APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2002, p. 5796–5803 Vol. 68, No. 12
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.12.5796–5803.2002

Microbial Source Tracking: Current Methodology and Future Directions†

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Maintenance of the microbiological quality and safety of water systems used for drinking, for recreating, and in the harvesting of seafood is imperative, as contamination of these systems can exact high risks to human health as well as result in significant economic losses due to closures of beaches and shellfish harvesting areas. Waters contaminated with human feces are generally regarded as a greater risk to human health, as they are more likely to contain human-specific enteric pathogens, including *Salmonella enterica* serovar Typhi, *Shigella* spp., hepatitis A virus, and Norwalk-group viruses. Animals can also serve as reservoirs for a variety of enteric pathogens (e.g., various serotypes of *Salmonella*, *Escherichia coli*, and *Cryptosporidium* spp.). Understanding the origin of fecal pollution is paramount in assessing associated health risks as well as the actions necessary to remedy the problem while it still exists. Traditional and alternative indicator microorganisms have been used for many years to predict the presence of fecal pollution in water; however, it is well established that the majority of these organisms are not limited to humans but also exist in the intestines of many other warm-blooded animals (55). Due to the ubiquitous nature of these organisms, the effectiveness of using traditional indicators to predict the presence of human or animal waste impact and subsequent health risks is limited. The usefulness of the microbial indicators as tools for risk assessment can be significantly enhanced by the development of testing methods and analysis techniques that can define specific sources of these organisms.

The concept that the origin of fecal pollution can be traced using microbiological, genotypic, phenotypic, and chemical methods has been termed microbial source tracking. This work will provide an overview of microbial source tracking methods that are currently being used to predict and identify sources of fecal pollution in the environment as well as provide insight into future directions in the field.

MICROBIAL INDICATORS OF FECAL POLLUTION

Indicator microorganisms are used to predict the presence of and/or minimize the potential risk associated with pathogenic microbes. Indicator organisms are useful in that they circumvent the need to assay for every pathogen that may be present in water. Ideally, indicators are nonpathogenic, rapidly detected, easily enumerated, have survival characteristics that are similar to those of the pathogens of concern, and can be strongly associated with the presence of pathogenic microorganisms.

Total and fecal coliforms have been used extensively for many years as indicators for determining the sanitary quality of surface, recreational, and shellfish growing waters. In recent years, scientists have learned more about the ways in which the coliforms' ecology, prevalence, and resistance to stress differ from those of many of the pathogenic microorganisms they are proxy for (18, 74). These differences are so great that they limit the utility of the coliforms as indicators of fecal pollution. Therefore, additional microbes have been suggested for use as alternative indicators, including *E. coli*, enterococci, and *Clostridium perfringens* (30).

***E. coli*.** *E. coli* has long been used as an indicator of fecal pollution (24). It has good characteristics of a fecal indicator, such as not normally being pathogenic to humans, and is present at concentrations much higher than the pathogens it predicts. However, recent studies have suggested that *E. coli* may not be a reliable indicator in tropical and subtropical environments due to its ability to replicate in contaminated soils (18, 75).

***Enterococcus* spp.** The enterococcus group is a subgroup of the fecal streptococci that includes at least five species: *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus gallinarum*, and *Enterococcus avium*. They are differentiated from other streptococci by their ability to grow in 6.5% NaCl and at high pH (9.6) and temperature (45°C). *E. faecalis* and *E. faecium* are the species most frequently found in humans. Enterococci have been used successfully as indicators of fecal pollution and are especially reliable as indicators of health risk in marine environments and recreational waters (10, 11). It is known, however, that environmental reservoirs of enterococci exist and that regrowth of these organisms may be possible once they are introduced into the environment (18).

***C. perfringens*.** *C. perfringens* is an enteric, gram-positive, anaerobic, spore-forming, pathogenic bacterium found in human and animal feces. Although there is considerable controversy surrounding the use of *C. perfringens* as a water quality indicator because of its persistence in the environment, a number of scientists continue to recommend its use, particularly in situations where the prediction of the presence of viruses or remote fecal pollution is desirable (21, 59).

