vaccine. However, most interventions including LLINs, IRS and other methods of vector control will have an indirect effect on κ, and such assays have limited value in community assessments of transmission.

2.2. Parasite rate in humans

Parasite prevalence in humans (PR) is the proportion of individuals with parasitaemia at a given point in time. PR, the metric that was designed to estimate prevalence, measures the proportion of individuals who are found to be carrying parasites in their blood, which varies by the method used. PR is intrinsically inaccurate because parasite densities fluctuate over the course of an infection and because there are methodological limits on the ability to find parasites in a sample of blood when they are present at low densities. PR has been the traditional metric for classifying malaria endemicity and was used to measure malaria transmission during the era of the Global Malaria

Eradication Programme, wherever PR exceeded 1–3% (Hay et al., 2008; Macdonald and Gockel, 1964). PR has been widely collected, with 22,212 estimates between 1985 and 2010 (888.5 estimates per year) (Gething et al., 2011).

2.2.1 How to collect

PR can be rapidly measured by examining blood from a cross-sectional survey of a representative sample of the population, for example, the whole community or school survey. Light microscopy is considered the gold standard assay for clinical diagnosis, but RDTs or polymerase chain reaction (PCR) are alternate assays with advantages over light microscopy in some contexts. These assays should ideally be double-read to improve accuracy. PCR and microscopy both distinguish between malaria species and RDTs to detect both *P. falciparum* and *P. vivax* have also now been developed (WHO-GMP, 2012).

2.2.2 Accuracy

The accuracy of PR is affected by (1) the distribution of parasite densities in a population at some point in time, (2) the method used for parasite detection, and (3) human factors of the blood donor. Parasite densities are observed to vary over the course of a simple infection (Eyles and Young, 1951), and they vary in some unknown way in populations where humans of various ages are being exposed to mosquitoes at different points in time. Parasite densities in an infection can vary from a single parasite to more than 10^{11} parasites in hyperparasitaemic patients. A parasite must be present in a blood sample to be counted by light microscopy, but field methods (typically a thick blood film) take approximately 5 μL of blood and then examine only a fraction of this blood volume. The ability to detect a parasite is thus linked to the number of parasites per microlitre, with the probability of detecting a parasite increasing with the number of parasites in the sample. With such a small amount of blood examined, many active infections will inevitably be missed, and these are called *subpatent*. While this is not the only factor, it is one of the most important factors, so PR patterns must be interpreted in light of other facts that are known about parasite infections.

Evidence suggests that parasite densities vary systematically by the age of the infection and thus vary seasonally (i.e. because 'older' infections are less likely to have high parasite densities). Parasite densities also tend to be lower in those with well-developed immunity, so they are lower in older patients (Smith et al., 2007b). Finally, parasite densities are strongly affected by the

recent history of antimalarial drug use and parasite resistance to those drugs. To accurately capture PR, sampling must be frequent since patent parasitaemia is dynamic and can be short-lived. Single cross-sectional surveys may therefore not accurately capture PR (Corran et al., 2007; O'Meara et al., 2007). O'Meara and colleagues enrolled 51 individuals known to have a primary infection of malaria (i.e. with a true overall infection prevalence of 100%) and sampled these individuals 400 times on random days. Observed prevalence by light microscopy was 80% (95% CI 64–92%) in this particular set of infected patients, a 20% underestimation of true PR, produced by fluctuations in parasite density (O'Meara et al., 2007).

The accuracy of PR estimates is also affected by the age group sampled. PR in children aged 2–10 years has been widely used as a metric of transmission intensity since PR remains fairly constant in this age group regardless of endemicity (Smith et al., 2007b). PR can follow alternative age patterns if the epidemiology of malaria diverges from the standard model of household biting, for example, where certain occupations such as gold miners or forest or agricultural workers are at greater risk of malaria. Highly seasonal transmission also produces variation in age-specific PR patterns (Carneiro et al., 2010). Given the *a priori* relationship between parasite densities and the sensitivity of a test, and given the relationship between transmission intensity and parasite densities, it is likely that PR is more accurate during periods of high exposure than low exposure (McElroy et al., 1994).

The accuracy of PCR and RDTs is related to the accuracy of the PR but with certain caveats. PCR measures the prevalence of parasite DNA in a sample, and it may be much more sensitive than light microscopy. While DNA will always be present when there are viable parasites present, DNA might also be present when there are no viable parasites present. It is currently unclear whether this is affecting the accuracy of PR determined by PCR; studies in rodent malaria suggest that nonviable parasites are rapidly removed from the circulation and false-positive PCR results are unlikely 48 h after injection with dead parasites. RDTs that are based on the detection of histidine-rich protein 2, currently the most commonly used RDTs, detect a parasite antigen that is secreted by the parasite into the bloodstream and may persist for several weeks after parasite clearance. This persistence of antigen after viable parasites have been cleared affects the accuracy of RDTs in measuring PR. This explains differences in PR estimates between microscopy and RDT (Batwala et al., 2010). The sensitivity of light microscopy, RDT and PCR is related to the number of parasites. While microscopy and RDT can miss submicroscopic infections at all transmission settings but

particularly at low transmission intensity, PCR is more sensitive and will detect subpatent infections. A recent review found that in 106 studies, PR measured by microscopy was 54.1% (95% CI 50.3–58.2%) that of PR measured by PCR, but the proportional differences were larger when the PR was smaller (Okell et al., 2012).

2.2.3 Precision

All of the factors affecting accuracy of the PR will also affect its precision but depending on the sampling design. Since PR varies with seasonality, the precision of estimates can be improved by measuring PR at the same time of the year, such as at the peak of the transmission season, or by conducting repeat surveys year-round. At small spatial scales, intensive sampling is needed to provide robust estimates, as a result of heterogeneity in PR within small areas (Stewart et al., 2009). Most cross-sectional surveys deploy a cluster-randomised design, but this can substantially reduce the precision of the estimates when the distribution of malaria is itself clustered.

Uncertainty around PR estimates relative to other metrics increases at high transmission levels (where EIR >10) (O'Meara et al., 2007; Smith et al., 2005). This is because the relationship between transmission intensity and PR is mediated by acquired immunity, antimalarial drug use, multiple infections and heterogeneous biting. Another constraint to the accuracy of PR is the sensitivity and specificity of the assay used. PR measured through school-based surveys may be subject to certain biases, for example, the relative prevalence of parasitaemia in school age children will be higher at lower transmission intensities, due to the peak age shift (Okiro et al., 2009a).

Despite these concerns about accuracy and precision, it is worth noting that the PR can have sufficient precision to be useful. If repeated surveys use the same methodology, the same population and the same time of the season, estimates are likely to have sufficient precision for valid comparisons between surveys. It was used during the GMEP to monitor progress towards elimination, and it may be highly useful, even if it is biased (Macdonald and Gockel, 1964).

2.2.4 Costs

The costs of measuring PR vary from setting to setting and according to the size of the sample and the assay used (Table 3.2). The cost of each RDT varies from US$1 to 10. In some settings, PCR will be the more expensive assay; however, the cost per sample will be relatively inexpensive when a

Table 3.2 Comparison of costs of survey-based metrics

	Measures of infection			Measures of exposure
Cost	**RDT**[a]	**Microscopy**[a]	**PCR**[a]	**Serology**[b]
Cost per sample	$1.50	$0.25	$2.00	$0.5
Detection limit	100–200 p/μL	4–100 p/μL	<4 p/μL (individual PCR); 100 p/μL (pooled PCR)	n/a
Point-of-care test?	Yes	Yes, if basic laboratory services are available	No	n/a
Capital equipment required	None	Microscope	PCR machine, pipettes, gel tanks	Microplate reader
Training and rigour	Minimal	Moderate	Very extensive	Extensive
Turnaround time per sample	15 min	30 min	2 days	3 days
Turnaround time for 1000 samples	n/a	Weeks	Typically a week for individual PCR and days for pooled PCR; however, the turnaround time depends on staff costs and the type of PCR conducted	Week

[a]Hsiang et al. (2012).
[b]Unpublished data.

large number of samples are processed and where labour costs are relatively high. Microscopy in some settings is relatively expensive; however, costs for all assays are laboratory- and country-specific, and in a hospital setting, good microscopists are needed for other reasons. Overall, PR may be a relatively inexpensive metric for making a rapid assessment of transmission intensity where nothing is known about endemicity. However, baseline PR should be carefully established if PR is to be used to rigorously measure a change in transmission.

2.2.5 Critique

PR is the most frequently actively collected metric (Hay and Snow, 2006) and therefore is easily interpreted by malaria control programme managers. It is the only metric sufficiently ubiquitous for large-scale mapping of malaria transmission (Gething et al., 2011). However, the suitability of PR for assessing changes in transmission intensity varies with the endemicity. While PR is useful for obtaining rapid initial estimates of endemicity, PR is not a direct indicator of transmission intensity and becomes saturated at higher transmission intensities (wherever EIR > 10), due to heterogeneous biting, multiple infections and acquired immunity. In other words, large changes in the EIR can lead to reasonably small changes in the PR. The utility of PR for measuring changes in transmission is also limited at very low endemicity due to the sample sizes required to achieve the appropriate degree of statistical power. In addition, at very low transmission intensity, microscopy will also have low discriminative value. Specifically, below a PR of 1–5%, it will be very difficult to use PR to detect an impact of interventions (Hay et al., 2008). This limits its utility for accurately measuring the efficacy of transmission-reducing interventions.

2.3. Entomological inoculation rate

The annual EIR is the number of infectious bites received per person per period of time (typically year) (Davey and Gordon, 1933; Onori and Grab, 1980a). It is the product of the human biting rate (*Ma*, the number of bites per person per year) and the SR (the proportion of mosquitoes with sporozoites in their salivary glands). Human biting rates are estimated by catching and counting the number of mosquitoes that attempt to feed on a human, and the SR is found by examining those mosquitoes for the presence of sporozoites. It is widely considered the gold standard metric of malaria transmission, though this is usually an *a priori* assertion made without regard to precision and accuracy. It has been relatively frequently collected, with 233 estimates between 1980 and 2000 (11.7 estimates per year) (Hay et al., 2000).

2.3.1 How to collect

EIR can be measured using direct field measurements of SR and *Ma*, supplemented by models of mosquito populations. Standard methods for measuring *Ma* are indoor or outdoor human-landing catches, pyrethroid spray catches, exit traps and CDC light traps (Table 3.3). The SR is then calculated by examining the caught mosquitoes for sporozoites. It is also possible to

Table 3.3 Frequency of use of methods to measure annual *P. falciparum* EIR (1980–2004)

	Year intervals					
Sporozoite detection and method of determining biting rate	**1980–1984**	**1985–1989**	**1990–1994**	**1995–1999**	**2000–2004**	**All years**
Dissection + HLC	13	19	18	11		61
Dissection + PSC	8					8
Dissection + exit trap		2				2
Dissection + ELISA + HLC				1		1
ELISA + HLC		9	14	13	2	38
ELISA + PSC			11	31		42
ELISA + light trap		13	10	9		32
ELISA + HLC + PSC			4			4
ELISA + HLC + PSC + light trap				3		3
ELISA + HLC + exit trap			5			5
ELISA + PCR + HLC				3		3
All methods	21	43	62	71	2	199

HLC: human-landing catch; PSC: pyrethrum spray catch; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction.
Table reproduced from Kelly-Hope and McKenzie (2009) and originally published by BioMed Central, London.

separately estimate many of the elements of EIR using mark-release-recapture methodology, such as the population density of the mosquito population and the interval between consecutive blood meals.

Direct field measurements of SR and Ma: The gold standard method for estimating EIR is to directly measure SR and *Ma* and to calculate the product of their values:

$$\begin{aligned}\mathrm{EIR} &= \mathrm{SRMa} \\ &= \frac{\text{total sporozoite positive ELISA tests}}{\text{total mosquitoes tested}} \times \frac{\text{total mosquitoes collected}}{\text{total catches}}.\end{aligned}$$

Using this method, it is reasonably difficult to calculate appropriate confidence intervals since *Ma* and SR are not independent of each other (i.e. in seasonal areas, there is an inverse association over time between SR and changes in population density), and an estimation of the covariance of these is required in order to carry out the calculation (Charlwood et al., 1995). An alternative method has been proposed in which it is possible to calculate confidence intervals (Drakeley et al., 2003). This method assumes that sporozoite data are available for all mosquitoes caught:

$$\mathrm{EIR} = \frac{\text{total sporozoite positive mosquitoes}}{\text{total catches}}.$$

Often, mosquito catch data conform to negative binomial distributions, suggesting an alternative method can be developed to assess the confidence intervals (Nedelman, 1983; Shilane et al., 2010).

Methods for collecting data on SR and *Ma* have not been standardised and improved biostatistics are needed to establish the appropriate intensity of sampling (Hay et al., 2000; Nedelman, 1983). A recent review of 230 georeferenced EIR estimates in Africa between 1980 and 2004 found a total of 11 different methods of measuring EIR (Table 3.3) (Hay et al., 2000; Kelly-Hope and McKenzie, 2009). *Ma* had been estimated most frequently using human-landing catch or pyrethrum spray catch, although light traps and window exit traps had also been used. While human-landing catches are deemed the gold standard, measurements may not accurately reflect exposure when personal protection is in place and there are ethical issues surrounding the risk to those conducting catches.

In the same review, SR was most commonly estimated through dissection of mosquito salivary glands or by enzyme-linked immunosorbent assay (ELISA); however, PCR has also been used (Kelly-Hope and McKenzie,

2009). Of these methods, PCR is the most sensitive yet is also most prone to contamination, resulting in false-positives. False-positivity in the ELISA may also affect the validity of EIR estimates (Durnez et al., 2011). Dissection is a visual method and is therefore subjective. All three approaches for assessing sporozoites rates therefore have their shortcomings. Some studies have attempted to quantify the relationship between different methods of measuring *Ma* (Lines et al., 1991; Mbogo et al., 1993) and SR (Adungo et al., 1991; Boudin et al., 1991); however, there remains uncertainty over how measures compare. Site-specific validation of alternative methods may be increasingly recommended.

Modelling of mosquito populations: EIR can be estimated through a detailed study of the mosquito population and through the formulation of models incorporating the reproductive age of the mosquito. For example, Killeen and colleagues adapted an existing cyclical model (Saul, 1990) to allow calculation of EIR as a product of (1) the potential of individual vectors to transmit malaria over their lifetime, (2) vector emergence rate in relation to the size of the human population size and (3) the infectiousness of humans to vectors (Killeen et al., 2000). This model of EIR gave predicted values in the same range as those observed in the field at four sites in Papua New Guinea, Tanzania and Nigeria (Killeen et al., 2000). However, although such models may have a use in predicting the effect of interventions, the gold standard for EIR remains direct field measurement.

2.3.2 Accuracy

One of the most important questions about the accuracy of the EIR concerns the relationship between the methods used to catch mosquitoes and the actual number of bites received by a person over any given interval of time. A major concern is that different methods for catching mosquitoes have different and poorly characterised biases in their ability to sample different mosquito populations and different mosquito species in different places (Silver, 2008). The number of mosquitoes that are caught differs considerably between different mosquito trapping approaches and sampling efficiency may differ between mosquito species (Wong et al., 2013). If traps are used to sample mosquitoes, the placement of trap—whether inside or outside—and the properties of the trap itself have been shown to differ in the number of vector species that are being caught (Jawara et al., 2011; Wong et al., 2013). Interindividual differences in attractiveness to mosquitoes (Knols et al., 1995) may in turn affect the accuracy of human-landing catches. Another concern about the accuracy of mosquito sampling methods

is the poor understanding of outdoor biting patterns and if these capture all the relevant vector species (Stevenson et al., 2012). Most methods have been developed and standardised for indoor sampling and might miss outdoor biting, whether outdoor biting comprises a proportion of the biting by a single vector species or type or many types. This will also influence the precision of any estimate.

2.3.3 Precision

Without precise measurements of *Ma* and SR, the uncertainty in EIR can be so large that it is difficult to measure a change in EIR, particularly at low transmission levels. *Ma* is difficult to measure precisely due to spatial, temporal and seasonal variability in vector density, which necessitates intensive sampling. Sampling methods have yet to be standardised, so it remains unclear what spatial and temporal schemes would give the most precise measures of *Ma*. SR is also difficult to measure precisely, since it is dependent on the initial infectiousness and average age or survival times of adult mosquitoes in a population. It may also be necessary to measure mosquito species-specific *S*. Unless all mosquitoes caught are examined for sporozoites, calculation of confidence intervals around the EIR is difficult, with several implications: (i) it is not possible to extrapolate to other sites, (ii) sample size calculations for entomological studies are difficult to carry out and (iii) in any study, it is unlikely that the number of catches used to calculate EIR will fully represent the total number of person nights within a village. It is therefore difficult to put results in context without confidence intervals. Another concern related to accuracy is the sampling approach. To improve yields, entomological sampling may purposefully select household where a high mosquito density is expected. This affects the extent to which findings can be extrapolated to other households.

2.3.4 Costs

Costs for measuring EIR are difficult to generalise since they are heavily dependent on the intensity of entomological sampling, the setting and the methods used. Table 3.3 summarises the number of times each sampling method for computing the *Ma* has been used to estimate the EIR. Table 3.4 shows the costs of different methods of determining SR, which do not account for manpower. Though exact costs are laboratory-specific, ELISA is the least expensive assay when manpower is taken into account, since salivary gland dissections are laborious and PCR requires extensive training and rigour.

Table 3.4 Comparison of costs of methods for determining the sporozoite rate

Cost	Dissection[a]	ELISA[b]	PCR[a]
Cost per sample (approximate)	$0.25	$0.50	$2.00
Capital equipment required	Microscope	Microplate reader	PCR machine
Training and rigour	Moderate	Extensive	Very extensive
Turnaround time per sample	30 min	3 days	2 days
Turnaround time per 1000 samples	Weeks	Week	Week (individual PCR); days (pooled PCR)

[a]Hsiang et al. (2012).
[b]Unpublished data.

2.3.5 Critique

Traditionally considered the gold standard, estimates of EIR are relatively commonplace and considered easily interpreted by policymakers and national malaria control programme managers alike. However, EIR is not suited to obtaining rapid estimates of transmission intensity. The large uncertainty inherent in measuring *Ma* and the need for standardised methods for measuring both *Ma* and SR (Hay et al., 2000; Kelly-Hope and McKenzie, 2009) limit the precision and accuracy of EIR and its potential for measuring a change in transmission. This is especially so at low transmission intensities, where it is difficult to catch sufficient mosquitoes. Furthermore, methods that do not require humans are not well developed for exophagic vectors. There is also small-scale spatial variability in vector abundance and EIR (Mbogo et al., 2003). Despite its limitations, EIR may be necessary in certain settings where serological age profiles or parasitological or clinical measures become insensitive to changes in transmission, for example, at very high levels of transmission.

EIR estimates the rate of human exposure to infectious bites, which does not directly translate into population measures of either incidence or clinical disease, nor does it accurately represent exposure when interventions are in place. There are also species- and site-specific discrepancies between the different methods of measuring *Ma*, including human-landing catches, light trap catches and pyrethrum spray catches (Lines et al., 1991). While the Ross–Macdonald model assumed a linear relationship between the FOI and EIR, the efficiency of transmission (FOI/EIR or SCR/EIR) actually

declines in high transmission settings (Najera, 1974; Smith et al., 2010). There are three main hypotheses to explain the lower transmission efficiency at high intensity: (1) immunity, (2) heterogeneous biting and (3) systematic bias in estimation of the EIR (Smith et al., 2006). Long-lasting immunity is unlikely to be able to account for short-term variation in transmission efficiency however, while bias could explain the nonlinear patterns in transmission efficiency only if the magnitude of the bias increased sharply with EIR. Heterogeneous biting may therefore be the most plausible explanation; if 20% of the population receives 80% of bites, this is consistent with temporal variation in transmission efficiency correlated with EIR, observed at several sites, for example, Saradidi in Kenya (Beier et al., 1999, Smith et al., 2010). The loss of transmission efficiency, in addition to the previously described concerns about accuracy and precision, raises concern that entomological measures may not be the most appropriate method for measuring malaria transmission.

2.4. Force of infection/molecular force of infection

The FOI is the number of infections per person per unit time. mFOI is the molecular FOI, the number of new parasite clones acquired per unit time (Mueller et al., 2012). FOI counts all patent incident human malaria infections (symptomatic or asymptomatic) during a given time period and also takes into account whether or not a person is already infected.

2.4.1 How to collect

FOI can be measured using cohort studies or repeat cross-sectional surveys.

Cohort studies: FOI can be measured in a naturally uninfected cohort (e.g. uninfected immigrants or infants) or by artificially creating a cohort of uninfected individuals through treatment with antimalarial drugs and following up the cohort to measure the attack rate over some time period (the proportion becoming infected) (Smith et al., 2010), as has been done in Kenya, Ghana and Senegal (Baird et al., 2002; Beier et al., 1994; Owusu-Agyei et al., 2001; Rogier et al., 1999). A few studies have estimated the FOI by observing the patterns of parasite positives and negatives over time, but some of these transitions may represent natural fluctuations in existing populations, rather than new infections (Bekessy et al., 1976; Charlwood et al., 1998). To resolve some of these questions, new methods have been developed to examine the FOI using genetic methods (see the succeeding

text) to type new infections, called the mFOI. Since the number of newly acquired infections can be measured in the presence of previously acquired infections, there is no need to clear infections prior to longitudinal measurements (Felger et al., 2012; Mueller et al., 2012).

Cross-sectional surveys: Cross-sectional surveys can be conducted and FOI estimated by fitting reverse catalytic models to the increase in PR with age, controlling for infections that have been cleared (Davey and Gordon, 1933; Davidson and Draper, 1953; Pull and Grab, 1974; Smith et al., 2010). For mFOI, these models can allow for the imperfect detection of all circulating parasite clones (Felger et al., 2012).

2.4.2 Accuracy

Since the density of parasites fluctuates within an infected individual, the sensitivity and specificity of microscopy in detecting infections varies. Sampling on 1 day only will lead to a small proportion of infections remaining undetected and imprecision in FOI (Koepfli et al., 2011). This may be less problematic for mFOI, since PCR methods involved have greater sensitivity than microscopy (Felger et al., 2003; Mueller et al., 2012). Estimates of mFOI may be biased if certain parasite clones are not detected when fluctuating below the PCR detection threshold (Felger et al., 2012; Koepfli et al., 2011; Mueller et al., 2012) and will also be affected by seasonality, age, ITN use and chemotherapy (Mueller et al., 2012).

2.4.3 Precision

Estimates from several different studies suggest that FOI is relatively consistent (Bekessy et al., 1976; Charlwood et al., 1998; Rogier and Trape, 1993; Smith et al., 2010). The efficiency of transmission declines as transmission intensity increases, partly due to heterogeneous biting. Therefore, FOI saturates above an EIR of around 10 (Smith et al., 2010). Where the FOI is very high, the frequency of sampling limits the maximum value of estimates. The strong association between mFOI seasonality, age and ITN use indicates that it is a reasonable measure of exposure to infection. Significant variation in mFOI between villages also reflects small-scale heterogeneity in transmission (Mueller et al., 2012). mFOI is a more realistic estimate of FOI, since it is possible to monitor natural superinfections in asymptomatic individuals and will also have higher discriminative power at higher transmission intensity.

2.4.4 Costs

Costs for FOI and mFOI depend on whether a cohort or cross-sectional survey is used. PCR, required for mFOI, is more expensive than microscopy and requires a higher level of training and/or technical capacity (Table 3.2).

2.4.5 Critique

mFOI has greater sensitivity and specificity than FOI and overall a relatively high precision and accuracy in areas of low transmission. Due to the decline in transmission efficiency at high transmission levels, FOI plateaus above a certain transmission intensity and is therefore not useful for measuring a change in transmission in highly endemic areas. mFOI is likely to reach a plateau later than FOI. A plateau in mFOI may not be reached if this metric is interpreted as a dynamic MOI, at least in age groups where infections reach blood stage and if the method of detection is highly inclusive.

2.5. Multiplicity of infection

MOI is the number of concurrent parasite clones per *P. falciparum*-positive host. It has only relatively recently been pioneered as a metric of malaria transmission (Arnot, 1998; Beck et al., 1999; Kolakovich et al., 1996; Mbugi et al., 2006; Schleiermacher et al., 2001). Mathematical theory suggests that, in a cohort of uninfected people acquiring infections and clearing infections independently and naturally, MOI would reach a Poisson distribution with mean given by the FOI divided by the clearance rates (Dietz, 1988). With heterogeneous biting, this would become a negative binomial distribution. All of these distributions would be affected by within-host competition among parasites (Dietz, 1988) and by sporadic treatment with antimalarial drugs. The general pattern expected is a higher MOI with local transmission intensity and with time since parasites were last cleared with antimalarial drugs.

2.5.1 How to collect

MOI can be measured by genotyping infections using polymorphic markers such as the merozoite surface protein-1 (MSP-1) (Atroosh et al., 2011), MSP-2 (Mueller et al., 2012; Vafa et al., 2008), glutamate-rich protein (Akter et al., 2012) or microsatellite markers (Guitard et al., 2010). MOI has been measured in cross-sectional or longitudinal samples.

2.5.2 Accuracy

Accurate molecular typing is essential for measuring MOI and the number of molecular markers and the number of sampling days that are needed for this depend on transmission setting. The more rounds of sampling conducted, the more clones collected and the more precise the MOI estimate (Koepfli et al., 2011). Sampling parasites from the same individual repeatedly will improve the accuracy of the MOI estimate, but as sampling frame grows longer, new infections may decrease the accuracy of MOI. The capacity of MOI to accurately reflect transmission is also likely to be dependent on the diversity of malaria clones in a particular setting; however, the relationship between this genetic diversity and transmission intensity is not yet well quantified. Where parasite populations are less diverse, estimates of transmission may be underestimated due to a saturation effect where multiple clones are not distinguished by the molecular markers used (Mueller et al., 2012). In addition, although methods for estimating haplotype frequencies have been developed (Li et al., 2009), haplotypes are not typically considered. This may lead to underestimation of the true MOI, a bias that will change with transmission intensity.

2.5.3 Precision

MOI will be affected by interventions such as chemotherapy or chemoprophylaxis that influence susceptibility to infection. In malaria-endemic regions, multiple infections are common, and MOI closely correlates with endemicity (Arnot et al., 1985; Beck et al., 1997; Mueller et al., 2012; Ntoumi et al., 1995; Paul et al., 1995). Reduced MOI has also been observed to be associated with increased ITN use, indicating that it is a reasonable indicator of transmission intensity (Mueller et al., 2012). In a study in children aged 0.9–3.2 years in Papua New Guinea, variation in average MOI over time and between and within villages was not statistically significant. Parasite densities show seasonal fluctuations and the detectability of parasite clones may therefore also depend on season. This will affect the precision of MOI estimates. If this is taken into account and if the extraction of DNA and efficiency of PCR amplification remain constant over time, MOI estimates are expected to be relatively precise.

2.5.4 Costs

While information can be obtained from single time-point surveys, in general, repeat sampling is required for accurate estimates of MOI, together

with sophisticated procedures including genotyping, which makes it a relatively expensive measure. Costs are also laboratory-specific.

2.5.5 Critique

MOI has been developed as a metric of malaria transmission only recently as tools for disentangling the molecular complexity of natural parasite infections emerge, as such methods for validation require development. Two limiting factors on the maximum value of MOI are (1) the frequency of sampling and (2) the local diversity of parasite populations. In the absence of these constraints, MOI shows promise as a robust measure of changing malaria transmission.

2.6. Seroconversion rate

The SCR is a function of antimalarial antibodies in the population and indicates exposure to infection. It is calculated by fitting a reversible catalytic model to age-specific malarial antibody prevalence (seroprevalence) data (Drakeley et al., 2005b; Grab and Pull, 1974). SCR takes into account malaria exposure (infection) over time (Corran et al., 2007), allowing temporal patterns in transmission to be studied (Cook et al., 2011; Stewart et al., 2009).

2.6.1 How to collect

Methods for ascertaining SCRs have been described in detail (Corran et al., 2007; Stewart et al., 2009). In brief, data on seroprevalence to malaria parasite-specific antigens (e.g. *Pf*AMA-1, *Pf*MSP-1_{19}, and *P. falciparum* schizont extract) are collected across all age groups and converted to SCR by fitting a simple reversible catalytic model to the seroprevalence data, stratified into yearly age groups, using maximum likelihood methods (Pull and Grab, 1974). Exclusion of individuals aged <1 year from estimates of SCR minimises the effect of maternally derived antibodies.

Types of assay: The antibody assay can be adapted to different transmission settings using different antigens (Corran et al., 2007). Historical methods for measuring seroprevalence include the complement fixation test, indirect haemagglutination assay and immunofluorescence antibody test, enzyme-linked immunosorbent assay (ELISA) and, most recently, protein microarray (Drakeley et al., 2005b). ELISA is simple and easily standardised and has been used in many recent seroepidemiological studies of malaria (Cook et al., 2011; Drakeley et al., 2005b).

Types of survey: Blood samples for generating seroprevalence data can be collected in cross-sectional surveys (Cook et al., 2010), school surveys or in health facilities (which necessitates sampling all individuals attending a facility over a fixed period of time or until sufficient samples have been obtained). In a comparison of the relative advantages of cross-sectional survey and health facility data in northeastern Tanzania, a lower PR was recorded in cross-sectional surveys than health facility data (4.7% vs. 2%, $p < 0.001$), a lower seroprevalence for *Pf*MSP-1_{19} recorded in the health facility (29.1% vs. 40.5%, $p = 0.005$) and similar AMA-1 antibody prevalence found between the two (46.9% vs. 47.9%, $p = 8$) (Drakeley et al., 2005a).

There are three major limitations to the use of health facility seroprevalence data that are relevant to other measures. First, children aged <5 years and women of child-bearing age may be overrepresented (which can be partly compensated for by sampling accompanying family members). Second, most health centre attendees are ill; therefore, a substantial proportion will have active malaria infections, which could influence seroprevalence rates (Stewart et al., 2009). Third, unless the village of residence is recorded, estimates may be skewed by the recruitment of individuals from outside the catchment area, especially at referral facilities. The advantages of using health facility data are its speed of collection and low cost (Drakeley et al., 2005a).

2.6.2 Accuracy

Seroprevalence represents cumulative exposure to infection. The accuracy of serology must be addressed on both the individual level and at the level of a population. While the accuracy of serology for one antigen in one individual is probably not a highly accurate measure of previous infection, SCR may become a very useful and accurate measure of transmission in a population when it is taken on many individuals of different ages and using multiple antigens. Indeed, one of the advantages of serology is the potential to measure seropositivity in humans of different ages, to multiple different responses to the parasite, all lasting different periods of time, to paint a fairly detailed profile of transmission in a population in the present and at various points in the past. One strong advantage of serology is that, due to the long duration of specific antibody responses, seroprevalence can be less affected by seasonality and short-term fluctuations in transmission than other measures (Cook et al., 2010). The long duration of antibody responses has important consequences for areas with changing transmission intensity where seroprevalence in older age groups may reflect historical rather than current transmission intensity.

2.6.3 Precision

At low transmission intensities, SCR has high sensitivity since the longevity of the antibody response generates higher seroprevalence rates than equivalent PR (Stewart et al., 2009). At very high transmission intensities, SCR is less sensitive due to saturation of infection in the population. Heterogeneous immunity (not all individuals respond to all antigens) produces a lower estimate of SCR; hence, higher SCR estimates (that are closer to FOI) are observed when two or more markers are combined (Smith et al., 2010). The use of antibody titres rather than prevalence data may increase the sensitivity of estimates to changes in transmission intensity (Stewart et al., 2009).

2.6.4 Costs

Health facility data are cheaper to collect than cross-sectional survey data (in Tanzania, health facility data were five- to tenfold cheaper than cross-sectional data (Drakeley et al., 2005a)). Collecting blood samples to generate seroprevalence data is relatively simple since antibodies can be eluted from filter paper, making sample collection and storage straightforward (Stewart et al., 2009). Using an ELISA-based antibody assay is relatively simple, cheap and quick (Table 3.2) (Corran et al., 2007).

2.6.5 Critique

SCR has high precision and accuracy, although its accuracy may decline at very low or high transmission levels. A strong advantage of SCR is the ability to reconstruct the history of exposure, which is especially useful in the common situation of missing baseline data (Corran et al., 2007). With regard to measuring a *change* in transmission, it has not been established whether SCR can measure less than log-fold differences in transmission (Drakeley et al., 2005b). Currently, SCR is not sensitive to short-term changes in transmission since antibodies can persist for years after the period of exposure, so it is necessary to wait for the population to age (Corran et al., 2007). However, some studies have reported SCR in the youngest children (<5 years) (Ceesay et al., 2010) and this is a method for making SCR estimates more useful for determining recent yearly reductions in transmission intensity. Antigens that prove useful components of malaria vaccines may become redundant in SCR assays if such a vaccine becomes widely used (Corran et al., 2007). Fluctuations in recent exposure can also be determined by examining antibody titres, since seropositivity can last for many years, with currently infected individuals having the highest antibody responses and levels slowly declining as parasites are reduced. The frequency distribution

of antibody titres can therefore be used to describe endemicity if titres are drawn from an age-representative cross-sectional survey (Cook and Drakeley, 2009; Kagan et al., 1969; Lobel et al., 1973). Compared to antibody prevalence, antibody titres will have greater discriminatory power where transmission is high and when a very sensitive assay is used (Cook and Drakeley, 2009).

2.7. Clinical surveillance

Metrics for clinical surveillance include the slide positivity rate (SPR) (also known as the CPR or test positivity rate), which is the proportion of those examined who test positive for parasitaemia. The annual parasite index (API) is a proxy measure of incidence that is derived from SPR and takes into account the total population at risk of malaria and the rate at which that population is examined. This differs from incidence of clinical malaria, the rate at which new cases of clinical malaria arise in a population over time, directly measured by active or passive case detection. Finally, the proportion of fevers with *P. falciparum* parasitaemia (PF*Pf*) represents the total number of febrile malaria cases as a proportion of all febrile cases.

SPR: SPR is the proportion of those examined by microscopy or RDT with parasitaemia. This differs from the annual blood examination rate (ABER), which is the proportion of the total population examined for parasitaemia.

Incidence of clinical malaria: Incidence of clinical malaria is the rate at which new cases of clinical malaria (fever plus parasitaemia) occur in a population (e.g. total number of cases per 1000 person years at risk) and it is therefore a direct measure of disease burden. The annual parasite incidence is the product of the SPR and ABER: API = (ABER*SPR)/10. Division by 10 is necessary because SPR and ABER are expressed per 100 and API per 1000. Incidence of clinical malaria has been relatively frequently collected; a recent review documented 83 estimates between 1985 and 2005 (4.2 estimates per year) (Snow et al., 2005).

PF*Pf*: PF*Pf* is the number of parasitologically confirmed cases divided by the total number of presumptive malaria (febrile) cases. This differs from the malaria-attributable fraction (AF, or proportion of febrile cases attributable to malaria), which is the proportion of fever morbidity that would be removed if malaria were eliminated. AF is defined as $p(fp - fp0)/fp$, where p is the proportion of febrile individuals with parasites, fp is the proportion of parasitaemic individuals with fever and fp0 is the proportion of

aparasitaemic individuals with fever (Smith et al., 1994). There are relatively few estimates of PF*Pf*; a recent review documented 39 studies measuring PF*Pf* between 1986 and 2007 (1.9 estimates per year) in sub-Saharan Africa (D'Acremont et al., 2010), while another study identified 67 estimates between 2000 and 2009 (7.4 estimates per year) (Gething et al., 2010). Differences between the two estimates may have arisen due to different review inclusion; in the first review, the PF*Pf* denominator was the total number of presumptive malaria cases (D'Acremont et al., 2010), while in the second, the PF*Pf* denominator was the selection criteria for detailed microscopy, which varied from definitions of fever to unspecified criteria such as 'suspected' or 'presumed malaria' (Gething et al., 2010).

2.7.1 How to collect

SPR: SPR is collected through routine health facility data, using RDTs or blood slides, which ideally should be double-read. The total number of positive slides or RDTs is divided by the total number of slides or RDTs (assuming one test per person).

Incidence of clinical malaria: Incidence can be measured by active or passive case detection or indirectly estimated using mortality data or spatial techniques.

Active case detection: Incidence can be measured by active case detection by following up a cohort of children artificially cleared of infection and recording new incident infections (Beier et al., 1994). Active case detection captures more cases than passive case detection (Utarini et al., 2007). However, it is much more expensive, requires many staff and is time-consuming.

Passive case detection: Incidence can also be measured by passive case detection where new incident infections are recorded once they present at (health) facilities. Measuring incidence through passive case detection makes three assumptions: (1) complete spatial coverage (every health facility reports and every incident infection has access to a facility), (2) complete temporal coverage (every month is reported by a health facility) and (3) all disease events present to/are reported by the health facilities (Snow et al., 2005). Hospital admissions data can be used to measure incidence; however, this does not always reflect malaria incidence in the wider community (Okiro et al., 2009b).

Indirect estimation: Malaria incidence has been estimated at the country level using reported malaria mortality data as raw starting data and extrapolating this to incidence using (1) an adjustment for under-reporting of mortality, (2) an estimate of the likely *P. falciparum* case fatality rate and (3) the

proportion of cases attributable to *P. falciparum* (to which nearly all mortality is attributed) for each set of national data (Carter and Mendis, 2006; Mendis et al., 2001). However, this method is likely to underestimate incidence (Snow et al., 2005). In this study, the global clinical malaria burden was estimated using evidence of the epidemiological risks of disease outcome from active case-detection studies together with estimates of populations at risk of various *P. falciparum* transmission conditions.

There has been debate over the relative merits of estimating incidence directly and indirectly (Cibulskis et al., 2011). Routine data have the dual advantages of being more immediately sensitive to changes in incidence, and since it is collected as part of malaria control programmes, it can be easily integrated with other types of data to help evaluate and improve programmes. However, its reliability depends on the coverage and quality of the surveillance system. Strong mandates for the notification of all malaria cases in both public and private sectors may improve reliability (malERA, 2011). Model-based approaches provide estimates for areas with poor or no surveillance and can be used to examine trends over wide areas and over time. However, extrapolation in areas of limited data may not be reliable. Combining both approaches may give stronger estimates overall (Mueller et al., 2011).

PF*Pf*: PF*Pf* can be directly calculated from routine health facility or cross-sectional survey data. The total number of cases with confirmed parasitaemia should then be divided by the total number of presumptive malaria cases. Indirectly, PF*Pf* has also been estimated by combining estimates of childhood fevers and treatment-seeking rates from MIS or other survey data with estimates of the risk of febrile children being infected when reporting to clinics within three classes of endemicity. Using this method, it was estimated that of 656 million fevers in children aged 0–4 years in Africa in 2007, 182 million presented to public health facilities of which 78 million (42.9%) had parasitaemia (Gething et al., 2010).

2.7.2 Accuracy

SPR: Although SPR applies a consistent case definition of malaria, it is calculated from routine health facility data and therefore estimates will be affected by any seasonal or other changes in the incidence of nonmalaria fever, which influence the presentation of febrile cases (Jensen et al., 2009). As such, there is likely to be variation in repeat estimates over time, limiting the value of SPR as a robust assay for measuring changes in transmission. Consistency in diagnostic practices, for example, the likelihood that

a febrile individual is referred for laboratory diagnosis, is essential for accurate and precise estimates. High quality and consistency in microscopy or the use of RDTs is also necessary for accurate data.

Incidence of clinical malaria: The incidence of clinical malaria increases rapidly with transmission intensity. However, at high transmission levels, incidence does not exceed that observed at intermediate transmission levels, partly due to acquired immunity and multiple infections (Ghani et al., 2009; Trape and Rogier, 1996). Therefore, as transmission falls, a threshold must be crossed before a significant reduction in cases and hospital admissions is observed (Smith et al., 2004; Trape et al., 1987). Incidence estimated using hospital admissions will be unreliable if overdiagnosis leads to an overestimation of the number of cases presenting to health facilities and cases in the community are missed. Incidence varies with immunity; some individuals may be infected yet asymptomatic and this proportion will differ according to endemicity. Especially at low transmission levels, there is considerable heterogeneity in incidence that results from heterogeneity in transmission between households and may also be affected by human genetic factors that influence the progression of infections to symptomatic disease (Mackinnon et al., 2005).

PF*Pf*: There is likely to be large variation in estimates of PF*Pf* since the causes of fever differ between populations and over time. Furthermore, if health facility data are used, then inaccuracy is introduced into PF*Pf* since a varying proportion of febrile individuals within a population will present at clinics. In areas of very high transmission intensity, estimates may be inaccurate since nearly all individuals have parasitaemia. In addition, non-malaria fevers may suppress malaria parasitaemia, resulting in biased estimates (Smith et al., 1994).

2.7.3 Precision

SPR: Changes in SPR have been used as evidence of a decline in malaria in parts of Africa (Ceesay et al., 2008), yet the accuracy of SPR as a metric of transmission is less well established than for other metrics. SPR can explain variation in incidence of malaria (Bi et al., 2012) and has been found to be directly associated with the relative change in malaria incidence, assuming that there is no sampling bias in the subgroup of suspected malaria cases undergoing laboratory testing and that the incidence of nonmalaria fevers is constant over time. Since SPR incorporates only laboratory-confirmed cases, the denominator very clearly represents the number of laboratory tests (Francis et al., 2012). The decision-making by which laboratory

confirmation is requested should remain consistent over time to allow precise and comparable estimates to be made. SPR cannot be used to estimate the incidence of clinical malaria (Jensen et al., 2009) since health facility data may not accurately represent the entire population, especially if the health facilities are chosen because they have laboratories with trained staff and high standards.

Incidence of clinical malaria: At low transmission levels, a very large sample size is required to precisely measure incidence. Similar to SPR, the precision of estimates of clinical malaria depends on the consistency in diagnostic practices. In addition, if there are substantial variations in health-seeking behaviour over time, estimates of clinical malaria that depend on passive case detection will be imprecise.

PF*Pf*: In a review of 39 studies measuring PF*Pf* in sub-Saharan Africa, a 50% reduction in PF*Pf* was observed in the periods pre- and post-2000 (22% vs. 44%) (D'Acremont et al., 2010). This mirrors the recent decline in malaria across sub-Saharan Africa (O'Meara et al., 2010), indicating that PF*Pf* may be useful as a rough indicator of transmission intensity. However, in a review of 67 independent estimates of the proportion of febrile children attending clinics with parasitaemia, the ranges of PF*Pf* overlapped in medium (5–40% PR2–10 years) and high (>40% PR2–10 years) classes of endemicity, indicating low precision of PF*Pf* as an indicator of malaria transmission. Furthermore, the pattern of causes of fever in malaria patients is not uniform within populations and between areas (D'Acremont et al., 2010; Gething et al., 2010). Additionally, fever will not be caused by malaria infection in a certain proportion of parasitaemic fever cases. Like the PR, the PF*Pf* likely saturates at high transmission.