While the aforementioned alternative microbial indicators can be useful for predicting the possible presence of fecal contamination in water, their shortcomings as tools for risk

† Florida Agricultural Experiment Station Journal series no. R-09018.

assessment are also becoming increasingly apparent. The advent of microbial source tracking technologies has enhanced the ability of these and traditional indicator organisms to be used as tools for predicting potential sources of fecal pollution as well as health risks associated with impaired water systems.

RATIONALE BEHIND MICROBIAL SOURCE TRACKING METHODOLOGY

Various microbiological, genotypic, phenotypic, and alternative methods have been proposed to characterize groups of microorganisms, usually indicator organisms, for the purpose of detecting the subtle differences present within different groups of microorganisms that can subsequently be used to identify the host or environment from which the organisms were derived.

Genetic methodology can be used to differentiate different lineages of bacteria found within different animal hosts. However, one must assume that within a species of bacteria, there are members or subgroups that have become more adapted to a particular host or environment for various reasons, including differences in pH, availability of nutrients, and receptor specificity. The second assumption is that once these organisms become adapted to a particular environment and establish residency, the progeny produced by subsequent replications will be genetically identical. Therefore, over time, a group of organisms within a particular host or environment should possess a similar or identical genetic fingerprint, which will differ from those organisms adapted to a different host or environment.

Similarly, microbial source tracking methodologies that focus on phenotypic differences within different lineages of bacteria usually focus on traits that may have been acquired from exposure to different host species or environments. Traditionally, these methods have targeted multiple antibiotic resistance (MAR) patterns, cell surface or flagellar antigens, or biochemical tests designed to identify variations in the utilization of various substrates that may be found within a particular host environment.

Direct monitoring for human pathogens, such as enteric viruses and parasites (*Cryptosporidium* and *Giardia* species), has also been used as a means of identifying the presence of human or high-risk fecal pollution in water. Monitoring for pathogens provides direct evidence of their presence and thus circumvents the need to assay for often-ambiguous indicator organisms; however, many of these pathogens are not readily detectable in the environment as they are often present in very low numbers. This is complicated by the fact that many of them have a considerably low infectious dose, which renders even a low presence in polluted waters hazardous to human health.

Various chemical compounds have also been proposed as indicators of human- or animal-derived fecal pollution. The use of chemical indicators is unique, as parallels between survival, transport, and persistence of these chemicals and the pathogens they are being used to predict are more difficult to discern. Therefore, this review will focus primarily on source tracking methods that utilize microorganisms. Nevertheless, certain chemicals and metabolites can be associated with various types of fecal pollution, assuming that human and animal communities utilize different substances or produce different by-products that can subsequently be traced back to the source of the pollution in the environment. Therefore, a brief overview of these methods is provided.

MICROBIOLOGICAL METHODS USED FOR MICROBIAL SOURCE TRACKING

Fecal coliform/fecal streptococcus ratio. To meet the challenge of identifying sources of fecal pollution, various microbiological methods have been proposed. Initially, the ratio of fecal coliforms to fecal streptococci was proposed, where a ratio of >4.0 would indicate human pollution and a ratio of ≤ 0.7 would indicate nonhuman pollution (25). The rationale behind the use of this method was the observation that human feces contain higher fecal coliform counts, while animal feces contain higher levels of fecal streptococci.

The advantage of using this method is its ability to provide rapid results. In addition, the assay requires minimal expertise to perform. However, this approach has proven to be unreliable due to variable survival rates of fecal streptococci species, variations in detection methods, and variable sensitivity to water treatments and has been abandoned as a viable approach to fecal source tracking (14, 62).