2.7.4 Costs

SPR: Measurement of SPR is relatively cheap since data are obtained from health facility records and routine diagnostic procedure. Table 3.2 summarises the comparative costs of RDTs and microscopy.

Incidence of clinical malaria: Indirect estimation of incidence using previously collected data is relatively inexpensive, apart from staff costs. Direct estimation through passive case detection is cheaper than direct estimation through active case detection. Both active and passive detection have the same costs per individual tested (i.e. cost of RDT and cost of ACT); however, active detection involves more frequent testing, which requires more RDTs or slides (Utarini et al., 2007), and is logistically more challenging since it will involve repeated scheduled visits of cohort members.

PF*Pf*: The cost of measuring PF*Pf* directly is dependent on whether it is collected via health facility or survey data. Costs for microscopy and RDTs are given in Table 3.2. Surveys are more expensive to conduct.

2.7.5 Critique

SPR: SPR has the advantage that it can be calculated from routine health data, and for this reason, it is used by WHO as an indicator of reduced transmission at the country level (WHO, 2011). SPR is therefore useful as a rapid indicator of broad trends in malaria transmission within a site. However, trends in SPR may be affected by confounding factors such as age, area of residence, testing frequency, access to health care and type of diagnostic tests used (Francis et al., 2012), so that it is much more difficult to interpret the SPR across sites. Overall, the relatively low accuracy and precision of SPR negate its use as a robust metric of changes in malaria transmission.

Incidence of clinical malaria: At high transmission intensities, acquired immunity limits the accuracy of incidence as a measure of transmission intensity. Therefore, incidence may not be appropriate for accurately recording a decline in incidence from high to medium endemicity. In the context of a trial of a transmission-reducing intervention, clearing baseline and incident infections with drugs at the beginning of a study will affect the subsequent susceptibility of an individual to infection and disease, complicating the measurement of incidence. The clinical presentation of malaria is independently influenced by age (Marsh, 1999; Reyburn et al., 2005) and it has not yet been established which age groups should be monitored for measuring the efficacy of different transmission-reducing interventions.

PF*Pf*: The costs of measuring PF*Pf* are low since it can be readily incorporated into existing routine data collection; therefore, PF*Pf* may be useful as a rough indicator of changes in transmission for malaria control programmes. However, it has low precision and accuracy and therefore should not be relied upon as a reliable assay of changing transmission.

2.8. Vectorial capacity (C) and the basic reproduction number (R_0)

Vectorial capacity: Vectorial capacity (C) was developed as part of the early work done by Macdonald to identify the entomological factors responsible for transmission (Macdonald, 1952). A few years later, it was modified slightly to include the human blood index, the methods for measuring it were described, and it was then named (Garrett-Jones, 1964). The quantity describes the potential intensity of transmission by malaria vectors and it is

defined as the 'daily reproductive rate' or more precisely, the expected number of infectious bites that could eventually arise (i.e. assuming perfect efficiency of transmission) from all the mosquitoes that bite a single human on a single day (Garrett-Jones, 1964). It is calculated as

$$C = \frac{Ma^2 p^n}{-\ln p},$$

where M is the density of adult mosquitoes, a is their feeding frequency on humans, p is their daily survival rate and n is the duration of parasite development in humans. It can also be understood as the product of the human biting rate (Ma is the human biting rate), the probability a mosquito survives through sporogony (p^n) and the expected number of human blood meals that would be given by a mosquito after it has become infectious ($S = a/-\ln p$).

When more than one vector species present, the total vectorial capacity is the sum of vectorial capacity of each vector: $C = C_1 + C_2 + \cdots$.

R_0: The malaria basic case reproduction number (R_0) is the 'expected number of hosts who would be infected after one generation of the parasite by a single infectious person who had been introduced into an otherwise naïve population'. It is found by multiplying vectorial capacity by the net efficiency of transmission and by the duration of an infection: $R_0 = bcCD$, where D describes the average infectiousness of a human infection expressed in the equivalent number of fully infectious days (Johnston et al., 2013). Where transmission is stable, the value of R_0 is high, exceeding the value ($R_0 = 1$) needed to sustain transmission. Vectorial capacity-based estimates of R_0 range from 1 to 3000 in 121 African populations (Gething et al., 2011; Smith et al., 2007a).

2.8.1 How to collect

Vectorial capacity: All of the components of vectorial capacity can, in theory, be collected through studies of mosquito populations, usually through studies that capture, mark, release and recapture mosquitoes. Vectorial capacity has been estimated in this way by collecting data on its individual components together with cyclical feeding models of transmission (Charlwood et al., 1997; Graves et al., 1990; Saul, 1990). However, measuring all the entomological components to calculate vectorial capacity is technically difficult, and the measurement of each term introduces error that is compounded by taking the product. An alternative method has been proposed,

where the dominant entomological variables only are included to be used as a comparative index (Dye, 1986). For example, the Garki project human biting rate data were combined with an amalgamation of the remaining components of vectorial capacity to create a comparative index of vectorial capacity in different Garki villages (Dye, 1986). However, this method produces estimates with a comparative use only.

R_0: R_0 can be estimated directly by taking the product of estimates of each parameter in the formula for vectorial capacity. Under the classical assumptions (that human populations are infinite and the human biting rate is constant), $R_0 = bVD$, where b is the probability that a human becomes infected after being bitten by an infectious mosquito, V is the vectorial capacity (the number of infectious bites by mosquitoes arising from all mosquitoes infected by one person during 1 day), and D is the mean duration of infectiousness of a single human.

One estimate of b has been made using the control arms of experimental challenge studies (Smith et al., 2010). Since transmission from infectious humans to mosquitoes is often inefficient, depending inter alia on the fluctuating density of gametocytes, it may be best expressed as the number of days an untreated person would be fully infectious; analysis of data from malariatherapy patients and from models of it suggests that a person is infectious, on average, for a period that amounts to approximately 36 infectious days (Johnston et al., 2013). The first estimates were done this way (Davidson, 1955; Davidson and Draper, 1953), but it is technically difficult and expensive (Smith et al., 2007a).

There are, however, several other methods available for estimating R_0 (Dietz, 1993), based on a data describing (1) the EIR, (2) the FOI (Najera, 1974), (3) the average age at which infection with malaria occurs and (4) the ratio of malaria infections during successive generations (Freeman et al., 1999). Most estimates of R_0 for malaria have been derived from estimates of the EIR, but the other methods have also been used.

A simple method for estimating vectorial capacity from EIR is based on a model of mosquito infections that is consistent with the Ross–Macdonald model. By the formulas, vectorial capacity is closely related to the EIR: $E \approx C\kappa$, so $E/\kappa \approx C$, or more precisely

$$C = \frac{E(1 + S\kappa)}{\kappa}.$$

Transmission is slightly more efficient when humans who are more frequently bitten are at increased risk of infection and consequently infect more

mosquitoes. This increases R_0 by a factor proportional to the squared coefficient of variation of biting rates $(1+\propto)$. Making the assumption that net infectiousness is proportional to the PR (i.e. $\kappa \approx cX$), and that the duration of a simple human infection is $(1/r)$, estimates of the EIR and the PR were used to estimate R_0 in 121 African populations (Smith et al., 2007a), based on the formula

$$R_0 = bCD(1+\propto) = E\frac{b}{r}\frac{(1+cSX)}{X}(1+\propto).$$

The underlying model provides a good fit to the observed empirical relationship between the EIR and the PR and the empirical relationship between the EIR and the FOI (Smith et al., 2005, 2010). This relationship has now been updated to use empirical estimates of κ and the revised method was used to produce a global map of the reproductive numbers for *P. falciparum* that reflects current levels of control (Gething et al., 2011).

R_0 may also be estimated using the initial growth rate of an epidemic in a previously unexposed population (Macdonald, 1956); this is not feasible in malaria-endemic regions (Smith et al., 2007a), but it has been applied to measure R_0 in at least one epidemic situation (Freeman). More generally, R_0 has also been estimated from equilibrium situations, using age-independent and age-specific prevalence data (Dietz, 1993).

2.8.2 Accuracy

Vectorial capacity: The formula for vectorial capacity makes a number of assumptions: (1) each parasite species has one type of invertebrate vector and one type of vertebrate host, (2) the vector daily survival rate is constant over time and with age, (3) mosquitoes randomly take blood meals, (4) mosquitoes take a fixed number of blood meals per unit time, and (5) an infective bite always leads to infection in a susceptible host. However, these assumptions frequently fail, for example, mosquitoes do not feed at random (Dye, 1986). Vectorial capacity is only as accurate as its constituent parameters. Methods for estimating all parameters for vectorial capacity have not been validated and any errors in collection are subsequently compounded during the final calculation, giving large sampling error (Dye, 1986). Mosquito survival through a day, or *p*, *in* particular, must be carefully measured since vectorial capacity is sensitive to changes in adult survival, and other factors such as the distribution of bites on humans are not well understood. An alternative method for calculating vectorial capacity that attempts to address these issues has been developed (Dye, 1986).

R_0: Like vectorial capacity, R_0 is only as accurate as the individual parameters used in its calculation, for which there are practical difficulties in obtaining reliable measurements. In particular, it is difficult to acquire an accurate value for the human biting rate. Any errors in the measurement of individual components of R_0 will be compounded.

2.8.3 Precision

Vectorial capacity: Vectorial capacity is closely related to EIR; vectorial capacity multiplied by the transmission efficiency gives the gradient of the relationship between EIR and PR at low transmission levels. Unlike EIR, vectorial capacity is not a function of the proportion of humans who are infectious or of the SR. Therefore, vectorial capacity gives an independent indication of human infection prevalence because its calculation does not use information on the proportion of humans who are infectious (Smith and McKenzie, 2004). The concerns about precision that are listed under EIR and PR are also concerns for the precision of estimates of vectorial capacity. Natural fluctuations in vector densities, biting patterns and variation in performance of trapping methods affect the precision of estimates of vectorial capacity. Similarly, the precision of PR in humans is affected by the chosen methodology, sampling population and timing of sampling.

R_0: R_0 is the gold standard, theoretical measure of malaria transmission, which all other metrics of transmission aim to reflect. When obtaining direct field measurements of R_0, systematic sampling bias is introduced if PR is used as a measure of the probability that a mosquito becomes infected after biting a human. This is due to heterogeneous biting and immunity, which reduces the infectivity of both mosquitoes to humans and humans to mosquitoes. Initially, at low transmission levels, the proportion of mosquitoes that become infected after biting a human is higher than PR, due to heterogeneous biting; however, at high EIR, while PR continues to rise, transmission-blocking immunity causes the proportion of mosquitoes becoming infected to decline. Smith et al. therefore proposed a revision to the classic formula for R_0, which takes into account this variation in the infectivity of humans and mosquitoes with variation in EIR (Smith et al., 2007a).

2.8.4 Costs

The costs of collecting vectorial capacity and R_0 constitute the cost of measuring its individual components and since this involves detailed clinical and entomological surveys to measure EIR and PR, these are high.

2.8.5 Critique

Vectorial capacity: Vectorial capacity is not as useful a measure as EIR for predicting malaria transmission intensity, since it is less meaningful epidemiologically and is not testable by direct field measurement (Killeen et al., 2000). It is also difficult to obtain precise field measurements of vectorial capacity.

R_0: While R_0 remains the gold standard, idealised measure of malaria transmission, it is difficult to measure accurately and precisely in the field, and the relatively small number of studies (Burkot et al., 1988; Davidson, 1955; Davidson and Draper, 1953; Hagmann et al., 2003) that have attempted to measure R_0 is testament to this. In particular, the percentage of infected humans that infect a mosquito, c, and the duration of human infections in populations with some level of immunity remain poorly parameterised. Since R_0 is derived from other metrics, it is not suitable for measuring changes in transmission, but it is more useful as a way of interpreting those metrics within a common framework for control.

3. SCALING RELATIONSHIPS BETWEEN MALARIA METRICS

The metrics of transmission are all causally interrelated: infectious mosquitoes transmit parasites to humans causing new infections, and infectious humans transmit the parasite back to parasites that eventually appear as sporozoites in the mosquito. The potential rate at which these events occur increases with vectorial capacity or R_0. The ease of measuring these metrics varies from place to place, depending in part on the value of these parameters.

In order to understand which metrics are generally more useful in which settings, and to correctly interpret the results of trials or programmes aimed at reducing malaria transmission, it is necessary to understand the underlying scaling relationships between metrics; for example, does a 50% change in one variable correspond with a 50% change in another, and if so, does a second 50% change in one also correspond with a 50% change in the other? The expectations of these relationships derive from three sources: first, from well-designed epidemiological studies or large-scale controlled trials; second, from the examination of studies where two or more of these metrics have been measured in the same place; and third, from the output of malaria transmission models that are consistent with these changes and that provide *a priori* expectations about the shapes of those curves. Using previously

published work, the relationships between five major metrics of malaria transmission, namely, the EIR, FOI, PR, SR and the basic reproductive number (R_0), are modelled in Fig. 3.2 (Smith et al., 2006). Additionally, it is useful to visualise what metric(s) might be most appropriate in terms

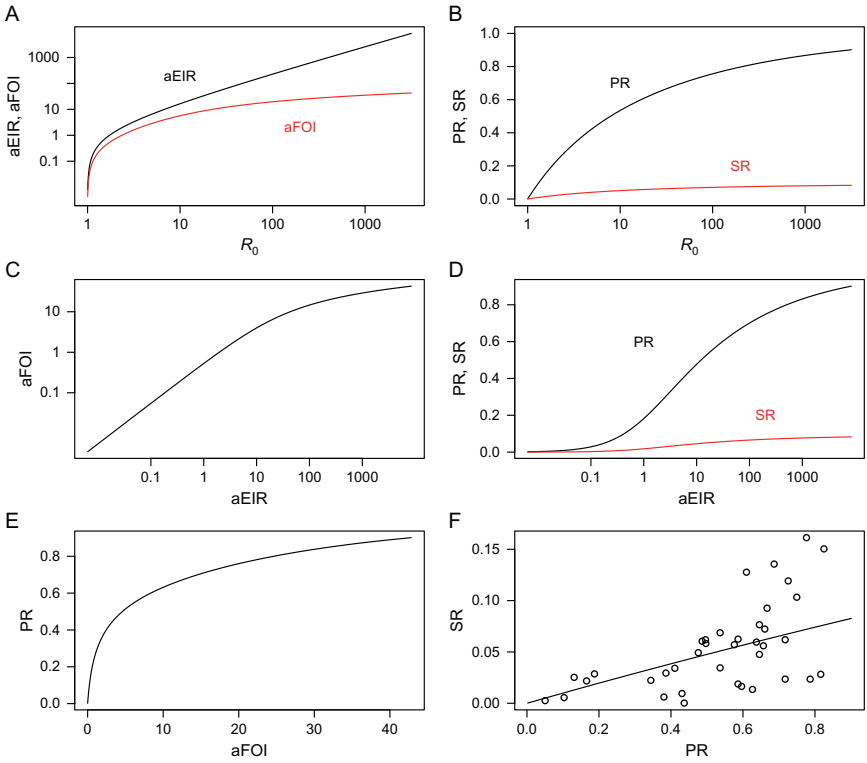

Figure 3.2 Relationship between metrics of malaria transmission. (A) Annual FOI versus R_0; annual EIR versus R_0: derived from a malaria transmission model, with assumptions of heterogeneous biting and superinfection, of *Pf*PR versus PfEIR (Smith et al., 2010). (B) SR versus R_0: derived from a malaria transmission model, with assumptions of heterogeneous biting and superinfection, of *Pf*PR versus *Pf*R_c (Smith et al., 2010), assuming sporozoite rate is linearly proportional to *PfPR*. PR versus R_0: malaria transmission model, with assumptions of heterogeneous biting and superinfection (Smith et al., 2010). (C) Annual FOI versus annual EIR: model of heterogeneous biting fitted to synthetic cohort data from Saradidi, Kenya (Smith et al., 2010). (D) SR versus annual EIR: derived from a log-linear model of *Pf*PR versus EIR (Gething et al., 2011), with the assumption that sporozoite rate is linearly proportional to *Pf*PR; PR versus annual EIR: log-linear model of *Pf*PR versus EIR (Gething et al., 2011). (E) PR versus annual FOI: derived from a log-linear model of *Pf*PR versus EIR (Gething et al., 2011). (F) SR versus PR: best-fit model for reported sporozoite rate-*Pf*PR pairs (Smith et al., 2005).

of accuracy and precision in measuring and detecting changes transmission at different levels of endemicity. To this end, the relative utility of PR, EIR, R_0 and SCR at different levels of transmission is modelled in Fig. 3.3A–C. This model does not explicitly incorporate any sampling variation around each particular measures nor the associated precision and accuracy. However, by expressing the measures on the same scales, it does illustrate the range of transmission over which different measures might be used and the likely optimal combination of these measures. The limitations pertinent to each measure are described in the respective sections in the preceding text, but the figures show that at all but the highest settings EIR and PR can be used, while at lower transmission levels, molecular and serological end points are likely to be most informative.

3.1. R_0, EIR, FOI, PR and SR

The bites of infectious mosquitoes give rise to new infections, and as a result of immune control and/or if not treated properly, these can resolve into a chronic infection. Given these causal relationships, the EIR must be related to both the FOI and to the PR. Ross developed the first model relating EIR and prevalence (Ross, 1911), and he also described the mathematical basis for the relationship between the EIR and the FOI (Ross, 1916). Ross's work motivated the first studies to examine these relationships (Davey and Gordon, 1933). In 1950, Macdonald assembled these data and examined the relationships. In theory, the FOI could increase linearly with the EIR, but Macdonald's analysis provided the first quantitative evidence they did not (Macdonald, 1950). The preponderance of evidence assembled since then suggests that there is a highly nonlinear relationship between the EIR and the FOI that holds regardless of the method used to measure the FOI (Najera, 1974; Smith, 2010).

Several mechanisms and models (Filipe et al., 2007; Smith et al., 2006, 2010) have been proposed to explain this relationship, including immunity, heterogeneous biting (Smith et al., 2005, 2010) and a reduction in the proportion of entomologically assessed inoculations leading to an infection (Smith et al., 2006). There is evidence, meanwhile, that biting frequency increases with age; that the proportion of infections becoming patent declines somewhat with age and exposure, possibly due to greater immunity in older children; and that there may be additional reasons why mosquitoes fail to feed at very high transmission intensity. Weighing the relative effects of these different putative causes is difficult because the associated models tend to give similar predictions.

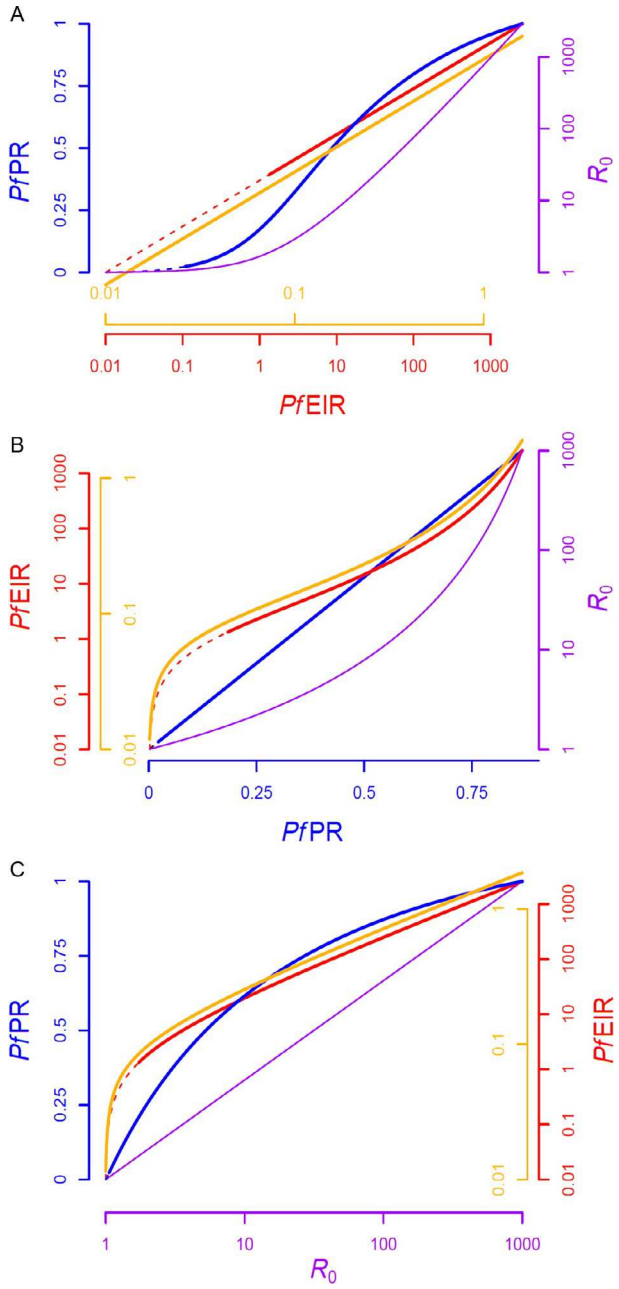

Figure 3.3 Potential utility of metrics at different levels of endemicity. (A) A plot of the logarithm of the annual *Pf*EIR against itself (red), the logarithm of the *Pf*SCR (orange), the $Pf\text{PR}_{2-10}$ (blue) and the logarithm of the PfR_0 (purple). These relationships are based

(Continued)

As part of baseline measurements for a malaria vaccine trial, Beier and colleagues conducted a cohort study in Saradidi, Kenya, in which 44 cohorts of children were cleared of infection before being followed up for 2 weeks. A linear model was fit to the relationship between EIR and FOI; however, this model made false assumptions about that rate of transmission when EIR equals zero (Beier et al., 1994). Smith and colleagues reanalysed these data from Saradidi, introducing heterogeneous biting (*gamma*-distributed biting rates) to refine the model:

$$\mathrm{FOI} = \frac{(\log(1 + bE\propto t))}{E\propto t},$$

where b = the proportion of infectious bites that cause a patent infection in a population previously unexposed to malaria, E = EIR, α = index of heterogeneous biting and t = number of days. Although the fit of this model was not as good as the unrooted linear relationship, it is not plausible that the relationship between EIR and FOI is linear; therefore, the model with heterogeneous biting is the most pragmatic (Smith et al., 2010) (Fig. 3.2C). More importantly, the nonlinear patterns showed up in cohorts drawn from the same population and changed with the EIR, a pattern that is broadly inconsistent with the hypothesis of immunity.