***Bifidobacterium* spp.** Bifidobacteria are obligate anaerobic, non-spore-forming bacteria that are a major component of the human intestine. These organisms have been investigated as potential candidates for use as indicators of human fecal pollution due to the fact that they are rarely found in animals (52, 65), and that certain species, when they are found, tend to be isolated at different frequencies from different animals (23, 48, 49, 67). In addition to their abundance in human feces, the ability of human isolates to ferment sorbitol has been used to further differentiate these organisms as being human-derived (67).

Human bifid sorbitol agar (HBSA) was developed by Mara and Oragui (52) specifically to identify sorbitol-fermenting bifidobacteria. This medium can be used in conjunction with established membrane filtration techniques for processing large volumes of water (14). Plates are incubated for 4 to 6 days under anaerobic conditions at 37°C, and yellow, raised colonies are presumed to be sorbitol-fermenting bifidobacteria. Colonies can then be confirmed by subculturing and additional anaerobic incubation on selective media. A membrane filtration method is preferred over direct spread plating due to cell stress exacted by direct exposure to selective agents present in the media.

The use of these organisms as indicators of human fecal pollution holds some promise due to the above observations; however, the survival of these organisms is highly variable and numbers can decrease by 3 to 4 orders of magnitude after as little as 2 weeks in the environment (66, 67). The advantage of using an anaerobic bacterium, however, is its inability to reproduce once deposited in the environment. Therefore, if detected, it can provide evidence of recent fecal contamination. Because survival issues tend to reduce or alter the numbers of bifidobacteria present in the environment, new techniques must be developed that increase both the specificity and sensitivity of detection of these organisms before this method can be used as a reliable indicator of fecal pollution.

***Bacteroides fragilis* bacteriophage.** *B. fragilis* is an obligately anaerobic, gram-negative, pleomorphic rod-shaped bacterium. The *Bacteroides* group of bacteria is present in high numbers in both human and animal intestines. Tartera and Jofre (79) examined 40 human fecal samples for the presence of different *Bacteroides* spp. and determined that one *B. fragilis* strain, HSP40, was found in 10% of the human samples but was not detected in samples from any other animal species. This finding prompted the idea that bacteriophage that specifically infected this strain could be used as indicators of human fecal pollution. Tartera et al. (80) reported a wide range of numbers of *B. fragilis* HSP40 bacteriophage present in water that was subject to impact by human fecal pollution and domestic sewage. However, they did not detect *B. fragilis* phage in slaughterhouse wastewaters or water containing fecal contamination from wildlife only. Because of the low numbers of *B. fragilis* phage present in some sewage and domestic wastewaters, Puig et al. (63) attempted to identify additional host strains of *Bacteroides* in order to detect additional phage originating from the human gut or the guts of different animal species that may have more far-reaching capacity than the HSP40 bacteriophage. They identified an additional strain of *B. fragilis*, RYC4023, which was almost phenotypically identical to strain HSP40 and which showed similar sensitivity to infection by bacteriophage. They also identified an additional strain, RYC2056, that detected greater numbers of phage in waters polluted with domestic sewage. In spite of the fact that this strain detected phage in animal feces, its ability to detect higher numbers of phage than strain HSP40 in waters with a known human impact makes it a potential candidate as an indicator of human fecal pollution in environmental waters.

Overall, the detection of *B. fragilis* bacteriophage has the advantage of being a highly specific method for tracking the source of human fecal pollution. In addition, these phage do not replicate in the environment, and their presence in the environment has been found to significantly correlate with the presence of human enteric viruses (41). The absence of *B. fragilis* phage in highly polluted waters and sewage in some areas (such as the United States) and the inherent difficulty in performing the assay limit the usefulness of this method, however (35, 39, 63).

F-specific RNA coliphage. Coliphages are viruses that infect *E. coli.* Investigators have also reported that animal and human feces contain specifically different serotypes of RNA coliphages, suggesting that phage can be used to predict sources of pollution (22, 37, 38).

There are two main groups of coliphages: somatic coliphages and male-specific (F+) coliphages. The somatic and male-specific bacteriophage are grouped taxonomically into several groups. The male-specific coliphages belong to two main groups (*Leviviridae* [RNA] and *Inoviridae* [DNA]). Somatic coliphages span four groups (*Myoviridae* [DNA], *Styloviridae* [DNA], *Poloviridae* [DNA], and *Microviridae* [DNA]). Somatic coliphages attach directly to the lipopolysaccharide of *E. coli*, whereas F+ coliphages attack only bacteria that possess an F plasmid, which codes for an F pilus and serves as the site of attachment for the virus. Although significant genetic differences are present between and within members of each group of bacteriophage, the F+ RNA bacteriophage have been more fully characterized. Therefore, the majority of microbial source tracking research has focused on the F+ RNA coliphages.

There are four main subgroups of F+ RNA coliphages (*Leviviridae*): group I, group II, group III, and group IV. Members of groups II and III have been shown to be highly associated with human fecal contamination and/or domestic sewage, while group IV coliphages have a higher incidence in wastes associated with animals and livestock. Group I coliphages are present in feces and sewage from both humans and animals. The apparent differences in host tropism for the various groups of F+ RNA coliphage have been utilized to predict the presence of fecal contamination based on the presence or absence of a particular group of coliphage.

The F+ RNA bacteriophage can be enumerated by a variety of methods (36, 73). Once detected, the phage can be further characterized as being human or animal derived by immunological or genetic methods (29, 38). Serotyping of phage has been shown to produce ambiguous results (5, 38). For this reason, genotyping of F+ RNA phage has been utilized using a nucleic acid hybridization approach. This method involves plating the phage on a particular host, transferring the plaques to a nylon membrane, denaturing the phage to expose the nucleic acid, cross-linking the nucleic acid to the membrane, and then detecting group-specific nucleic acid sequences with ^{32}P- or digoxigenin-labeled oligonucleotide probes. This technique has been shown to be successful in identifying the four groups of F+ RNA bacteriophage and subsequently for use in tracking sources of fecal pollution (29, 38).

Because the number of bacteriophage present in the environment is often considerably lower than that of traditional bacterial indicators, it is important that detection be sensitive and include both enrichment procedures and direct assay. Furthermore, if a mixed contamination event occurs, then water samples must be collected and assayed immediately so that die-off of a particular group of coliphage does not occur, which would falsely indicate the presence of only one group or another. Although the host specificity (or at least the apparent general association of particular groups of coliphage with either humans or animals) is well documented, efforts to isolate F+ RNA coliphage have revealed that only a small percentage of human fecal samples contain these phage (27, 37). F+ RNA bacteriophage predominate in domestic sewage, however, which suggests an ability of coliphage to proliferate or be released in the sewage environment. Overall, more research into the differential survival characteristics of the various groups of coliphage is warranted. In addition, further genetic characterization of coliphage from the different groups as well as F+ DNA bacteriophage is warranted so that differences may be identified within or between groups that are capable of differentiating pollution from humans and specific animal hosts.

Human enteric viruses. Over 100 different enteric viruses are associated specifically with the human gastrointestinal tract. Many of these viruses are not easily cultivated in environmental samples; however, methods have been developed to concentrate and cultivate these organisms and are useful for directly detecting the presence of human fecal contamination and public health risk. Studies have shown that outbreaks of gastroenteritis have been associated with water supplies with acceptable coliform counts (16), and bacterial

indicators have been shown to be unreliable indicators of the presence of enteroviruses (26, 53). By monitoring directly for human enteric viruses, the uncertainty associated with the use of fecal indicators can be avoided. Jiang et al. (40) used a nested PCR protocol to detect the presence of adenovirus in waters off the California coast, and she and others have suggested routine monitoring for adenoviruses as an index of human pollution (60). In addition, Lee and Kim (46) recently detected infectious enteroviruses and adenoviruses in 47.8 and 39.1% of drinking water samples in Korea, respectively, and suggested a broad survey of viral pollution in tap water using a broad range of samples and wide spectrum of target viruses.