The model by Ross had been shown to work poorly in the African Savannah (Najera, 1974; Smith et al., 2005), and an analysis from 31 sites in Africa found that the relationship between the EIR and the PR was

Figure 3.3—Cont'd on one particular model for the steady-state relationships. The annual EIR is difficult to measure when the annual EIR is less than one because of the large sample sizes required to catch sufficient vectors both infected and noninfected. Similarly, the PR is difficult to measure when it is less than about 1% because of the large sample population sizes that need to be screened. For both measures, the solid line indicates those values where each metric can be measured accurately with reasonable effort, while the dashed line illustrates where the accuracy will wane. The cutoffs for 'reasonable effort' could vary depending on costs and priorities. The SCR has some advantages because it can be measured across the spectrum. Nothing is implied about the effort required to measure R_0, since it is generally inferred from the other metrics, based on some transmission model. The shapes of the curves have all been standardised to have the same minimum and maximum over the observed range of values to illustrate how the shapes of these curves affect the relative amount of information about transmission at different points along the spectrum. The steeper the curve, the more information that is conveyed about one metric relative to another. Panels (B) and (C) are the same curves with the *Pf*PR_{2-10} and the logarithm of the R_0 on the *x*-axis, respectively. We have not attempted to incorporate any estimates of error into these plots.

approximately log-linear. An immediate consequence of this formula is that the PR is not likely to fall until EIR is less than 1 per person per year (Beier et al., 1999). Smith and colleagues found that a simple model with heterogeneous biting fitted the patterns of an expanded dataset at least as well as the log-linear model (Smith et al., 2005). One hundred and nineteen estimates of EIR matched to PR in African children aged 0–15 years were identified. The best overall model was a simple extension of the Ross–Macdonald model, with heterogeneous biting:

$$\mathrm{PR} = 1 - \left(1 + \frac{b\varepsilon}{rk}\right) - k,$$

where b = transmission efficiency, k = variance of infection rate distribution, ε = annual EIR and $1/r$ = expected time to infection clearance. The dataset of paired EIR–*Pf* PR estimates was recently updated and a log-linear model of EIR versus PR fitted (Fig. 3.2D) (Gething et al., 2011).

Since gametocytes arise from asexual parasites and give rise to infections in mosquitoes, there should be a causal relationship between the PR and κ, and κ and the SR. Far more often, studies have focused instead on the relationship between the EIR and the SR, which is also not only of interest but also partially confusing since the EIR is the product of the SR and the HBR. Studies of the EIR and the SR have not found any statistically significant relationship. There is, however, a statistically significant relationship between the PR and the SR.

3.2. SCR and other metrics

Much of the attraction of measuring PR and SCR is that they can be ascertained as part of the same sample collection process and measured using the same blood sample. However, while measures of current infection and past exposure can be derived concurrently, the relationship between the two parameters has to date not been examined in a systematic manner. In two cross-sectional surveys of 250 people conducted in each of 12 villages in Tanzania, the correlation between seroprevalence to merozoite antigens and PR was found to be highly correlated in children aged 4 years or less but decreased with increasing age (MSP-1_{19}, $r = 0.41$, 0.29 and 0.17; MSP-2, $r = 0.24$, 0.20 and 0.13; and AMA-1, $r = 0.28$, 0.19 and 0.05 in individuals aged 0–4, 5–14 and 15–45 years, respectively) (Drakeley et al., 2005a). This is to be expected, since although the antibody responses measured may not be directly linked to immune protection, they will likely

reflect increased immunity in older individuals, who have a lower parasite burden. Indeed, where both parasitological and serological measures are age-adjusted, there is good correlation between the two (Figs. 3.3B and 3.2F). For example, in a cross-sectional survey of 7387 people across 18 sentinel sites across Bioko, Equatorial Guinea, site-specific *Pf* PR_{2-10} was positively correlated with SCR ($r=0.85$) (Cook et al., 2011). The absence of parasitaemia observed in Tanzania in individuals with antibodies to merozoite antigens may also be due to seasonality, since a correlation between SCR and incidence of infection was observed at the same site (MSP-119 $r=0.78$, AMA-1 $r=0.91$) (Stewart et al., 2009). Similarly, in Bioko, there was good correlation between the reduction in SCR, PR and incidence pre- and postintervention (Cook et al., 2011).

4. DISCUSSION

The goal of measuring malaria transmission and changes in its intensity has many challenges. To critically compare the most commonly used methods for measuring malaria transmission, this chapter evaluates the (a) methods of collection (b) accuracy, (c) precision and (d) costs of collection of 11 major metrics of transmission. The chapter highlights some of the most important questions about the accuracy and precision of each metric of transmission and discusses differences in the utility of these metrics in light of these shortcomings. On one hand, there are open questions about the accuracy and precision of all these metrics; specifically, how well do these metrics measure the true value and with what fidelity? On the other hand, there is another and closely related question about how well these metrics can be used as measures of transmission. Each metric has some utility, but to be useful as a measure of the current level of or a change in transmission, a metric must be sufficiently precise. When all else is equal, precision can usually be increased by increasing the sampling effort. Yet this becomes a challenge for measuring some of the metrics at low intensities when the number of events (infected people or mosquitoes) is low and when the inherent heterogeneity in transmission is likely to be most pronounced. With regard to accuracy, however, there are many open questions about the biases contributing to each one of these metrics at every level of transmission. The importance of these biases across the spectrum remains to be evaluated robustly. This chapter does not make any attempt to quantify the accuracy or precision for each metric, and while these have to some extent been characterised for PR (Gething et al., 2012; Smith et al., 2007b), there is

a need for future work to address this for other metrics. A pragmatic approach is to continue to measure transmission, subject to the relative costs of different metrics, while being cognizant of the possibility that bias can also change and undermine the findings of a study.

With respect to the interpretation of these metrics, other issues become more important, most notably the nonlinear relations of these metrics across the spectrum of transmission. Because of these issues, together with concerns about accuracy and precision, it is prudent and necessary to measure malaria transmission in several different ways to paint a robust picture of transmission and to compare these with mechanistic models of transmission. We have illustrated the patterns for EIR, FOI, SR, PR and R_0, in order to understand how these relationships scale across the spectrum of transmission intensity.

Traditionally, the EIR has been considered the gold standard measure of transmission intensity (Hay et al., 2000). While the precision and accuracy of EIR have not been well quantified, these are likely to be low because there are intrinsic difficulties in obtaining precise measurements of the human biting rate (*Ma*) and the SR, due in large part to the highly variable nature of mosquito populations (Mbogo et al., 2003), together with the lack of standardised sampling methods (Hay et al., 2000; Kelly-Hope and McKenzie, 2009). In addition, the accuracy of EIR is mediated by transmission efficiency, which declines as transmission increases (Fig. 3.2).

Another widely collected metric is PR, with over 22,000 estimates documented to date (Gething et al., 2011). PR is useful for obtaining a rapid initial estimate of transmission, however, and has limited precision and accuracy at higher transmission intensities and its accuracy is determined by the sensitivity of the assay used. At low endemicity, PR is likely to lose accuracy where EIR < 0.5, since malaria becomes more focal and population sampling may miss infections (Guerra et al., 2008) (Fig. 3.3). PCR-PR estimates, once they become more widely available, may improve sensitivity at very low endemicity but are unlikely to improve the value of PR at high endemicity (Okell et al., 2012). Genotyping approaches can also be used to make cross-sectional and longitudinal surveys more informative by determining the MOI and the mFOI. mFOI and MOI show great promise for assessing changes in malaria transmission. MOI is a single time-point quantification of the complexity of infections and increases the dynamic range over which infection estimates are informative: MOI saturates at a higher transmission intensity than PR. Similarly, mFOI has greater precision and accuracy than FOI. FOI can be seen as a dynamic PR where newly acquired

infections are measured over time; mFOI can be interpreted as a dynamic MOI. mFOI may not lose accuracy when transmission efficiency declines with increasing transmission intensity (Koepfli et al., 2011; Mueller et al., 2012). While methods for validation of MOI and mFOI need developing, this metric could prove a robust measure of changing malaria transmission in the absence of limits on sampling frequency and the local diversity of parasite populations (Mueller et al., 2012).

Metrics derived from clinical surveillance, including SPR, incidence of clinical malaria and PF*Pf* have the advantage of being estimable by routine health facility data, which keeps costs low and is useful for obtaining rapid or routine measures of transmission intensity (D'Acremont et al., 2010). Yet the precision and accuracy of both SPR and PF*Pf* is affected by factors such as access to and frequency of testing and, in the case of PF*Pf*, the incidence of other febrile illness. Incidence of clinical malaria remains the gold standard outcome for clinical trials of malaria control interventions; however, it may have limited accuracy at higher transmission intensities for assessing changes in transmission, due to acquired immunity and superinfection, which limit the efficiency of transmission.

SCR has high precision and accuracy at all but the extremes of the transmission spectrum (Fig. 3.3) and is useful for assessing long-term changes in transmission. In its current form, SCR has limited use for assessing the short-term effects of an intervention (Cook et al., 2011; Drakeley et al., 2005b); however, estimating SCR in children aged less than 5 years or measuring antibody levels rather than prevalence may allow these short-term changes to be measured (Cook et al., 2011; Corran et al., 2007).

For interventions that directly reduce the infectiousness of humans to mosquitoes, including transmission-blocking vaccines or gametocyte-reducing chemotherapy, κ may have the most relevance. However, within the context of a Phase III trial, this must be measured from field data in order to take into account the effect of mosquito densities and biting rates. Field measurements of κ will have low precision due to natural variation and measurement error, yet methods to address this have not been overcome. Increased vector sampling will go some way to improving these limitations (Chaki et al., 2012), but these approaches have yet to be validated in different settings. Lastly, while it is important to consider the theoretical effects of a transmission-reducing intervention on vectorial capacity and R_0, neither metric has practical utility due the difficulties inherent in achieving precise and accurate field measurements (Burkot et al., 1988; Davidson, 1955; Davidson and Draper, 1953; Hagmann et al., 2003; Killeen et al., 2000).

This chapter highlights the considerable variation in both the costs and ease of collection of different metrics, which are setting-specific and difficult to generalise. Although the expertise, equipment and materials required drive costs, the scale and frequency of sampling is also important. For example, although MOI requires sophisticated procedures including genotyping, single time-point measurements may be taken, meaning it is cheaper overall than FOI. Similarly, since EIR requires intensive sampling, it is likely the most expensive metric overall. The mode of sampling also affects costs; health system data likely to be less expensive than cohort or survey data since the majority of costs are already covered. The costs of survey-based metrics, such as PR and SCR, are roughly comparable, with some variation arising from the differing expense of the assays required (Hsiang et al., 2012). The fact that these metrics can be collected from the same sampling procedure is an additional advantage. The relative costs of metrics are an important consideration for routine monitoring by malaria control programmes although may be less relevant to the design of internationally funded intervention trials. It is also important to consider that ancillary information not directly relevant to measures of transmission may be collected for most measures such as speciation of both mosquito and parasite, molecular characterisation of insecticide and drug resistance.

Understanding how metrics vary across the transmission spectrum, and how they relate, can also help pinpoint which metrics are useful for measuring transmission in different settings. This chapter illustrates that two basic kinds of nonlinearity arise from the simple dynamics in the Ross–Macdonald model: thresholds and saturating responses. Other kinds of nonlinearities are introduced by heterogeneous biting and by human immunity (Fig. 3.2) (Smith et al., 2005, 2010). These four basic kinds of nonlinearities play out differently in different transmission contexts, and the design of large-scale interventions should be cognizant of them. For example, the relationship between EIR and prevalence is fairly linear below a threshold value of EIR (typically where EIR is less than 10, where transmission is 'unstable' or 'low to moderate'); therefore, malaria control results in an almost proportionate reduction in prevalence. When EIR exceeds this threshold, the relationship between EIR and prevalence is nonlinear and a reduction in EIR will not reduce prevalence (Beier et al., 1999; Griffin et al., 2010; Smith et al., 2005).

Based on the findings of the review, we advocate that at low transmission levels, appropriate metrics for measuring a change in transmission will generally include PR, although where EIR < 0.5, where PR by microscopy

becomes very low, molecular methods such as PR by PCR or mFOI may have greater precision and accuracy. SCR also becomes increasingly suitable at lower transmission intensities. At very high levels of transmission, EIR may be necessary since serological age profiles or parasitological or clinical measures can become insensitive to changes in transmission, although it is important to recognise its low precision and accuracy and the need to standardise methods for sampling variable mosquito populations. However, when assessing a specific intervention, the most relevant effects may be detected by also examining those metrics most directly affected by an intervention and/or tailoring these to focus on age groups likely to have benefitted most from the intervention (e.g. reduction of exposure to infection defined by antibody levels in children aged less than 5 years).

In conclusion, the precision and accuracy of malaria metrics has not been well quantified, nor have robust sampling methods to address low precision and accuracy been developed. This has important ramifications both for ongoing malaria surveillance and for the evaluation of malaria intervention and control programmes, especially given that the lack of consensus on precision and accuracy extends to some of the most widely used metrics, including EIR, PR and incidence of clinical malaria. Generic issues affecting the precision and accuracy of all malaria metrics include measurement issues, bias (especially for processes that are observed passively) and seasonality or interannual variability in the values of these parameters, including trends that are attributable to other factors. In order to measure a change in transmission, a baseline must first be established, which is problematic against a background of seasonal, trending and otherwise variable signals and this complicates the attribution of effects. Further work is required to establish which metrics are most appropriate in which settings and the most robust and inexpensive methods for their collection.

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CHAPTER FOUR

A Review of Molecular Approaches for Investigating Patterns of Coevolution in Marine Host–Parasite Relationships

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Contents

Abstract

Parasites and their relationships with hosts play a crucial role in the evolutionary pathways of every living organism. One method of investigating host–parasite systems is using a molecular approach. This is particularly important as analyses based solely on morphology or laboratory studies of parasites and their hosts do not take into account historical evolutionary interactions that can shape the distribution, abundance and population structure of parasites and their hosts. However, the predominant host–parasite coevolution literature has focused on terrestrial hosts and their parasites, and there still is a lack of studies in marine environments. Given that marine systems are generally more open than terrestrial ones, they provide fascinating opportunities for large-scale (as well as small-scale) geographic studies. Further, patterns and processes of genetic structuring and systematics are becoming more available across many different taxa (but especially fishes) in many marine systems, providing an excellent basis for examining

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whether parasites follow host population/species structure. In this chapter, we first highlight the factors and processes that challenge our ability to interpret evolutionary patterns of coevolution of hosts and their parasites in marine systems at different spatial, temporal and taxonomic scales. We then review the use of the most commonly utilized genetic markers in studying marine host–parasite systems. We give an overview and discuss which molecular methodologies resolve evolutionary relationships best and also discuss the applicability of new approaches, such as next-generation sequencing and studies utilizing functional markers to gain insights into more contemporary processes shaping host–parasite relationships.

1. INTRODUCTION

Parasites and their relationships with hosts play a crucial role in the evolutionary pathways of every living organism and 'have long been recognized as fundamental drivers of macro-evolutionary patterns of diversification' (Nieberding et al., 2008). This is because they can, among others, affect host behaviour and reproduction, invasions, regulation of the host immune system and the evolution of immune genes (Paterson and Piertney, 2011). Notably, it is estimated that around 30% of eukaryote taxa are represented by parasitic species (Poulin and Morand, 2004), and as such, parasites form integral components of both marine and terrestrial systems (Hoberg and Klassen, 2002). Therefore, their importance in contributing to the evolutionary fate of species is becoming better understood, although there are major questions remaining to be answered in the evolution of host–parasite systems (Rigaud et al., 2011). Theoretically, the evolutionary pathways of the host (including population genetic structuring and phylogenetic relationships) should be reflected by their parasites, but testing hypotheses around coevolution has been difficult using more traditional approaches based on, for example, morphological analyses.

One method of investigating host–parasite systems is using a molecular approach. This is particularly important as analyses based solely on morphological or laboratory studies of parasites and their hosts do not take into account historical evolutionary interactions that can shape the distribution, abundance and population structure of species. Therefore, a molecular approach in combination with more traditional methods that also takes into account metapopulation structure, historical population structuring, gene flow, cryptic speciation, vicariance effects and changes in population demography is more likely to resolve host–parasite relationships than studies based purely on morphological or ecological characteristics.

The majority of the host–parasite coevolution literature has focused on terrestrial hosts and their parasites with a paucity of studies within marine environments. In these systems, relatively few barriers exist that might characterize dispersal and migration compared to those in terrestrial or freshwater environments (Waples, 1998; Waples et al., 2008), and a lack of local adaptations was assumed for a long time (Palumbi, 2003). For instance, only a few molecular-based studies on the distribution of parasites in coastal fish exist (Table 4.1), although this group is probably the most well studied and understood of marine taxa using molecular methods. Nevertheless, marine fish stock identification (on which management plans are often based) has been challenging, since relatively few barriers are present in the marine environment causing a generally weaker underlying genetic structure compared to terrestrial landscapes (Hauser and Carvalho, 2008; Selkoe et al., 2008; Waples, 1998). However, numerous papers also show that large-scale phylogeographic breaks exist across multiple taxa, suggesting that historical events (such as changes in sea levels) influence diversification and speciation processes in the sea (see, e.g. Ayre et al., 2009; Hubert et al., 2012; Kuo and Avise, 2005; Toonen et al., 2011; von der Heyden, 2009).

The use of parasites as natural tags to delimit host populations has therefore gained attention in the past (Costa et al., 2013; MacKenzie, 2002), as the prevalence of parasite species can be used to acquire information about host demographic patterns and to assess influences of environmental gradients. However, it is only quite recently that scientists are discovering the additional enhanced value of molecular approaches. Parasites may be used as a 'biological magnifying glass' and help unravel the evolutionary processes that shape extant population patterns. For example, previously undetected phylogeographic information in the host may be highlighted by a higher evolutionary resolution from its parasite (Nieberding et al., 2004). This is because parasites usually have shorter generation times than their hosts, and therefore, genetic differentiation and demographic changes may be detected sooner as more mutations are fixed over time, leading to more rapid lineage sorting (Huyse et al., 2003). The increased expected genetic variance of parasites relative to their hosts may reveal the hosts' evolutionary history before the host DNA has coalesced (Rannala and Michalakis, 2003; Whiteman and Parker, 2005). If a parasite is more finely subdivided than its host, then one could potentially use the genotypes of a parasite species to assign hosts to their population of origin with higher accuracy than by using the host's own genotypes alone (Criscione et al., 2005; Huyse et al., 2003); equal or higher genetic structure in parasites compared to their

Table 4.1 Genetic marine studies that emphasize coevolutionary host–parasite processes, including euryhaline Salmonidae and sticklebacks

Host–parasite system	Molecular marker used	Molecular approach	Pattern observed	References
Oncorhynchus mykiss and *Oncorhynchus tshawytscha* (Teleostei) + *Ceratomyxa shasta* (Myxozoa)	SSU rDNA (p) + ITS1 (p)	Pop gen	Disparate parasite infection patterns in hosts correlate with ITS1 sequence variation in the parasite	Atkinson and Bartholomew (2010)
Salmonids (Teleostei) + *Parvicapsula minibicornis* (Myxozoa)	SSU rDNA (p)	Pop gen	Population structure of parasite was resembled in geography and host species	Atkinson et al. (2011)
Sardinops sagax (Teleostei) + *Anisakis* sp. (Nematoda)	Complete ITS, COII	Pop gen	Panmictic distribution of parasite haplotypes cannot be used to confirm population subdivision of Pacific sardines	Baldwin et al. (2011)
Thunnus albacares (Teleostei) + *Nasicola klawei* (Monogenea)	ITS1 + LSU rDNA + AFLP (p)	Pop gen	Levels of intraspecific genetic diversity among *N. klawei* but no infrapopulation monophyly	Bullard et al. (2011)
18 Teleostei species + *Kudoa thalassomi* (Myxozoa)	SSU rDNA + LSU rDNA (p)	Phylogeny	*K. thalassomi* has various levels of host specificity	Burger and Adlard (2011)
Chaetodon unimaculatus and *Gymnocranius audleyi* and *Lethrinus harak* (Teleostei) + *Kudoa* sp. (Myxozoa)	SSU rDNA (p)	Phylogeny	Host specificity and geographical distribution showed some correlations with genotype. Relationships reflect host tissue sites more than host identity	Burger et al. (2007)

Paralichthys lethostigma (Teleostei) + *Philometra overstreeti* and *Philometroides paralichthydis* (Nematoda)	COI (p)	Phylogeny	Four distinct genetic clades correspond to different habitats within the host	de Buron et al. (2011)
Merluccius merluccius (Teleostei) + *Anisakis* sp. (Nematoda)	ITS1–5 + 8S-ITS2 + COII (p)	Phylogeny	Variability of COII gene was higher compared to ribosomal genes	Ceballos-Mendiola et al. (2010)
Elasmobranchs (Condrichtyes) + *Staphylorchis cymatodes* (Digenea)	ITS2 + D1–D3 regions of LSU rDNA (p)	Barcoding	Low host specificity, found in at least eight elasmobranch species	Cutmore et al. (2010)
Linckia laevigata + *Protoreaster nodosus* (Asteroidea) and *Thyca crystallina* (Gastropoda) + *Periclimenes soror* (Decapoda)	COI (h, p)	Phylogeography	All four species show little common genetic structure, perhaps because of species-specific responses to past climate change events	Crandall et al. (2008)
Oncorhynchus spp. (Teleostei) + *Plagioporus shawi* (Trematoda)	Microsatellites + mtDNA (p)	Phylogeography	Broad-scale phylogeographical patterns of parasite can be predicted by the biogeographical history of hosts	Criscione and Blouin (2007)
Oncorhynchus spp. (Teleostei) + *Deropegus aspina* and *Nanophyetus salmincola* and *Plagioporus shawi* (Trematoda)	ITS1 + NADH1 (p)	Pop gen	Predicted, autogenic species had more highly structured populations and lower gene flow among subpopulations than an allogenic species sampled from the same locations	Criscione and Blouin (2004)

Continued

Table 4.1 Genetic marine studies that emphasize coevolutionary host–parasite processes, including euryhaline Salmonidae and sticklebacks—cont'd

Host–parasite system	Molecular marker used	Molecular approach	Pattern observed	References
Oncorhynchus mykiss (Teleostei) + *Plagioporus shawi* (Trematoda)	Microsatellites (h + p)	Pop gen	Genotypes of parasites are more accurate than the ones of fish to assign individuals to their population of origin	Criscione et al. (2006)
Conger conger (Teleostei) + *Lecithochirium fusiforme* (Trematoda)	Microsatellites (p)	Pop gen	Panmixia with co-transmission of three clusters of clones	Criscione et al. (2011)
Salmo salar (Teleostei) + div. Bacteria	MHC class II + microsatellites (h)	Coadaptation	Increases MHC diversity in regions with warmer temperature, maybe inferred by higher bacterial diversity. Microsatellites did not show this pattern	Dionne et al. (2007)
Pagellus (Teleostei) + *Lamellodiscus* (Monogenea)	ITS1 + SSU rDNA (p); cytochrome *b* (h)	Phylogeny	Old divergence time or rapid molecular evolution among parasite species; coevolutionary host–parasite interactions occur	Desdevises et al. (2000)
Sparidae (Teleostei) + *Lamellodiscus* (Monogenea)	16S + cytochrome *b* (p); SSU rDNA (h)	Phylogeny	Absence of widespread cospeciation process; association more due to ecological factors than coevolutionary processes	Desdevises et al. (2002a)
Sparidae (Teleostei) + *Lamellodiscus* (Monogenea)	16S (h) + cytochrome *b*; partial SSU rDNA (p)	Phylogeny	Host specificity in *Lamellodiscus* species constrained by phylogeny and also linked to host size	Desdevises et al. (2002b)

Oncorhynchus tshawytscha, (Teleostei) + div. Bacteria	16s rDNA (p) + MHC class I–A1 and class II–B1 + microsatellites (h)	Coadaptation	Bacterial community similarity was not related in genetic MHC or microsatellite variety	Evans and Neff (2009)
Rhinobatid rays (Chondrichtyes) + *Branchotenthes octohamatus* and *Calicotyle australis* and *Pseudoleptobothrium aptychotremae* (Monogenea)	ND4 + cytochrome *b* (h, p) + EF1a (p)	Phylogeny	Monogeneans under study are not strictly host specific although some host preferences are indicated	Glennon et al. (2008)
Atlantic salmon (Teleostei) + *Lepeophtheirus salmonis* (Copepoda)	Microsatellites (p)	Pop gen	Weak, but significant population variation throughout northern Atlantic	Glover et al. (2011)
Div. marine crustaceans (Crustacea) + *Hematodinium* sp. (Alveolata)	ITS1 + ITS2 secondary structures (h, p)	Phylogeny	*Hematodinium* isolates from North Atlantic crustaceans fall into distinct genotypic groups that correspond to host species, but there is no geographical correlation with geographical origin	Hamilton et al. (2010)
Pomatoschistus sp. (Teleostei) + *Gyrodactylus* sp. (Monogenea)	V4 region of SSU rDNA + complete ITS (p)	Phylogeny	Allopatric speciation seems to be the dominant mode of speciation with a possible case of sympatric speciation	Huyse et al. (2003)
Pomatoschistus sp. (Teleostei) + *Gyrodactylus* sp. (Monogenea)	V4 region of SSU rDNA, complete ITS (p) ITS1, and 12S and 16S mtDNA (h)	Phylogeny	Phylogenetically conserved host-switching mimics the phylogenetic signature of cospeciation	Huyse and Volckaert (2005)

Continued

Table 4.1 Genetic marine studies that emphasize coevolutionary host–parasite processes, including euryhaline Salmonidae and sticklebacks—cont'd

Host–parasite system	Molecular marker used	Molecular approach	Pattern observed	References
Meoma ventricosa and *Plagiobrissus grandis* (Echinoidea) + *Dissodactylus primitivus* (Decapoda)	Microsatellites (h, p)	Phylogeny	No spatial or host-dependent differentiation detected	Jossart et al. (2013)
Diplodus (Teleostei) + *Macvicaria crassigula* and *Monorchis parvus* (Digenea)	ITS1 (p) + cytochrome *b* (h)	Phylogeny	Distribution of parasites are not due to coevolutionary interactions but perhaps associated with host ecology	Jousson et al. (2000)
Zeacumantus subcarinatus (Gastropoda) + *Maritrema novaezealandensis* and *Philophthalmus* sp. (Trematoda)	COI (p)	Pop gen	Contrasting diversity and population structure between trematode species which may result from distinguished effective population sizes and/or life history traits	Keeney et al. 2009
Gammarus duebeni (Amphipoda) + Myxozoa	SSU rDNA (h, p)	Phylogeny	Multiple infections of host populations by different microsporidian strains of the same species, phylogeographical associations not assessed	Krebes et al. (2010)
Div. marine hosts including whales, crustaceans, fish and cephalopods + *Anisakis* sp. (Nematoda)	Complete ITS (p)	Phylogeny	Anisakid nematodes might be useful as biological indicators for their final host distribution and abundance as they follow trophic relationships among hosts	Kuhn et al. (2011)

Salmo solar and *Oncorhynchus mykiss* and *Thymallus thymallus* (Teleostei) + *Gyrodactylus salaris* (Monogenea)	ITS1 + ITS2 + COI (p)	Phylogeny	Parallel to allopatric mode, host switch and instant isolation by host specificity can be operated as a speciation mechanism	Meinilä et al. (2004)
Lutjanidae + Haemulidae (Teleostei) + *Retrovarium* (Trematoda)	LSU rDNA + ITS1 + ITS2; 16S + cytochrome *b*	Phylogeny	High host specificity in most species but absence of strict coevolution or codescent	Miller and Cribb (2007)
Thunnus orientalis and *T. thynnus* (Teleostei) + Didymozoidae (Trematoda)	LSU rDNA + ITS2 + COI	Phylogeny	Habitat is the leading driver of shaping didymozoid phylogenetic relationships within their hosts	Mladineo et al. (2010)
Pagrus pagrus (Teleostei) + *Pleistophora* sp. (Myxozoa)	SSU rDNA (p)	Phylogeny	Small subunit rDNA sequence determines a likely new Myxozoa species	Morsy et al. (2012)
Gasterosteus aculeatus and *Pungitius pungitius* (Teleostei) + *Schistocephalus* sp. (Cestoda)	COI + NADH1 (p)	Phylogeny	Antagonistic coevolution between two cryptic parasite species	Nishimura et al. (2011)
Five teleost host species + *Tentacularia coryphaenae* (Cestoda)	LSU rDNA + mtDNA (COI-16S) (p)	Phylogeny	All cestode morphotypes belonged to the same species with only few genetic differences	Palm et al. (2007)
Lithognathus mormyrus (Teleostei) + *Ceratothoa italica* (Isopoda)	Microsatellites	Pop gen	Both host and parasite populations were not differentiated for neutral genetic variation and were likely to exchange migrants	Sala-Bozano et al. (2012)

Continued

Table 4.1 Genetic marine studies that emphasize coevolutionary host–parasite processes, including euryhaline Salmonidae and sticklebacks—cont'd

Host–parasite system	Molecular marker used	Molecular approach	Pattern observed	References
Anguilliformes, Siluriformes, Cypriniformes, Osmeriformes, Salmoniformes, Gasterosteiformes, and Perciformes (Teleostei) + *Proteocephalus* (Cestoda)	SSU rDNA (h, p)	Phylogeny	Lack of congruency between parasite and host phylogenies; rather host-switching than cospeciation events	Skerikova et al. (2001)
Conger conger (Teleostei) + *Lecithochirium fusiforme* (Trematoda)	Allozymes (p)	Pop gen	Low genetic differentiation between populations	Vilas et al. (2003)
Kudoa thyrsites (Myxozoa) (p)	SSU rDNA (p)	Phylogeny	Four phylogenetic separations broadly corresponded to geographic regions and low host specificity and high gene flow within each region	Whipps and Kent (2006)
Cyprinidae + Salmonidae + Percidae + Esocidae + Gasterosteidae + Gobitidae (Teleostei) + *Gyrodactylus* (Monogenea)	Complete ITS (h, p)	Phylogeny	Molecular and ecological evolution rate of *Gyrodactylus* parasites is manyfold in comparison to hosts. Phylogenies largely independent and disconnected	Ziętara and Lumme (2002)

h = host; p = parasite.

hosts has been revealed in several studies (e.g. Criscione et al., 2005; Jobet et al., 2000; McCoy et al., 2005; Mulvey et al., 1991; Nieberding et al., 2004; Prugnolle et al., 2005).

Unravelling the historical biogeography of populations and identifying genetic subdivisions within species are major objectives of phylogeographic studies, and such an approach often provides baseline information on host–parasite coevolutionary relationships (Avise, 2000). The identification of distinct evolutionarily units is important in management strategies (Bermingham and Moritz, 1998), and genetic tagging is a valuable but thus far underexploited resource for conservation biologists (Nieberding and Olivieri, 2007). For example, identifying the accurate host range of parasites can be of enormous value for predicting the threat of introduction of potential pathogens and for managing existing diseases (Burger and Adlard, 2011). At the same time, understanding the determinants of host specificity is essential for the control of parasitic zoonoses (Secord and Kareiva, 1996). Furthermore, in light of emerging diseases, the spread of drug resistance, and the potential effects of habitat alterations and climatic changes on parasite transmission (Crompton, 1999; Daszak et al., 2000; Dobson and Foufopoulos, 2001; Harvell et al., 2002; Roper et al., 2003), it will be critical to develop predictors of gene flow among populations of parasites. A molecular approach of investigating host–parasite systems provides a new and stimulating avenue in answering these currently important research questions.

This chapter aims to bring together relevant papers dealing with molecular studies of marine organisms (inclusive of the euryhaline Salmonidae and Gasterosteidae for which a significant portion of papers are available) and their parasites. Wherever possible, we have included papers aimed at resolving population structure for both the host and the parasite, but some papers only included population structuring in the parasite. Although these do not shed light into host–parasite evolutionary relationships, they nevertheless add vital insight into the genetic structure of parasites, some of which are defined by diverse life history stages. Such a growing body of information may well allow us to predict expected genetic structuring for parasites with similar life histories.

We first discuss possible confounding factors of population genetic patterns and then review currently utilized genetic markers in marine host–parasite systems. We give an overview of which molecular methodologies resolve host–parasite relationships best and also provide insight into recently developed approaches using functional markers, which allow resolution at contemporary, rather than historical timescales. Such approaches can also

provide direct information on selective processes of individuals with their environment.

2. FACTORS THAT MAY CONFOUND ELUCIDATION OF COEVOLUTIONARY PATTERNS

It has been suggested that genetic information of parasites can complement genetic data from their host species (Nieberding and Olivieri, 2007; Wirth et al., 2005). Since parasites are dependent on their hosts, speciation in the latter is likely to induce speciation in the parasite, resulting in mirror-image phylogenies referred to as Fahrenholz's rule (Page, 1994; Page and Hafner, 1996; Poulin, 1998). Examining patterns that show congruence in the evolutionary trajectories of their hosts is complex, because 'evolutionary congruence' between host and parasite associations is often lacking or unresolved and many associations represent a combination of cospeciation and host switching with subsequent colonization events (Caira and Jensen, 2001; Paterson and Banks, 2001; Rannala and Michalakis, 2003). Host-switching events are common (e.g. Huyse and Volckaert, 2005; Meinilä et al., 2004; Ziętara and Lumme, 2002) and the extinction of a parasite in some lineages but not others may also confound extant genetic structuring (Johnson et al., 2003).

However, a foundation for any coevolutionary study of a host–parasite system is the correct identification of the study species (Caira and Jensen, 2001); therefore, the occurrence of cryptic species is a definite obstacle to unravelling true evolutionary relationships. Cryptic species are those in which the genetic variation between related species is less conserved than morphological characteristics (Jousson et al., 2000), and thus, they cannot be separated by morphological studies alone. Cryptic species have been detected in several parasite taxa (de León and Nadler, 2010), and parasitologists have recently put more emphasis on their discovery with the help of molecular markers (Mladineo et al., 2010). Genetic approaches have helped resolve species questions in parasites, as they are able to utilize measures of sequence distance/divergence between lineages and therefore aid in the definition of distinct genetic lineages that may correspond to cryptic species. Several papers give sequence distance as a percentage or discuss certain guidelines, which can point the researcher in deciding between scenarios of cryptic species or deep genetic breaks between populations (e.g. Johns and Avise, 1998; Palumbi et al., 2001, but see Cognato, 2006; Hudson and Turelli, 2003; Knowles and Carstens, 2007). However, there are no

explicit rules that define the amount of divergence in any DNA segment to justify a new species (Meinilä et al., 2004) and the debate is still open. Specifically for parasite species, there is a distinct lack of baseline data, and as such, there is a distinct need for approaches that include morphological characteristics and molecular analyses for elucidating parasite taxonomy and systematics.

Another prominent obstacle in understanding the coevolutionary patterns of parasites and their hosts is probably the paucity of knowledge of parasite ecology, including their life cycles (Criscione et al., 2005). This is particularly important as the depth of the signal of molecular association varies significantly between parasites that have direct life cycles and utilize a single host, compared to parasites with indirect life cycles that depend on several intermediate hosts, but for which only the association between the final host and the parasite might have been studied (Nieberding and Morand, 2006; Page et al., 2003). The intermediate hosts of parasite larval stages are generally invertebrates, which are more sessile in their adult phase and thus have more limited dispersal abilities compared to the final host species, which are often vertebrates with much wider geographic dispersal capabilities (Cribb et al., 2001). The genetic tree structure of the intermediate host is therefore likely to differ from that of the parasite because parasite gene flow is actually defined by the most dispersive host (Keeney et al., 2009; Prugnolle et al., 2005); this can confuse and obscure phylogenetic signals and thus hinder coevolutionary inferences in the host–parasite system of interest. As such, resolving the biological life history of the parasite, including the number of host species required in completing a life cycle, makes it significantly easier to interpret signals of population structure (Fig. 4.1).

Most cases of congruence between host and parasite genetic structure have been observed in highly specific interactions, whereas generalist parasites seem to evolve more independently from their hosts (reviewed in Nieberding and Morand, 2006 and references therein). However, obtaining a reliable assessment of specificity and generalism can be difficult because both can be pure transitional states instead of fixed traits (Nosil, 2002). A specialist parasite species, which colonizes a new host, might first become a generalist to increase its host range and subsequently still become a specialist on the new host(s) (Huyse and Volckaert, 2005). Moreover, host specificity can vary between different parasite development stages. The free-swimming miracidial stage of a trematode, for instance, infects a molluscan first host usually with strict specificity, while the degree of specificity of the metacercarian stage and definitive host are usually broader (Gibson

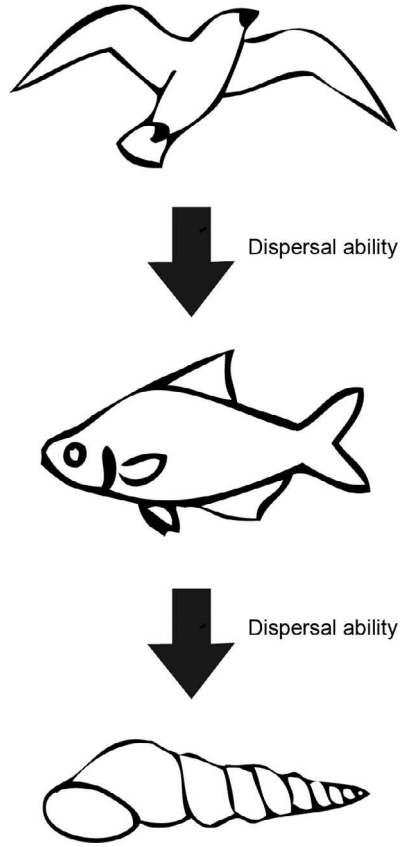

Figure 4.1 Understanding the life history of parasites is crucial, especially for species with multiple hosts. Population genetic patterns of the parasite should be defined by the most dispersive host, since even in a nondispersing host (where one might expect a pattern of high population genetic structure), population genetic signals may reveal little or no structuring.

and Bray, 1994; Jousson et al., 2000; Nunez and De Jong-Brink, 1997). It has been argued that host specificity is usually linked with cospeciation processes (Kearn, 1994; Poulin, 1992) and that specificity is inversely related to host range. Therefore, a decrease in specificity might be mirrored by a range increase of the host. In addition, specialization can also predominantly be under the influence of ecological factors (Bentz et al., 2001; Desdevises et al., 2002a,b). For example, the association between digenean families and their vertebrate taxa is often defined by ecophysiological similarity rather than phylogenetic relationships (Cribb et al., 2001), which may mask insights into coevolutionary processes.

Furthermore, sampling effort can confound reliable assessments of specificity. Assessing the full extent of host and parasite ranges is dependent on research effort; parasite (and host) sampling can sometimes be difficult and dangerous, which additionally hampers sampling effort. Therefore, the true range of hosts and their parasites may not be adequately represented (Burger and Adlard, 2011). Low parasite prevalence and intensity may also obscure the true host range, and inaccurate host–parasite identification will further add to the problem (Glennon et al., 2008; Poulin and Morand, 2000). Cribb et al. (2001) reviewed the association among digeneans, molluscs and fishes and highlighted that all the available data to be analysed are biased in the way it has been collected. Thus, 'digeneans of tetrapods are better known than those of fishes; digeneans of freshwater and terrestrial systems are better known than those of marine systems; and temperate systems are better known than tropical systems' (Cribb et al., 2001).

It is important to consider the scale (temporal, spatial and taxonomic level) at which coevolutionary associations between hosts and parasites are assessed (reviewed in Hoberg and Klassen, 2002). Parasite taxa, which are initially identified as species-specific, may be shown to belong to a parasite clade that actually parasitizes a larger clade of hosts when subsequent additional analysis is conducted. Additionally, scale can affect the biogeographic component of coevolutionary interactions (Hoberg and Klassen, 2002; Klaasen, 1992). Investigations on a global scale may, for instance, reveal repeated biogeographic patterns of coevolutionary events across different host groups that could not be detected in single species analyses (Klaasen, 1992).

Attempts have been made to understand the processes leading to speciation of parasites, and as for many taxa, the dominant mode of speciation in most host–parasite systems appears to be allopatric (Hoberg and Klassen, 2002; Huyse and Volckaert, 2005), but sympatric speciation may also occur (Jousson et al., 2000; ŜImková et al., 2004). Allopatric speciation events due to sea-level change and related shifts to habitats have been widely documented for many marine species (see, e.g. Hubert et al., 2012; Shafer et al., 2010). However, whether such large-scale changes also affect population divergence and speciation rates in marine parasites has yet to be thoroughly tested. Additional difficulties concern cryptic microgeographic population structures, that is, cryptic parasite subgroups within a host (Criscione et al., 2011; Glennon et al., 2008). For example, a recent study on the relatedness in the Kudoidae pointed out that relationships are rather reflected by the site of infection within the host rather than host identity *per se* (Burger et al., 2007); therefore, the presence of one or more parasitic lineages on the same

host can result in divergent host and parasite phylogenies (Criscione et al., 2011; Page, 1993). Moreover, coinfecting parasite strains can increase or decrease the effect of each other's selection impact on the host (de Roode et al., 2005; Maitland et al., 1997; May et al., 2000), and antagonistic effects of different parasite communities can cause deviant structures through cross-immunity or immunosuppression (Lello et al., 2004).

Mixed parasite assemblages on a single host species can also add to confounding effects in resolving coevolutionary patterns of marine parasites and their hosts. For example, an infrapopulation may actually contain several parasite populations (Jarne and Theron, 2001), and if different allele frequencies are pooled and considered as a single unit, this may cause a heterozygote deficit due to the Wahlund effect (Criscione et al., 2011; Vilas et al., 2003). Important to note is that single populations of parasites are often temporary systems and can be patchy, with low effective population sizes due to genetically identical individuals as a result of asexual reproduction (Keeney et al., 2009; Vilas et al., 2003). As effective population size influences the relative rate of gene flow (because genetic drift is usually more pronounced in species with a smaller effective population size), discrepancies between effective population sizes in different parasite species and hosts may therefore result in differences in genetic diversity. Since effective population sizes may vary even at small scales, this should be taken into account, as it may skew interpretations of population genetic structure.