Monitoring directly for human pathogens provides valuable information as to the quality of the water system being evaluated. However, many viruses can be present in a water system, while only a few can be detected by cultivation methods that distinguish viable from nonviable organisms. Molecular methods (reverse transcription-PCR) can be used to detect noncultivable viruses; however, nonviable viruses are also detected by this procedure, which provides no information as to potential risk to human health. This problem is partially remedied by using cell culture cultivation followed by reverse transcription-PCR. This allows the detection of viruses that propagate in cell culture but do not cause cytopathic effects (1). Finally, as with any presence-absence test, the inability to detect an enteric virus cannot be construed as evidence of its absence. Therefore, this method should be used in conjunction with one or more additional methods for predicting the presence of fecal pollution and enteric pathogens.

PHENOTYPIC METHODS USED IN MICROBIAL SOURCE TRACKING

Numerous phenotypic methods have been suggested for use in discriminating among various groups of bacteria. These include biochemical tests (54), phage susceptibility (87), outer membrane protein profiles (3), antibody reactivity (84), fimbriation (43), bacteriocin production and susceptibility, and other methods. However, these systems have serious disadvantages, including unstable phenotypes, low sensitivity at the intraspecies level, and limited specificity. However, a few phenotypic methods have been used successfully as bacterial source tracking (BST) methodologies.

MAR analysis. MAR analysis is a method that has been used to differentiate bacteria (usually *E. coli* or fecal streptococci) from different sources using antibiotics commonly associated with human and animal therapy, as well as animal feed (15, 34, 56, 85, 86). The use of this method is based on the underlying principle that the bacterial flora present in the gut of various types of animals are subjected to different types, concentrations, and frequencies of antibiotics. Over time, selective pressure within a specific group of animal selects for flora that possess specific "fingerprints" of antibiotic resistance.

This procedure involves the isolation and culturing of a target organism, then replica plating the isolates on media containing various antibiotics at various concentrations. The plates are then incubated and the organisms are scored according to their susceptibilities to various antibiotics to generate an antibiotic resistance profile. These fingerprints are then characterized, analyzed by discriminate (or cluster) analysis, and compared to a reference database to identify an isolate as being either human or animal derived.

The MAR technique has been shown to be successful in discriminating *E. coli* or fecal streptococci isolated from specific animal species, including wildlife, various livestock (cattle, pigs, horses, and chickens), and humans (34, 85, 86). In a direct application, Hagedorn et al. (31) used antibiotic resistance patterns of fecal streptococci to identify cattle as the predominant source of fecal pollution in the Page Brook watershed in rural Virginia, which resulted in the implementation of restricted access of the cattle to the stream and a 94% reduction of fecal coliform bacteria in the watershed. This method has received significant attention as a viable tool for tracking the sources of fecal pollution; however, antibiotic resistance is often carried on plasmids, which can be lost from cells via cultivation and storage or by changes in environmental conditions. More research is needed to determine if this factor could potentially change the apparent origin of an organism after its persistence in the environment. In addition, strains from different locations may show variations in specific sensitivities due to variable antibiotic use among humans and livestock species. For these reasons, large databases may need to be compiled that contain antibiotic resistance profiles from multiple organisms from a large geographic area. Furthermore, antibiotic sensitivity is not useful in situations where the isolates under study show no significant resistance patterns yet come from different animal species.