All the factors mentioned earlier result in complex interplays between life history and environmental variations over different spatial and temporal scales (Fig. 4.2), which define the genetic coevolutionary structures of

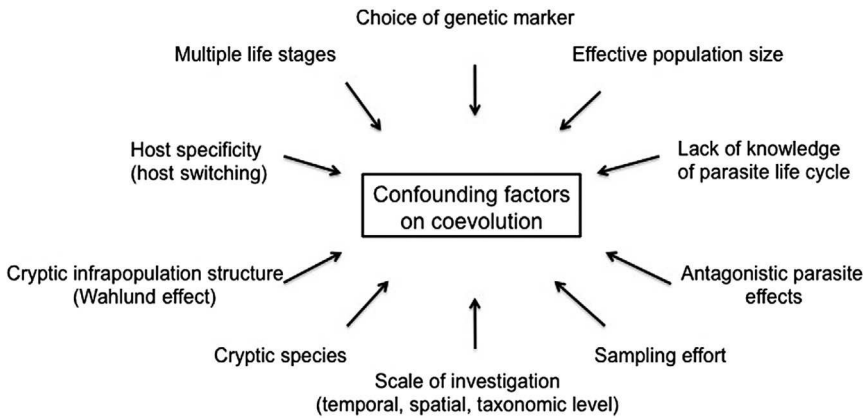

Figure 4.2 Factors confounding the elucidation of host–parasite coevolution.

parasites and hosts (Chopelet et al., 2009; Sala-Bozano et al., 2009). Caira and Jensen (2001) therefore proposed five key points of considerable importance to any study of coevolution, which are as follows: (1) both the host and the parasite groups are monophyletic; (2) both the hosts and parasites have been correctly identified to species; (3) reasonably accurate estimates of host and parasite phylogenies are available; (4) the hosts have been sampled comprehensively for parasites; and (5) the parasites exhibit a considerable degree of specificity for their hosts. In marine systems, this represents a considerable challenge, given the broad geographic distributions of many host species. Therefore, good candidates for host–parasite coevolutionary studies might focus on narrow-range, endemic species as a starting point.

3. WHAT TYPES OF MARKERS RESOLVE MARINE HOST–PARASITE EVOLUTIONARY RELATIONSHIPS THE BEST?

Molecular ecological studies on parasites in marine systems have lagged far behind those on free-living organisms, and there is a paucity of population genetic studies given the taxonomic and life history variation found among them (Criscione et al., 2005, 2011). Thus, the exploration of molecular markers is still very limited in parasite species, and based on the existing literature, it is difficult to generalize which are the most suitable for resolving relationships between host and parasites. An important first typical step is the choice of the appropriate marker to disentangle cryptic species in different parasite groups—one of the most prominent obstacles and confounding factors in most marine host–parasite studies. For subsequent additional analyses, in general, markers should be chosen based on the research question being asked (Lymbery and Thompson, 2012; Monis et al., 2002; Sunnucks, 2000). Furthermore, the choice of marker is often dependent on data already available, on which a new study may be built. Here, we review the most popular markers and their applicability from recent marine host–parasite studies.

3.1. Nuclear DNA

3.1.1 Internal transcribed spacer

The internal transcribed spacer *(ITS)* is part of the rDNA array and lies between the *SSU* rDNA and the *LSU* rDNA coding regions. It is divided into *ITS1* and *ITS2*, which are separated by the gene coding for *5.8S* rDNA. In the past, it had been assumed that the *ITS* had no function, but Lalev and

Nazar (1998) proposed that *ITS1* may affect the efficiency or play a supporting role in ribosome biogenesis. Additionally, conserved regions in internal transcribed spacers have been found among diverse eukaryotes that indicate a function of *ITS2* in pre-rDNA maturation (Coleman and Mai, 1997; Jospeh et al., 1999; Peculis and Greer, 1998). The *5.8S* rDNA is relatively slow-evolving, while the *ITSs* are less conserved. Different studies reported that *ITS1* is more variable than *ITS2* (van Herwerden et al., 1999; Tkach et al., 2000; Vilas et al., 2005). This is likely because of the presence of variable repeat units that are generally missing in the *ITS2* (Nolan and Cribb, 2005). Relatively conserved regions within the *LSU*, *SSU* and *5.8S* rDNA have made the development of primers possible that amplify across many eukaryote groups and *ITS* is a popular nuclear marker; it was the most commonly used nuclear gene fragment in our reviewed studies (Table 4.1).

The internal transcribed spacer 1 has been successfully used to test for cryptic species in digeneans. Jousson et al. (2000), for instance, discovered cryptic diversity among digenean parasites of Mediterranean *Diplodus* fishes, while Criscione and Blouin (2004) used *ITS1* sequences to detect cryptic species in salmon trematodes. It could be also applied to identify worm species within monogeneans (Raeymaekers et al., 2008; Ziętara and Lumme, 2002) and presents the greatest documented variability of all markers explored to date for this class of parasites (Olson and Tkach, 2005).

Desdevises et al. (2000) used *ITS1* sequences of *Lamellodiscus* spp. (Monogenea) to assess their level of interspecific differences from fish host species from the genus *Pagellus* in the north Mediterranean Sea. They discovered high variability, which suggests a divergence time in ancient history or a rapid molecular evolution within this genus. In a study by Huyse and Volckaert (2005), *ITS1* delivered information about the phylogenetic relationships between monogenean species and goby hosts. Marine nematoda of the genus *Anisakis* showed two fixed differences in *ITS1* between species (Ceballos-Mendiola et al., 2010), yet no variability was found in other studies, which used *5.8S* or *ITS2* fragments. Ceballos-Mendiola et al. (2010) claimed that identifications of *Anisakis*, which are only based on *5.8S* or *ITS2*, are not justified and recommend the use of more variable markers, including mitochondrial DNA (mtDNA).

The internal transcribed spacer 2 has been used extensively in trematode identification, because it is usually conserved within species but more variable among species (Cutmore et al., 2010; Nolan and Cribb, 2005) and it has been successfully used to interpret trematode species identity within certain elasmobranchs (Cutmore et al., 2010). Both *ITS1* and *ITS2* sequences

were applied for phylogenetic reconstruction (Ziętara et al., 2002) and population dynamics (Meinilä et al., 2004) of *Gyrodactylus* species (Monogenea). The genetic diversity of the crustacean parasite from the genus *Hematodinium* (Alveolata) was investigated by Hamilton et al. (2010), who used *ITS1* and *ITS2* secondary structures. They argue that in alveolates, sometimes highly variable but almost identical *ITS* sequences may not prove same species and conclude that more conserved *ITS2* secondary structures can be a good tool for distinguishing among organisms with highly similar morphologies. Analyses showed that *Hematodinium* from the east and west North Atlantic comprised distinct ribotypes, which however did not correspond to a specific area but varied in host specificity.

Parasites, such as trematodes, have proven to be useful research subjects, especially regarding sequence divergence studies. There are more cryptic species of trematodes found than in other helminth taxa (Poulin, 2011), supposedly because of their lack of morphological structuring, which makes this group particularly interesting in the light of understanding genetic and morphological divergences in parasites. As such, marine trematodes have been relatively well studied using molecular markers. For example, Ziętara and Lumme (2002, 2003) suggested a divergence of 1% in *ITS* for *Gyrodactylus* (Monogenea) as a practical species break, since species of this genus are morphologically difficult to distinguish. Hayward et al. (2001) used the whole ribosomal *ITS* region (*ITS1*, *5.8S* rDNA and *ITS2*) and discovered that within *Gyrodactylus anguillae*, it was extremely conservative across wide geographic ranges. Desdevises et al. (2002a,b) reported extremely high overall *ITS* variability among closely related monogenean species, which made it difficult to align sequences. Therefore, they recommend the use of *SSU* rDNA instead of *ITS* for better resolution of phylogenetic relationships. In contrast, Jousson et al. (1999) used the entire *ITS* region and discovered a rather low variation among digeneans but also detected that some genera were more variable than others. The ribosomal *ITS* region (in addition to *LSU* rDNA, see the succeeding text) was furthermore applied to match life-cycle stages of the trematode *Phyllodistomum folium* recovered from their intermediate hosts (bivalves) and from the three-spined stickleback (*Gasterosteus aculeatus*; Petkeviciuite et al., 2004). However, studies based on *ITS* also need to be mindful of several drawbacks that have been identified with this marker class. Vilas et al. (2005) discuss that 'undetected paralogy, incomplete lineage sorting and introgressive hybridization could mislead a prospective study using *ITS* sequences'. They additionally comment on the obstacle of possible pseudogenes, which can be difficult to

detect as *ITSs* are not protein-coding genes. Therefore, unlike the widely used mtDNA genes, which can be translated, there is greater uncertainty regarding their usage (Benasson et al., 2001). Furthermore, platyhelminths, for example, accumulate nucleotide substitutions at a much higher rate in mtDNA *COI* and *NADH1* sequences than *ITS* (up to 10% in mtDNA vs. about 1% at *ITS*), and it is suggested that a difference of 5% at mtDNA markers should drive further investigations in testing for cryptic species (Vilas et al., 2005).

3.1.2 Small (SSU rDNA) and large (LSU rDNA) subunit ribosomal gene

The *SSU* rDNA mediates interactions between anticodons of the tRNAs and the codons in the mRNA to determine the order of amino acids in the protein being synthesized (Steitz, 2008). Unlike *ITS*, they are longer and have extremely conserved regions interspersed with more (sometimes highly) variable regions. As for *ITS*, several primers exist that are able to amplify these gene regions (or parts thereof) across a wide range of taxa. Desdevises et al. (2000) applied the *SSU* rDNA marker to investigate phylogenetic relationships among *Lamellodiscus* (Monogenea) species, and their *Pagellus* fish hosts and found signs for coevolutionary host–parasite interactions. The *SSU* rDNA was also used by Karlsbakk and Nylund (2006) for clarifying the identity of marine teleost trypanosomes from cod (*Gadus morhua*) in the North Atlantic. In this study, the authors strongly advocate trypanosome taxonomy with molecular methods like *SSU* rDNA sequencing. Molecular analysis of the diversity of myxozoan parasites is currently based on investigations of the *SSU* rDNA (Krebes et al., 2010; Morsy et al., 2012; Whipps and Kent, 2006 and references therein). The *SSU* rDNA marker could even be used to detect cryptic myxozoan species within the same trout host (*Percopsis omiscomaycus*) but in different tissue types (Easy et al., 2005). Burger et al. (2007) questioned the suitability of *SSU* rDNA in resolving species issues by showing that morphologically distinct forms of *Kudoa* sp. did not show any variances in that region. In contrast, Whipps and Kent (2006) used the *SSU* rDNA marker for the identification of distinct regional representatives of the cosmopolitan marine parasite *Kudoa thyrsites* (Myxozoa) on a global scale. Using a phylogenetic approach, they recovered four major groups, which broadly corresponded to the geographic regions of Japan, Australia, the eastern Pacific and the eastern Atlantic. Within these four regions, *SSU* rDNA showed little population subdivision, suggesting low host specificity and high gene flow. *SSU* rDNA sequences were also used to screen amphipod species (*Gammarus duebeni*)

from marine and freshwater populations and detected multiple infections with different microsporidian strains; however, their phylogeographic associations could not be assessed (Krebes et al., 2010). In a recent study by Atkinson et al. (2011), population structure based on *SSU* rDNA of the myxozoan *Parvicapsula minibicornis* could be detected. Structure was associated with river system and salmonid fish host species, but the variation was less than the 2–10% generally published for morphologically distinct species of myxozoans (Arsan et al., 2007; Ferguson et al., 2008), suggesting population genetic differentiation based on geography and salmonid host species. Krebes et al. (2010) suggest the largest subunit of RNA polymerase II (*RFB1*) might be a good marker to distinguish between closely related species myxozoans. This region evolves approximately four times faster than *SSU* rDNA (Cheney et al., 2001).

The *LSU* rDNA acts as a peptidyl-transferase ribozyme, which catalyses the formation of peptide bonds in the growing polypeptide (Steitz, 2008; Steitz and Moore, 2003). Burger and Adlard (2010) showed that *LSU* rDNA has a greater resolving power to genetically distinguish morphological variations of *Kudoa* sp. compared to *SSU* rDNA. Whipps and Kent (2006) utilized *LSU* rDNA in their *K. thyrsites* study, and results corresponded to the four major phylogenetic grouping gained from the *SSU* rDNA analyses mentioned earlier. The *LSU* rDNA was also applied in a study by Palm et al. (2007), which investigated intraspecific genetic variation of the cestode species *Tentacularia coryphaenae* in four teleost host species. Large subunit ribosomal gene results confirmed that all specimens belonged to the same cestode species and showed similar patterns to mtDNA markers, which were additionally used in this investigation (see Section 3.2). Mladineo et al. (2010) revealed with the *LSU* rDNA marker (among others) that habitat selection has been a leading force in shaping didymozoid (Trematoda) phylogenetic relationships within Pacific (*Thunnus orientalis*) and Atlantic (*T. thynnus*) bluefin tunas. Large subunit ribosomal genes have also been used to examine trematodes from the Gorgoderidae family within six different species of elasmobranchs (Cutmore et al., 2010). Although trematode specimens were morphologically variable, *LSU* rDNA (which showed coherent results to *ITS2*) revealed identical sequences from parasites collected from three host families and two host orders, which implies remarkably low host specificity and showed morphological plasticity within these parasites. Miller and Cribb (2007) applied *LSU* rDNA (among *ITS1* and *ITS2*) to explore the host and geographic distribution and integrity of trematode species belonging to *Retrovarium* from various Lutjanidae and

Haemulidae species and discovered that the sequence results correlated perfectly with the separation of species supported by morphology. Petkeviciuite et al. (2004) used the D1–D3 region of the *LSU* rDNA sequences (in addition to *ITS*) to successfully identify and match life-cycle stages of the trematode *P. folium*.

3.1.3 Microsatellites

Microsatellites are codominant makers with repetitive sequences of DNA, which are widely dispersed along and among chromosomes (Chistiakov et al., 2006; DeWoody and Avise, 2000). They are known for high mutation rates of 10^{-2} to 10^{-6} mutations per generation (Ellegren, 2000) in contrast to regular nonrepetitive DNA (10^{-9}; Li, 1997). Because of their high mutation rates, microsatellites are more likely to disentangle fine-scale population structuring, which probably reflect contemporary rather than deeper evolutionary events (Hewitt, 2004; but see also Karl et al., 2012). Microsatellites are generally considered to be neutral markers but are often linked to genomic regions, which are under natural selection (Ellegren, 2004; Laine et al., 2012). Another complication for studies using microsatellites can arise from potential homoplasy, the independent mutation of microsatellite markers to the same size (Estoup et al., 2002) and the regular occurrence of null alleles (Brown et al., 2005). Importantly, although microsatellites have been used extensively to determine population structures of marine species and to delineate marine fish and invertebrate stocks (see review by Chistiakov et al., 2006), so far, they have received limited attention in marine parasite phylogeographic research.

In a recent study, microsatellite analysis of trematode parasites has been used to identify the origins of steelhead trout (*Oncorhynchus mykiss*; Criscione et al., 2006). Here, the likelihood of correct assignment was four times greater with the parasite's genotypes, which was greater genetically structured than with the host's genotypes. Criscione and Blouin (2007) also chose microsatellite markers in a study of a trematode species (*Plagioporus shawi*) and found congruent population genetic patterns with its salmonid hosts (*Oncorhynchus* spp.). In a different investigation, seven microsatellites were used successfully to resolve cryptic microgeographic population structures of a marine trematode (*Lecithochirium fusiforme*) within and among individual conger eel (*Conger conger*; Criscione et al., 2011). Glover et al. (2011) used 14 microsatellite loci to reveal significant population genetic variation throughout the Atlantic Ocean in the sea louse copepod (*Lepeophtheirus salmonis*) within Atlantic salmon (*Salmo salar*). Salmonids and especially

Atlantic salmon lend themselves as model organisms for these types of studies given their population genetic structuring due to habitat shifts associated with glaciations, 'landlocking' of fjords or lakes previously open to the sea as well as various sources of anthropogenic changes (Bourret et al., 2013; Verspoor et al., 2007). Whether patterns of congruent genetic signals from microsatellites of both hosts and parasites also hold for obligate marine species remains largely unexplored and requires future investigations. A recent study by Jossart et al. (2013) applied microsatellites to investigate the potential host specialization in a parasitic pea crab (*Dissodactylus primitivus*) in two echinoid species from different geographic regions. However, microsatellite analyses did not detect spatial differentiation or differentiation according to host species, which points to random mating and high gene flow between parasitic crabs from the two hosts. A further study by Sala-Bozano et al. (2012) applied microsatellites to investigate neutral genetic variation in an isopod mouth parasite (*Ceratothoa italica*) and its fish host, *Lithognathus mormyrus*. Here both host and parasite populations were not genetically differentiated and thus likely exchanged migrants.

An additional constraint of the successful application of microsatellites to parasite studies has been the high cost of developing libraries. We anticipate however that new technologies like next-generation sequencing will improve on the current lack of microsatellite data (Ekblom and Galindo, 2011) and their usage in marine parasite and host–parasite coevolutionary studies. New microsatellite discovery of parasites, pathogens and other non-model organisms in the terrestrial literature has already begun (see, e.g. Santana et al., 2009; Schoebel et al., 2013), and we hope that marine studies will not lag far behind.

3.2. mtDNA

The mitochondrial genome (mtDNA) and its different functional gene units are popular molecular markers for invertebrate and vertebrate studies because of their general lack of recombination (Hickerson et al., 2010). Nevertheless, the substitution rates among genes are variable (10^{-1} to 10^{-3} mutations per generation; Baldwin et al., 2011; Vilas et al., 2005), and its putative neutrality, its smaller effective population size and also its limited size, which makes it relatively simple to determine homologies or gene rearrangements (Boore and Brown, 1998; Hickerson et al., 2010), are of advantage. Several generic primers are available that amplify across a wide variety of phyla; further mtDNA genes can be used for both phylogeographic and phylogenetic studies. mtDNA has been used, for

instance, in phylogenetic analysis as operational taxonomic units (Avise et al., 1987; reviewed in Hickerson et al., 2010), as molecular clocks (Bermingham et al., 1997) and to gain insights into sex-biased phenomena due to the nature of its inheritance (Avise, 2000). Furthermore, it has been applied extensively for elucidating population genetic patterns in marine species, but it has been pointed out that it may not be suitable for detecting more recent population or species-level divergences (reviewed by Baldwin et al., 2011; Hewitt, 2004, but see also Karl et al., 2012). However, Vilas et al. (2005) showed that the higher rate of evolution and the smaller effective population size of two mtDNA loci detected diagnostic characters between cryptic platyhelminth parasite species better than *ITS* regions.

Most studies that have used mtDNA in marine organisms utilize the *cytochrome oxidase c subunit I gene (COI)*, which encodes a key enzyme that catalyses the reduction of oxygen to water in aerobic metabolism. The *COI* is also the standardized marker of choice for DNA bar coding in animals and many other eukaryote taxa. DNA barcoding is a newly applied method for species for which there is no recorded gene sequence in any database. This method has been recently used to distinguish parasite species (de Buron et al., 2011; Elsasser et al., 2009; McGowin et al., 2011) in combination with morphological taxonomy and is useful since the nucleotide sequence is identical at every developmental stage. Meinilä et al. (2004) used the mitochondrial *COI* marker to increase phylogenetic resolution compared to *ITS1* and *ITS2* and inferred that in a salmonid pathogen *Gyrodactylus salaris* (Monogenea), parallel to an allopatric mode, host switching and instant isolation by host specificity drives speciation. The *COI* also gave better resolution compared to *ITS* at the intraspecific level of different trematode species from the family Didymozoidae in bluefin tunas (*Thunnus* spp.; Mladineo et al., 2010). Keeney et al. (2009) applied *COI* to examine the population structures of the marine gastropod *Zeacumantus subcarinatus* and its trematode parasites. They discovered that the genetic homogeneity of the detected trematode species (*Maritrema novaezealandensis*) might be obscured by demography or natural selection effects acting on the *COI* since neutrality was rejected. However, they argue that high levels of gene flow are a more likely scenario, which was further supported by microsatellite markers of the host in an earlier study (Keeney et al., 2008). In another study, the *COI* gene was used to reveal four distinct genetic clades in two nematode species of the southern flounder (*Paralichthys lethostigma*), which corresponded to four locations of the parasite in the host (de Buron et al., 2011). Crandall et al. (2008) chose *COI* to examine the

genetic structure of two sea star species and their ectosymbionts, a gastropod parasite snail (*Thyca crystallina*) and a shrimp (*Periclimenes soror*). Despite their close physical and ecological associations, all four species showed little common genetic structure, possibly explained by species-specific responses to historical climate change events.

Another applied mtDNA marker in marine host–parasite studies is the *cytochrome oxidase* c *subunit II gene (COII)*. It encodes for subunit II, which transfers the electrons from cytochrome *c* to the catalytic subunit I. Sequences of *COII* were used to investigate the genetic variability of *Anisakis* nematode species within the European hake (*Merluccius merluccius*) and resolved three different haplotypes (Ceballos-Mendiola et al., 2010). The authors furthermore pointed out that the *COII* gene unit would be a potentially good candidate to design PCR-RFLP to be able to differentiate the genus in more detail. They remarked that the usage of *COII* would be an advantage over *ITS* because of higher copy numbers and thus simpler amplification (Ceballos-Mendiola et al., 2010). Jousson et al. (2000) applied *cytochrome* b mtDNA sequences in *Diplodus* hosts and revealed that the distribution of trematode parasites is not a consequence of coevolutionary interactions but rather associated with the ecology of the host.

The *NADH dehydrogenase subunit 1 (NADH1)* encodes for NADH dehydrogenase I, which is necessary for electron transfer during oxidative phosphorylation. This marker was chosen (in combination with *ITS1*) by Criscione and Blouin (2004) to identify cryptic salmon trematode species. A combination of *COI* mtDNA and *NADH1* was used on cestode species within three-spine (*G. aculeatus*) and nine-spine (*Pungitius pungitius*) stickleback hosts (Nishimura et al., 2011). Here, the divisional pattern of mtDNA sequences delivered strong evidence for the presence of two cryptic cestode species, where one exclusively infects three-spine sticklebacks and the other one only the nine-spine stickleback.

Larger regions of mtDNA (*COI-16S*) were applied by Palm et al. (2007) to detect intraspecific genetic variation of cestode species in a variety of different teleost hosts. The results corresponded well with the information received from *LSU* rDNA, which was additionally used in this study. In a study conducted by Criscione and Blouin (2007), a combination of various mtDNA sequence genes of the trematode *P. shawi* showed discordance with results gained from microsatellite loci. Therefore, they advise that mtDNA should not be applied in isolation to analyse the population history of this trematode species. Olson and Tkach (2005), for example, reported that because of fast evolutionary rates, mtDNA genes are not as useful for

resolving platyhelminth systematics, due to the presumed antiquity of the group. Also, Huyse and Volckaert (2005) discussed that the *COI* gene could be too variable for reliable phylogenetic reconstruction of *Gyrodactylus* (Monogenea) species (but see Vilas et al., 2005).

4. WHAT CAN FUNCTIONAL MARKERS TELL US ABOUT LOCAL ADAPTATIONS IN HOST–PARASITE SYSTEMS?

In this chapter, we mainly focused on broader evolutionary relationships between marine hosts and their parasites, but we should not overlook the increasing evidence of finer-scale patterns of local adaptations of parasites and their host species, which take place across contemporary, ecological timescales (Conover et al., 2006).

Most studies investigating host–parasite relationships at evolutionary timescales were primarily based on allegedly neutral markers because they revealed genetic structure of populations or lineages without the confounding effects of selection or environmental influences. For a long time, it was widely believed that local adaptations of marine species with highly dispersive or mobile life stages could only occur on broad geographic scales due to high connectivity among widely distributed populations (Palumbi, 2003; Waples, 1998; Waples et al., 2008). Hence, our knowledge of local adaptation in highly dispersive marine species remains poor.

Only recently, new evidence is emerging that suggests local adaptation is probably quite common in marine organisms and that such adaptation takes place at contemporary timescales (Conover et al., 2006). To gain insights into adaptive coevolutionary processes at ecological timescales and moreover to gain direct information on selective processes involving the interactions of individuals with their environment, higher resolution can often be achieved with functional markers that are under selection. Several studies have shown that the proportion of quantitative trait variation at the population level is much higher than it is for neutral markers (Conover et al., 2006; Glover et al., 2011; Hemmer-Hansen et al., 2007; Nielsen et al., 2009; Westgaard and Fevolden, 2007). Thus far, nonneutral markers have only been applied in potential fish host species; there are no data available on parasites. For example, Hemmer-Hansen et al. (2007) revealed local adaptation in showing that genetic differentiation at an insertion–deletion associated with the heat-shock cognate protein gene *Hsc70* in the European flounder (*Platichthys flesus*) vastly exceeded neutral genetic differentiation. A study by Westgaard and Fevolden (2007) suggested that ongoing selection

over generations led to significant divergence between two nonneutral microsatellite loci and was thus able to deliver additional information for discriminating northeast Arctic and Norwegian coastal cod stocks. Also, single-nucleotide polymorphism (SNP) markers, which have been developed from the alignment of expressed sequence tag for cod and salmon (Hayes et al., 2007; Moen et al., 2008), might be able to deliver new insights into adaptive divergence. Additionally, new general SNP databases for Atlantic herring (*Clupea harengus*), European hake (*M. merluccius*) and common sole (*Solea solea*) have recently been established for fishery control and management purposes (Zelenina et al., 2011). It is therefore important to also consider nonneutral markers for investigating contemporary parasite patterns to the same extent as host species to gain fine-scale resolution into coevolutionary processes.

However, it is encouraged to choose a 'candidate gene approach' and thus markers for genes of a known function as 'this would not only allow recognition of population structure, but also identification of genetically based adaptations to local environmental conditions' (Nielsen et al., 2009). A popular functional marker, which has been under extensive investigation for host–parasite coevolutionary processes of several terrestrial (e.g. Froeschke and Sommer, 2005, 2012; Schwensow et al., 2010) and marine (e.g. Dionne et al., 2007; Evans and Neff, 2009) vertebrate species, is the major histocompatibility complex (MHC). The MHC encodes cell-surface glycoproteins that bind antigens derived from pathogens and parasites and present them to T lymphocytes, thereby initiating the appropriate immune response (Klein, 1986). Genes of the MHC are the most polymorphic loci known for jawed vertebrates (Geraghty, 2002; Trowsdale and Parham, 2004), and varying parasite-mediated selection has been proposed as a major evolutionary force for maintaining such polymorphisms (Eizaguirre et al., 2012). There are several hypotheses that try to explain the mechanism behind the extraordinary variability based on parasite-mediated selection, and they have been part of several reviews already (e.g. Bernatchez and Landry, 2003; Eizaguirre and Lenz, 2010; Milinski, 2006; Piertney and Oliver, 2006; Sommer, 2005; Spurgin and Richardson, 2010; Wegner, 2008).

More recently, the MHC marker has been used to examine selection processes in contemporary generations. For this, studies compared patterns of variation at functional MHC genes with those expected under neutrality (Piertney and Oliver, 2006; Vassilakos et al., 2009). Loci under selection usually show higher differentiation under different local selection pressures or lower differentiation under balancing selection (Schierup, 1998). Overall,

studies on teleosts have yielded mixed results (reviewed in Wegner, 2008), but investigations in salmonids have demonstrated elevated heterozygosity at MHC compared with neutral loci (Aguilar and Garza, 2006; Dionne et al., 2007; Landry and Bernatchez, 2001; Miller et al., 2001). Here, the authors concluded that pathogen-mediated selection across differing ecological environments maintains MHC diversity. An Atlantic salmon study by Dionne et al. (2007) revealed that MHC class II allelic diversity and specifically the diversity at the MHC pathogen-binding region increased with temperature in contrast to neutral microsatellite patterns and linked this with elevated bacterial diversity in warmer thermal regimes. Evans and Neff (2009) conducted another investigation on Chinook salmon (*Oncorhynchus tshawytscha*) to examine whether bacterial infections are the source of selection on the MHC. They found susceptibility associations between a few MHC alleles and specific bacterial parasites and evidence for heterozygote advantage. No bacterial community similarity, which was related to population genetic similarity at either the MHC or microsatellite loci, was reported. Several other recent studies suggested that a mosaic of habitat shapes MHC composition in neighbouring populations, probably facilitated through variations in the parasite community (Alcaide et al., 2008; Babik et al., 2008; Blais et al., 2007; Bonneaud et al., 2006; Eizaguirre et al., 2011; Ekblom et al., 2007; Loiseau et al., 2009; Wegner et al., 2003). Evidence is emerging that locally adapted MHC allele pools are driven by parasite-mediated selection pressures, which may ultimately lead to host speciation (reviewed in Eizaguirre and Lenz, 2010). However, there still is a lack of studies that explicitly demonstrate that the pathogen fauna does or does not vary across populations (reviewed in Spurgin and Richardson, 2010). Specifically in marine and aquatic ecosystems, relationships between MHC adaptations of hosts to multiple parasite communities are still understudied and parasites need to be better characterized to complement host studies.

5. WHICH METHODOLOGIES REVEAL COEVOLUTIONARY RELATIONSHIPS IN MARINE HOST–PARASITES THE BEST?

A tree drawn from a single gene reflects the phylogeny of that gene and not necessarily that of the species (Constantine, 2003). Single-locus DNA data sets are generally insufficient and can be misleading (Criscione and Blouin, 2007), and therefore, it is strongly advised to apply more than one locus or data set and to be aware of the interpretations that can be made

from such studies (Karl et al., 2012). Different molecular markers can be influenced by different responses to evolutionary forces and as such accumulate homoplasies, which can be problematic in phylogenetic and phylogeographic inference. Hence, it is recommended to use a combination of several independent genes with different rates of evolution (Edwards and Bensch, 2009; Hypsa, 2006; Toews and Brelsford, 2012), although we recognize the methodological complexity of this for parasitological studies. Multilocus data can dramatically improve the results (reviewed in Hickerson et al., 2010), and generally, the more independent neutral markers are used, the better the resolution of genealogical history (reviewed in Nieberding and Olivieri, 2007). Several recent studies have used more than one marker, and often, nuclear and mtDNA sequences were chosen to unravel phylogenetic host–parasite structures and thus resolve their evolutionary relationships (Table 4.1). Frequently, analyses of different marker types showed congruent patterns (e.g. Mladineo et al., 2010), but genetic patterns of nuclear genes and mtDNA can also contrast each other (Criscione and Blouin, 2007; Toews and Brelsford, 2012). Several examples show high levels of divergence between mtDNA and nuclear markers, which almost certainly leads to ambiguous interpretations if the genealogical history of only one marker is considered (Criscione and Blouin, 2007; see Toews and Brelsford, 2012 for an in-depth review about discordances in mtDNA and nuclear DNA and how it may influence biogeographic analyses).

Hypsa (2006) recommends the usage of 'idiosyncratic markers' (Avise, 1994; Murrell et al., 2003) that are 'unique features with presumably low probability of homoplasy, such as insertion of mobile elements, gene rearrangements and secondary structure features'. These can provide additional phylogenetic information and resolve relationships; however, their frequency may constrain them to only some taxa (reviewed in Hypsa, 2006). This is because the occurrences of genome rearrangements seem to be random with unknown background and thus not necessarily evenly distributed among taxa.

In the absence of known rare genomic changes or other idiosyncratic markers, whole-genome analyses provide a useful tool for moving away from single or low numbers of marker studies. Teske et al. (2011) encouraged the generation of more multilocus genetic data sets like nuclear sequence data, AFLPs, SNPs and microsatellite libraries for future marine phylogeographic studies. Bullard et al. (2011) successfully used amplified fragment length polymorphism (AFLP) analysis in addition to *ITS1* to investigate the genetic

variation and hence the origins of infrapopulations of the flatworm *Nasicola klawei* (Monogenea) within yellowfin tuna (*Thunnus albacares*). AFLP is a highly sensitive method for analysing genome-wide DNA polymorphism without prior knowledge of the genome. It has been used, for example, in species identification of salmon (Gwo et al., 2008), to analyse phylogenetic patterns (Mendelson and Wong, 2010) and to examine infrapopulation genetic variability (Techaprasan et al., 2008). AFLP was applied for population genetics of nematodes (Gruijter et al., 2006) and flatworms (Bakke et al., 2007; Bullard et al., 2011), although its use has been limited thus far in parasitological studies, especially those in the marine environment.

SNPs are biallelic and occur about every 200–500 bp across the genome in coding and noncoding regions (Morin et al., 2004) and have been shown to have applicability across a large number of research fields such as genome-wide association studies, breeding programmes and disease treatments. They have also been successfully used for the fine-scale assessment of fish stocks and in fisheries forensics (Zelenina et al., 2011). Their use in parasitological studies has thus far remained limited, although SNPs have been employed in parasite strain characterizations (Atkinson and Bartholomew, 2010; Atkinson et al., 2011) and assessments of parasite communities (Rellstab et al., 2011).

The advent of new sequencing technologies (e.g. pyrosequencing) is already advancing the development of molecular markers on a large scale (e.g. Ekblom and Galindo, 2011; Oomen et al., 2013; Schoebel et al., 2013). Yet the use of pyrosequencing in parasitological studies is still rare (Sreekumar et al., 2005; Troell et al., 2003). A recent study by Rellstab et al. (2011) showed that pyrosequencing already allows reliable allele quantification of SNPs in pooled samples of closely related trematodes of the genus *Diplostomum* in freshwater fishes. Furthermore, a study with two species of sea stars (*Meridiastra calcar* and *Parvulastra exigua*) demonstrated that targeted 454 pyrosequencing was simultaneously possible at five different nuclear DNA loci across 16 different populations of 20 individuals of each species (Puritz et al., 2012). The study demonstrated that targeted 454 sequencing is more time- and cost-effective than traditional Sanger sequencing-based methods and that it enables the incorporation of nonmodel organisms into phylogeographic studies. To be able to characterize whole parasite species, communities in pooled DNA samples at once would be an immense technological advancement for future studies. If this could be achieved in parallel for both host and parasite species, host–parasite relationships could be examined at much larger scales, in more detail and at lower cost.

6. CONCLUDING REMARKS

Our chapter shows that the choice of molecular markers may seem complex for the study of host–parasite relationships in marine systems, especially as there does not seem to be a 'one marker fits all' scenario like there is for many more well-studied vertebrate and invertebrate species. The choice of marker usually depends on already existing gene sequences, databases and previous experiences (Table 4.2) for a certain host and/or parasite group under investigation. However, the choice of marker must be relevant to the research question, that is, is it an investigation into phylogeny, gene flow, phylogeography or adaptive selection processes (Monis et al., 2005)? Further, several possible confounding factors that will hinder the interpretation of coevolutionary results must be carefully considered. Specifically for many parasites, it still has to be debated how much sequence divergence is mandatory to define a new species at the molecular level. Several research gaps exist that would strengthen the interpretations that can be made from molecular investigations of host–parasite relationships and that will allow the advancement of the molecular ecology of marine host–parasite interactions. The first pertains to a lack of multispecies data sets. For example, there are no marine studies that focus on single parasite species with multiple hosts, where the hosts have different life history stages and dispersal abilities. This scenario would be ideal for testing phylogeographic congruence between hosts and their parasites. Secondly, few studies have used molecular tools to understand demographic processes in parasite populations. Many marine molecular studies show signals of population expansions and contractions, but unravelling historical and long-term evolutionary changes in parasite species with short generation times remains unexplored. Thirdly, phylogenetic approaches that examine the evolutionary relationships of parasites or their hosts should be further developed to examine patterns of congruence in evolution, which ultimately will allow us better insight into the processes driving speciation of marine parasites, which is currently poorly understood. With the development of several new and exciting technologies and their first applications in nonmodel species, we hope that the field of coevolution of hosts and their parasites does not remain far behind. This also promises to focus the attention on thus far largely understudied parasite groups and will provide new and stimulating avenues in marine parasitological research.

Table 4.2 Purposes of recent host–parasite marine studies and their successfully applied markers

Purpose	Host–parasites	Applied markers
Taxonomy (cryptic species)	Digenea/Trematoda	ITS1 + 2, LSU rDNA, COI, NADH1
	Monogenea	ITS1 + 2, Cyt-b, EF1a
	Cestoda	ITS 1 + 2, LSU rDNA, COI, NADH1, RFLP
	Nematoda	ITS1, COII
	Myxozoa	SSU rDNA, LSU rDNA, RfB1
	Amphipoda	SSU rDNA
Phylogeography	Teleostei	Microsatellites, COI
	Digenea/Trematoda	Microsatellites, mtDNA
	Monogenea	AFLP, SNP
	Myxozoa	SSU rDNA, LSU rDNA
	Gastropoda	COI
	Decapoda	COI
	Asteroidea	COI
Phylogeny	Teleostei	ITS1 + 2, Cyt-b, SSU rDNA, mtDNA
	Trematoda/Digenea	ITS1 + 2, LSU rDNA, COI, Cyt-b
	Monogenea	ITS1 + 2, SSU rDNA, SSU rDNA, COI, 16S rDNA
	Cestoda	LSU rDNA, SSU rDNA, COI, NADH1, div. mtDNA
	Nematoda	ITS1 + 2, COI, COII
	Myxozoa	SSU rDNA, LSU rDNA
	Amphipoda	SSU rDNA
	Echinoidea	Microsatellites
	Decapoda	Microsatellites

Table 4.2 Purposes of recent host–parasite marine studies and their successfully applied markers—cont'd

Purpose	Host–parasites	Applied markers
Population genetics	Teleostei	Microsatellites
	Trematode/Digenea	Microsatellites, ITS1, COI, NADH1
	Monogenea	AFLP, ITS1, LSU rDNA
	Nematoda	ITS 1 + 2, COII
	Myxozoa	ITS1, SSU rDNA
	Copepoda	Microsatellites
	Isopoda	Microsatellites
Contemporary adaptation	Teleostei	MHC class I + II, SNP, nonneutral microsatellites, HSC70

ITS: internal transcribed spacer; EF1a: elongation factor 1-alpha; COI, II: cytochrome oxidase *c* subunit I and II; Cyt-b: cytochrome *b*; LSU rDNA: large subunit ribosomal DNA; SSU rDNA: small subunit ribosomal DNA; NADH1: NADH-dehydrogenase subunit 1; AFLP: amplified fragment length polymorphism; RFLP: restriction fragment length polymorphism; SNP: single-nucleotide polymorphism, MHC: major histocompatibility complex.

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CHAPTER FIVE

New Insights into Clonality and Panmixia in *Plasmodium* and *Toxoplasma*

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Contents

Abstract

Until the 1990s, *Plasmodium* and *Toxoplasma* were widely considered to be potentially panmictic species, because they both undergo a meiotic sexual cycle in their definitive hosts. We have proposed that both parasites are able of clonal (nonrecombining) propagation, at least in some cycles. *Toxoplasma* was soon shown to be a paradigmatic case of clonal population structure in North American and in European cycles. But the proposal provoked an outcry in the case of *Plasmodium* and still appears as doubtful to many scientists. However, the existence of *Plasmodium* nonrecombining lines has been fully confirmed, although the origin of these lines is debatable. We discuss the current state of knowledge concerning the population structure of both parasites in the light of the recent developments of pathogen clonal evolution proposed by us and of new hypotheses presented here.

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1. INTRODUCTION

The population structure of pathogens is a fundamental parameter, not only for understanding the biology of these organisms but also for applied studies. The population structure of pathogens needs to be ascertained for molecular epidemiology (strain typing), vaccine and drug design, follow-up of genes of interest, and control measures. Discussions of pathogen population structure have focused on the "clonality/sexuality debate" (Tibayrenc et al., 1990). Clonality is here understood as scarcity or absence of genetic recombination, a definition widely accepted for all kinds of pathogens (Tibayrenc and Ayala, 2012), including *Plasmodium* (Anderson et al., 2000; Annan et al., 2007; Beck et al., 2009; Conway, 2007; Griffing et al., 2011; Heitman, 2006; Mu et al., 2005; Nkhoma et al., 2013; Razakandrainibe et al., 2005; Volkman et al., 2012a) and *Toxoplasma* (Beck et al., 2009; Heitman, 2006; Khan et al., 2011; Rajendran et al., 2012; Sibley and Ajioka, 2008; Smith, 2009; Su et al., 2003, 2010; Wendte et al., 2011). Clonality does not amount to genetic monomorphism, an erroneous definition still used by some researchers (Tibayrenc and Ayala, 2012). A clonal species can have considerable genetic diversity, such as *Trypanosoma cruzi*, while some sexual organisms can be extremely monomorphic, after a drastic bottleneck effect, for example.

Clonality strongly influences the population structure of pathogens. If a species is potentially panmictic (freely recombining), its multilocus genotypes (MLGs) are transient individual variants that vanish in the common gene pool in each generation. A panmictic pathogen behaves like sexual metazoa (*Drosophila* and humans), with the peculiarity that pathogens have very short generation times. If clonal evolution predominates in a given species, its MLGs are stable in space and time and can persist over years and over wide geographical ranges. Even when clonal evolution has a limited impact, it introduces a stratification component and discontinuities in the pathogen's population structure, which must be taken into account in association studies and in surveys dealing with the dynamics of genes of interest. Importantly, all systems that restrain genetic recombination amplify the impact of natural selection, by generating coadapted multigenic complexes (Avise, 2004).

Plasmodium and *Toxoplasma* were widely considered panmictic until the early 1990s (Grigg and Sundar, 2009; Walliker, 1991), an apparently logical inference from the well-known feature that they undergo sexual recombination and meiosis in their definitive hosts (anopheline mosquitoes for the

Plasmodium species that infect humans and felids for *Toxoplasma*). However, *a priori* inferences may not be correct. The only adequate means to settle the issue is population genetic analysis of natural populations.