Immunological methods. Serogrouping of microorganisms based on the presence of different somatic (O) antigenic determinants has been used by several investigators to differentiate *E. coli* from various sources (17, 28). It has been reported that different serotypes of *E. coli* are associated with different animal sources, although many serotypes are also shared among humans and animals (8, 33, 55). Parveen et al. (58) tested a total of 100 human source and nonhuman source *E. coli* isolates for the presence of various O antigens. Of these, 77% were successfully typed. Human-derived isolates exhibited 19 serotypes, with 48% being classified within 7 serotypes. Animal-derived isolates spanned 26 serotypes, with 36% being classified within 7 of those serotypes. Overlap between predominant serotypes of human- and animal-derived isolates was not significant, which indicates that serotyping may be useful in discriminating *E. coli* from human and animal sources. One of the drawbacks to this method, however, is the necessity for a large bank of antisera. Parveen et al. (58) suggested the use of this method in conjunction with another method, such as ribotyping, which would allow the testing of a limited number of serotypes. The possibility of testing for only certain serotypes makes this a potentially valuable method to be included in the microbial source tracking "toolbox."

GENOTYPIC METHODS USED IN MICROBIAL SOURCE TRACKING

PFGE. Pulsed-field gel electrophoresis (PFGE) is a method of DNA fingerprinting whereby DNA fingerprints are generated after treatment of genomic bacterial DNA with rare-cutting restriction endonucleases. PFGE has been a very useful

technique in determining bacterial relatedness and in epidemiological studies (4, 42). Parveen et al. (58) analyzed 32 *E. coli* isolates by PFGE and found no association between PFGE profile and isolate source. However, Simmons et al. (71) used PFGE to match 51% of 439 *E. coli* isolates from a stream in an urban watershed, and classified the majority of isolates as being from wildlife (especially raccoons) and dogs. Additional published research using this technique for BST is lacking, and its usefulness for this purpose has not been fully determined.

Repetitive element PCR. Repetitive element PCR uses primers corresponding to interspersed repetitive DNA elements present in various locations within the prokaryotic genome to generate highly specific genomic fingerprints. Three methods of repetitive sequence analysis have been used, with each targeting a specific family of repetitive element. These methods include repetitive extragenic palindromic sequence PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequence PCR, and PCR with extragenic repeating elements (BOX-PCR). The REP primer set generally generates a lower level of complexity, while the ERIC primer set is more sensitive to suboptimal PCR conditions, such as the presence of contaminants in the DNA preparation (64). Generally, the BOX primer is used in cases where a detailed characterization is needed, as this primer generates robust fingerprints and generally yields a highly complex pattern of amplified fragments. This method has been used previously to differentiate between closely related strains of bacteria (82, 83). For these reasons, BST research has initially focused on the use of the BOX primer in performing REP-PCR (19).

The genetic fingerprint generated using BOX-PCR contains several bands, which can subsequently be analyzed, categorized by host source, and used to construct a database to which fingerprints from unknown isolates can be compared. Successful identification of an unknown bacterial isolate also requires that a reference database be established, and additional known isolates must be fingerprinted from a large geographic region in order to assess the potential universal application of this procedure. Questions have also arisen as to the reproducibility of this method.

Ribotyping. Ribotyping is a method of DNA fingerprinting whereby highly conserved rRNA genes are identified using oligonucleotide probes after treatment of genomic DNA with restriction endonucleases. The method is a labor-intensive procedure that involves bacteriological culture and identification, DNA extraction, gel electrophoresis, Southern blotting, and discriminant analysis of the resulting DNA fingerprints. Ribotyping has proven to be a very useful epidemiological technique for use with various bacteria, including *E. coli* (77), *S. enterica* (54), *Vibrio cholerae* O1 (61), and *Vibrio vulnificus* (2, 78).

Ribotyping has also been reported to effectively track human and nonhuman sources of pollution (12, 32, 57, 69). Parveen et al. (57) examined 238 *E. coli* isolates from the Apalachicola National Estuarine Research Reserve in Florida and additional human and animal sources and were able to correctly classify 97 and 67% of animal- and human-derived isolates, respectively. Similarly, Carson et al. (12) analyzed 287 *E. coli* isolates collected from humans, various livestock (cattle, swine, horses, poultry, and turkeys), pets (dogs), and wildlife (geese) and correctly classified 95 and 99% of human- and animal-derived isolates, respectively.