2. INITIAL PROPOSALS

Early proposals of clonal population structure emerged from linkage disequilibrium (LD) analyses of natural populations of *Plasmodium falciparum* (Tibayrenc et al., 1990, 1991) and *Toxoplasma* (Tibayrenc et al., 1991). LD analysis seeks nonrandom association of genotypes at different loci. In a panmictic species, genotypes from different loci recombine at random (except in the case of physical obstacles to genetic exchange: isolation by space and time, the so-called Wahlund effect). Knowing the genotype at a given locus provides no information about which genotypes will occur at other loci. Such information may become apparent if recombination is rare or absent (clonality). LD is therefore taken as a strong circumstantial evidence of clonality, when a sufficient number of loci are surveyed and the significance of statistical tests is high (Tibayrenc and Ayala, 2012; Tibayrenc et al., 1990). In an initial survey (Tibayrenc et al., 1990), *P. falciparum* was chosen as a counterexample. Yet, it was surprising to observe significant LD in the populations surveyed. The inference followed that "uniparental and biparental lineages may coexist within this species, for which a sexual cycle has been a classical notion" (Tibayrenc et al., 1990). Although this inference was corroborated by further studies (Ben Abderrazak et al., 1999; Urdaneta et al., 2001), it resulted in a vehement outcry (Dye et al., 1990; Walliker, 1991; Walliker et al., 1990). Our proposal was widely rejected. The "panmictic prejudice" about *Plasmodium* was kept for long and still has not been rejected by all. The sample we analysed in *Toxoplasma* was less limited, which allowed us to suggest more firmly that "not only that a uniparental cycle exists in *T. gondii* (which was already known), but also that it is common and perhaps predominant" (Tibayrenc et al., 1991).

3. INDISPENSABLE RECALLS

The model of preponderant clonal evolution (PCE) proposed by us includes, as again recently stated (Tibayrenc and Ayala, 2012, 2013), the following: (i) It refers to a pathogen's population structure, not to a particular cytological mechanism of propagation; (ii) its definition is based on restrained recombination (see Section 1); (iii) it affirms that recombination

is severely restricted, not that it is completely absent; and (iv) it definitely includes selfing and strong inbreeding and considers them as particular cases of clonality. That selfing and inbreeding amount to clonality is a view shared by most authors working on *Plasmodium* (Anderson et al., 2000; Annan et al., 2007; Beck et al., 2009; Conway, 2007; Griffing et al., 2011; Mu et al., 2005; Mzilahowa et al., 2007; Nkhoma et al., 2013; Razakandrainibe et al., 2005; Volkman et al., 2012a) and *Toxoplasma* (Beck et al., 2009; Grigg and Sundar, 2009; Lehmann et al., 2004; Sibley and Ajioka, 2008; Su et al., 2012; Wendte et al., 2010). However, clonality does not limit itself to selfing and inbreeding and can have other origins (mitotic propagation and several cases of parthenogenesis, gynogenesis, and hybridogenesis).

4. RECENT DEVELOPMENTS

We have proposed (Tibayrenc and Ayala, 2012) that the two main features of PCE are (i) strong LD and (ii) near-clading.

LD analysis has been criticised as a measure of genetic exchange (De Meeûs et al., 2007). However, (i) it is the very and only statistic appropriate for testing rarity or absence of recombination, on which the definition of PCE is based; (ii) when enough loci are surveyed, its power of resolution is high (Tibayrenc and Ayala, 2012; Tibayrenc et al., 1990); and (iii) it is widely used as a circumstantial evidence for clonality by many authors working on *Plasmodium* (Anderson et al., 2000; Arnott et al., 2012; Branch et al., 2011; Chenet et al., 2012; Conway, 2007; Ferreira et al., 2007; Imwong et al., 2007; Iwagami et al., 2009, 2012; Karunaweera et al., 2008; Mu et al., 2005; Nkhoma et al., 2013; Orjuela-Sánchez et al., 2010; Rezende et al., 2010; Volkman et al., 2007, 2012a,b) and *Toxoplasma* (Khan et al., 2011; Lehman et al., 2004; Rajendran et al., 2012; Su et al., 2006), as well as on other pathogens (Tibayrenc and Ayala, 2012). As asserted here, only the LD approach has been able to refute the panmictic prejudice in *Plasmodium* and *Toxoplasma* (Tibayrenc et al., 1990, 1991).

Near-clading refers to the tendency of pathogen natural populations to differentiate into discrete genetic subdivisions that are stable in space and time. This is evidenced by a clear phylogenetic signal. The term "near-clade" has been coined (Tibayrenc and Ayala, 2012) because some recombination nearly always interferes with PCE in pathogens, which renders the term "clade" improper (there is no recombination between true clades). We have proposed to ascertain whether PCE is apparent within near-clades, which leads to a "Russian doll pattern" (Tibayrenc and Ayala, 2013): one

observes a miniature picture of the whole species within each near-clade, which includes LD and lesser near-clades (Fig. 5.1).

The expectations of a strict cladistic approach are not fulfilled in pathogens due to occasional recombination. Near-clading and a Russian doll pattern should be therefore explored by means of a flexible phylogenetic approach relying on the congruence principle (Avise, 2004), which states that evidence increases when additional suitable data, coming from various sources, are considered. For example, in the case of multilocus sequence typing, evidence accumulates when additional loci are considered. Also, the evidence becomes stronger when microsatellite and multilocus enzyme electrophoresis data are jointly considered. The phylogenetic signal should be looked for by genes that do not undergo strong selective pressure (neutral genes or nearly so). Antigen genes, which are strongly selected, are not an appropriate population genetic tool.

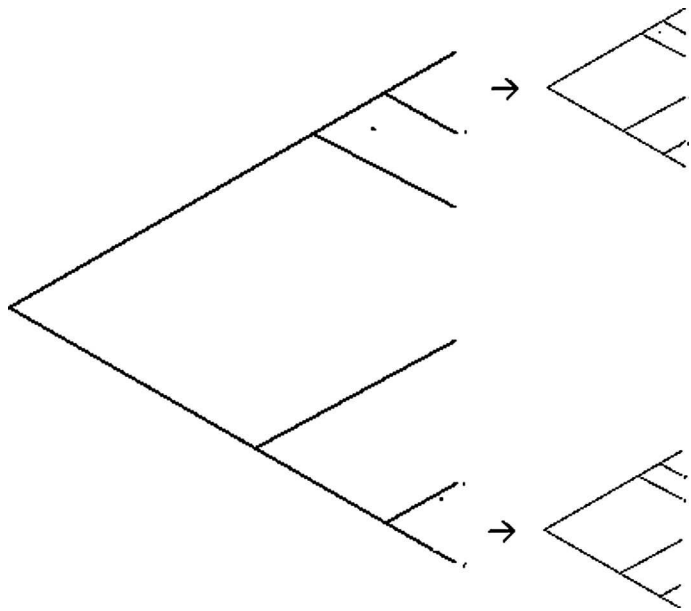

Figure 5.1 "Russian doll" model (Tibayrenc and Ayala, 2013). When population genetic tests are practised with appropriate markers (of sufficient resolution) within each of the near-clades that subdivide the species under study (large tree, left part of the figure), they evidence within these near-clades a miniature picture of the whole species, with the two main PCE features, namely, linkage disequilibrium and lesser near-clades (two small trees, right part of the figure). This is an evidence that the near-clades do not correspond to cryptic, biological species that are potentially panmictic, and that they undergo predominant clonal evolution too.

5. POPULATION STRUCTURE OF *PLASMODIUM* AND *TOXOPLASMA* IN THE LIGHT OF THE PCE MODEL

5.1. *Plasmodium*

A drastic reduction in the panmictic prejudice was caused by a seminal paper (Anderson et al., 2000) dealing with microsatellite variability in a large number of *P. falciparum* worldwide strains. LD analysis led to the inference that *P. falciparum* exhibits a broad spectrum of population structures, ranging from panmictic to highly inbreeding (clonal) populations. Stable, widespread MLGs were observed. For example, the same MLG was sampled in Bolivia in 1994 and in Brazil in 1997–1998. Identical genotypes were obtained from different individuals in Bolivia, Brazil, Colombia, Thailand, Papua New Guinea, and Zimbabwe. The hypothesis advanced to account for restrained recombination was that, in low transmission areas, multiclonal infections are rare, so that identical MLGs frequently mate together (selfing and inbreeding). In other words, recombination is restrained by the absence of different MLGs. We will call it here the "starving sex hypothesis". It has been accepted by many authors in order to account for the observed population structure of *Plasmodium* (Arnott et al., 2012; Branch et al., 2011; Chenet et al., 2012; Conway, 2007; Ferreira et al., 2007; Gupta et al., 2012; Imwong et al., 2006, 2007; Iwagami et al., 2009; Mobegi et al., 2012; Mu et al., 2005; Mzilahowa et al., 2007; Neafsey, 2013; Neafsey et al., 2008; Nkhoma et al., 2013; Schultz et al., 2010; Volkman et al., 2012a,b). It can be challenged, as we will further see, since many observations are at odds with it, both in *P. falciparum* and in *Plasmodium vivax*.

Many studies have ascertained evidence for clonality and restrained recombination, based on the analysis of LD in many populations of *P. falciparum* (Annan et al., 2007; Branch et al., 2011; Chenet et al., 2012; Griffing et al., 2011; Iwagami et al., 2009; Manske et al., 2012; Nkhoma et al., 2013; Razakandrainibe et al., 2005; Volkman et al., 2007, 2012a) and *P. vivax* (Chenet et al., 2012; Imwong et al., 2007; Iwagami et al., 2012; Karunaweera et al., 2008; Rezende et al., 2010). Consequently, the panmictic model was considered to be "oversimplified" in *Plasmodium* (Heitman, 2006). In confirmation of Anderson et al.'s (2000) observations, persistent MLGs were observed in *P. falciparum* for as long as 8 years (Nkhoma et al., 2013). Many authors have come to use the very terms "clones" and "clonal population structure" for describing the population structure of *Plasmodium* (Annan et al., 2007; Chenet et al., 2012; Ferreira

et al., 2007; Griffing et al., 2011; Heitman, 2006; Karunaweera et al., 2008; Nkhoma et al., 2013; Razakandrainibe et al., 2005), which would have been unthinkable 20 years ago. In some populations, restrained recombination manifests itself, not only by LD and ubiquitous MLGs but also by a tendency of natural populations to be structured into lasting, stable clusters, which cannot be accounted for by isolation by distance and time. This has been observed in *P. falciparum* (Branch et al., 2011; Griffing et al., 2011) and *P. vivax* (Gupta et al., 2012; Iwagami et al., 2009; Orjuela-Sánchez et al., 2010; Rezende et al., 2010). However, *Plasmodium* clusters cannot be equated to near-clades similar to the ones observed in *T. cruzi* and *Leishmania* (Tibayrenc and Ayala, 2013), because their stability is limited in time and is blurred by genetic recombination from 1 year to another. Still, the fact remains that they evince a major factor of population structuration and heterogeneity. This is all the more the case, because LD can be observed, not only in whole populations but also within the clusters that subdivide them (Griffing et al., 2011), like a nascent and labile "Russian doll" pattern (Tibayrenc and Ayala, 2013) (Fig. 5.1). In several cases, the strength of LD and structuration is not linked to transmission intensity (see further).

5.2. *Toxoplasma*

The proposal that *Toxoplasma* could undergo clonal evolution in certain cycles (Tibayrenc et al., 1991) did not face strong opposition such as it did in *Plasmodium*, although "a clonal population structure was entirely unexpected, especially since cats are both highly prevalent and widely distributed" (Grigg and Sundar, 2009). Clonality in *Toxoplasma* has been confirmed by numerous papers (Boothroyd, 2009; Dubey et al., 2011; Khan et al., 2009, 2011; Sibley and Ajioka, 2008; Sibley and Boothroyd, 1992; Smith, 2009; Su et al., 2003; Wendte et al., 2010). It is preponderant in Europe, North America, and Africa, while recombination has a greater impact in South America (Lehman et al., 2004, 2006; Mercier et al., 2011; Su et al., 2006, 2010). *Toxoplasma* clonality is common also in North American wildlife (Dubey et al., 2011; Wendte et al., 2011). Authors use the terms "clonal" and "clonal population structure" in the case of *Toxoplasma* more so than for *Plasmodium* (Boothroyd, 2009; Grigg and Sundar, 2009; Khan et al., 2011; Sibley and Ajioka, 2008; Smith, 2009; Su et al., 2003, 2010; Volkman and Hartl, 2003; Wendte et al., 2011). Even in South America, *Toxoplasma*'s population structure is far from "panmictic" (Grigg and Sundar, 2009). The signal of clonal propagation is not "largely absent" (Minot et al., 2012); rather, it is obvious. One MLG isolated from sheep

in Brazil is identical to a MLG isolated years apart in France from a human congenital case (Da Silva et al., 2011). Identical MLGs have been isolated in French Guiana from humans, cats, dogs, and chickens 50 km apart (Mercier et al., 2011). The "clonal types" I, II, and III, preponderant in Europe and North America, are present in Africa (Mercier et al., 2010) and have been also recorded in Central and South America (Da Silva et al., 2011; Mercier et al., 2011; Rajendra et al., 2012; Su et al., 2012).

Near-clading in *Toxoplasma*

Toxoplasma natural populations show, in contrast to *Plasmodium*, a strong tendency for persistent and widespread clustering patterns that meet our definition of near-clades (Tibayrenc and Ayala, 2012). The three major "clonal lineages" (Boothroyd, 2009; Sibley and Boothroyd, 1992) can definitely be equated to near-clades. This near-clading pattern in *Toxoplasma* is not limited to the three major clonal genotypes. When a sufficiently broad sampling of genetic markers and stocks is used (Su et al., 2012), the whole species appears to be subdivided into six major "clades" (near-clades). Three of them (clades A, B, and F) exhibit a distinctive Russian doll pattern (Tibayrenc and Ayala, 2013): LD and lesser near-clades are observed within them. Within-near-clade clonality (Russian doll pattern) has been also observed within "group 12" (Khan et al., 2011). The term clade (Su et al., 2012) is improper, since hybridisation is ubiquitous in *Toxoplasma* evolution, hence the relevance of the new term "near-clade" (Tibayrenc and Ayala, 2012). The three main *Toxoplasma* clonal lineages, although they are stable in space and time and propagate clonally, are thought to have a hybrid origin (Minot et al., 2012; Sibley and Ajioka, 2008; Su et al., 2010, 2012; Volkman and Hartl, 2003). They show striking similarities to some *T. cruzi* near-clades that also have a hybrid origin (Zingales et al., 2012). Clonal propagation of successful hybrid genotypes may represent a specific adaptation to human environments. *T. cruzi* hybrid near-clades are indeed widespread in human cycles of the southern range of Chagas disease (Zingales et al., 2012). Similarly, *Toxoplasma* clonality and major clonal genotypes are obviously associated (although not exclusively) with human environments (Boothroyd, 2009; Mercier et al., 2011).

6. PASSIVE CLONALITY (STARVING SEX) VERSUS IN-BUILT CLONALITY IN *PLASMODIUM*

We have seen that the existence of clonal propagation and a clonal population structure in some *Plasmodium* populations (Tibayrenc et al.,

1990) is now widely accepted, although many researchers still consider that clonality is doubtful in *Plasmodium* and clashes with the notion of an obligatory sexual cycle in these parasites. We propose to move the debate from panmixia versus clonality, to passive clonality versus active (in-built) clonality. As we have noted, passive clonality (starving sex hypothesis) is the widely accepted working hypothesis to account for restrained recombination in *Plasmodium*. It is epidemiologically highly relevant. Indeed, if clonality is strictly correlated to low transmission, LD could be used as a measure of transmission intensity (Volkman et al., 2012a).

Starving sex can be challenged by the hypothesis that clonality in *Plasmodium* cannot be purely passive, "mechanical", but could be due in some cases to in-built biological properties of the parasite. This would allow it to actively restrain recombination and escape the recombinational load (Agrawal, 2006), an evolutionary strategy that could be common in many pathogens (Tibayrenc and Ayala, 2012). The presence of putative meiotic genes, evidenced in many eukaryotic pathogens (Heitman, 2006), could be attributed to a "clonality/sexuality genetic machinery" (Tibayrenc and Ayala, 2012). The hypothesis of active clonality in *Plasmodium* is grounded on the fact that many observations are at odds with starving sex in *P. falciparum* and even more so in *P. vivax*. In Anderson et al.'s article (2000), African populations show no LD, which fits the starving sex hypothesis. However, a strong LD is observed in *P. falciparum* populations of Papua New Guinea and Zimbabwe, even though transmission is high in New Guinea and multiclonal infections are frequent in Zimbabwe (Anderson et al., 2000). LD is stronger in Zimbabwe than in Brazil, although transmission rate is lower in Brazil (Anderson et al., 2000). Weak sample size in Brazil could bias the power of LD analysis in this country. However, this does not lower the strength of the many other observations that challenge the starving sex hypothesis. Strong inbreeding (clonality) with high transmission has been confirmed in Papua New Guinea (Manske et al., 2012). Strong indications for clonality in spite of high transmission rates have been found in Kenya and Cameroon too (Annan et al., 2007; Razakandrainibe et al., 2005). In Brazil, high inbreeding has been evidenced, although multiclonal infections seem to be "highly prevalent", a result considered as "puzzling" by Ferreira et al. (2007). Departures from starving sex expectations are even more obvious in *P. vivax*. In this species, low transmission is frequently associated, not only with a strong LD but also with high genetic diversity (Gupta et al., 2012), which does not favour the starving sex hypothesis. In Brazil, strong LD and high levels of inbreeding in *P. vivax* are "at odds" with high

genetic diversity and prevalent multiclonal infections (Ferreira et al., 2007; Rezende et al., 2010). The same pattern has been observed in Sri Lanka (Karunaweera et al., 2008).

We do not claim that the starving sex hypothesis should be rejected. However, considering the many cases that are at odds with it, the alternative hypothesis (in-built, active clonality) deserves to be considered and is highly falsifiable. The two hypotheses are, in fact, not mutually exclusive.

7. ARE CLONALITY AND NEAR-CLADING IN *PLASMODIUM* AND *TOXOPLASMA* MAINLY DUE TO NATURAL SELECTION?

It has been proposed that natural selection, strongly favouring some variants to the detriment of others, plays a major role in the stability of the main clonal lineages in *Toxoplasma* (Su et al., 2003). It is most probable that natural selection, both diversifying and purifying, plays a significant role in the population structure of pathogens, as it does in other organisms. The PCE model specifically proposes that LD, clonality, and near-clading are mainly due to in-built genetic properties of the pathogens, rather than to the drastic elimination by selection of most possible variants in an otherwise panmictic species (Tibayrenc and Ayala, 2012). The proposal of a preponderant role of natural selection would indeed imply a heavy burden of elimination of most genotypes at each generation (Lehman et al., 2004).

8. ARE THE NEW *PLASMODIUM* "SPECIES" NOT MERE NEAR-CLADES?

In recent years, impressive sets of data have been gathered on *Plasmodium* parasites closely related to *P. falciparum* isolated from apes (Duval et al., 2010; Liu et al., 2010; Prugnolle et al., 2010, 2011a,b). Due to severe technical constraints, the description of these new taxonomical entities has relied on limited sets of genes, except for Prugnolle et al. (2011b). Proposals to describe several new "species" based on limited phylogenetic evidence (Liu et al., 2010; Rayner et al., 2011) are questionable (Tibayrenc and Ayala, 2012; Valkiunas et al., 2011). If the same level of evidence were used for *T. cruzi*, there would be even stronger arguments to describe six or seven "species" within the agent of Chagas disease! We have warned (Tibayrenc and Ayala, 2012, 2013) against the inflation of various different terms used for many pathogens ("clades", "clonal lineages", "clusters", and "haplogroups",

among many others) to designate what is probably the same evolutionary entity, namely, the near-clade. The new *Plasmodium* "species" described using limited phylogenetic evidence could be possibly equated to near-clades, evolutionarily equivalent to the near-clades described in *T. cruzi* and other pathogens (Tibayrenc and Ayala, 2012, 2013). Near-clading seems to be omnipresent in most pathogen species (Tibayrenc and Ayala, 2012). Describing new "species" based only on phylogenetic data would lead to a misleading inflation of new species.

The question whether or not the new *Plasmodium* entities deserve to be described as new species will only be settled by additional phylogenetic, population genetic, and phenotypic analyses of these "species".

9. CONCLUDING REMARKS

The panmictic prejudice in *P. falciparum* and *Toxoplasma* teaches us that "logical" *a priori* inferences (here based on a known sexual cycle in both species) have severe limitations. Suitable evidence should rather be gathered with adequate tools—in this case, population genetic analysis of natural populations (Tibayrenc et al., 1990). Population genetic analyses refute the panmictic prejudice in these parasites and, at the same time, evince clear differences between *Plasmodium* and *Toxoplasma*. Although *P. falciparum* has a spectrum of population structures (Anderson et al., 2000; Tibayrenc et al., 1990) and shows a clonal population structure in many populations, its clonal genotypes and near-clades are made unstable by frequent recombination, even in the populations where clonality is apparent. The same obtains for *P. vivax*. The fact remains that clonality introduces a major stratification factor in *P. falciparum* and *P. vivax* populations, a parameter that must be taken into account in all studies dealing with epidemiological surveillance, vaccine and drug design, and pathogenicity. Additional analyses should aim at falsifying the starving sex versus active clonality hypotheses, since their epidemiological and biological implications are dramatically different.

The present study focused on *P. falciparum* and *P. vivax* for two reasons: (i) They are medically the most relevant ones and (ii) more population genetic data are available for these two species than for others. Further studies should test the hypotheses proposed here on *Plasmodium malariae* and *Plasmodium ovale*, as well as in the many *Plasmodium* species that do not affect humans.

Toxoplasma exhibits a more typical PCE pattern, with stable clonal MLGs and near-clades, not only preponderant in the Northern Hemisphere and Africa but also present in South America.

In *Plasmodium* and in *Toxoplasma*, prior to all applied studies, a reliable population genetic framework should be firmly ascertained, by means of phylogenetic character mapping (Avise, 2004; Tibayrenc and Ayala, 2012). New powerful tools based on whole genome sequencing and the use of large sets of SNPs will help to determine such a robust population genetic framework.

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