Variations of the ribotyping procedure are present in the literature and usually involve the use of different restriction enzymes, the use of alternative detection methods during the Southern blotting procedure (colorimetric or radioactive), or variations in analysis and interpretation of ribotype profiles (discriminant analysis versus 100% similarity). Investigators using different methods have also reported differences in the ability of the ribotyping procedure to discriminate between bacteria from various animal hosts (32, 57, 69, 70; T. M. Scott, S. Parveen, K. M. Portier, J. B. Rose, M. L. Tamplin, S. R. Farrah, and J. Lukasik, submitted for publication). As with other DNA fingerprinting methodologies, the success of this procedure depends on the size of the "known-source" reference fingerprint database to which a ribotype profile from an unknown isolate must be compared. The inability of many laboratories to compile a database that contains enough isolates to which unknown profiles can be compared may be one limitation of this procedure, as ribotyping has been shown to lose its effectiveness when isolates are collected from a broad geographic area (32, 51; Scott et al., submitted). Additional factors such as differences in the diet of the host animal have also been suggested as a reason for variations in ribotype profiles. Therefore, databases either may need to be extremely large and contain isolates from a very broad geographic region or must be designed exclusively for a specific watershed with defined potential impacts (51). Finally, although this method has proven successful in some aspects, it is expensive and labor-intensive, unless the procedure is streamlined and performed routinely.

Host-specific molecular markers. Detection of host-specific molecular markers in raw water samples holds promise as an effective method for characterizing a microbial population without first culturing the organisms in question. Rapid tests that discriminate human fecal pollution from human and bovine fecal pollution are currently in the literature and use length heterogeneity PCR and terminal restriction fragment length polymorphism analysis to characterize members of the *Bacteroides-Prevotella* group and the genus *Bifidobacterium* (6, 7). In addition to this method, assaying for a battery of specific toxin genes or additional host-specific genes (such as various fimbriae) by PCR has shown some promise for differentiating bacteria based on their pathogenic properties and the hosts they target (70).

This approach offers the advantage of circumventing the need for a culturing step, which allows a more rapid identification of target organisms. In addition, the use of *Bacteroides* spp. is desirable, as anaerobic bacteria are less likely to reproduce once introduced in the environment. However, little is known about the survival and persistence of *Bacteroides* spp. in the environment, which raises questions as to its utility as an indicator organism. Assaying for toxin or adhesion genes has not been thoroughly investigated and is complicated by the fact that many organisms do not contain these genes regardless of their host specificity.

CHEMICAL METHODS USED IN MICROBIAL SOURCE TRACKING

Caffeine. Caffeine is present in several beverages, including coffee, tea, soft drinks, and in many pharmaceutical products.

TABLE 1. Advantages and disadvantages of current methods used for microbial source tracking

Method	Advantage(s)	Disadvantage(s)
Fecal coliform/fecal streptococous ratio	Easy to perform; may be useful for recent contamination	Variable survival rates of fecal streptococci can alter ratio
Bifidobacterium sp.	Sorbitol fermenters may be human specific	Low numbers present in environment; variable survival rates; culture methods not well-defined
B. fragilis HSP40 bacteriophage	Very human specific; easy to perform	Not present in sewage in some areas
F+ RNA bacteriophage	Groups are well-correlated with source; easy to perform	Unreliable in marine and tropical waters due to variable survival rates
Human enteric virus	Human specific; Direct monitoring for pathogen circumvents need to use indicators	Low numbers in environment; labor-intensive; more sensitive methods needed
MAR	Rapid; can be used to discriminate isolates from multiple animal sources	Requires reference database; may be geographically specific; isolates that show no antibiotic resistance cannot be typed
PFGE	Extremely sensitive to minute genetic differences	May be too sensitive to broadly discriminate for source tracking
BOX-PCR	Rapid; easy to perform	Reproducibility a concern; reference database required; may be geographically specific
Ribotyping	Highly reproducible; some methods useful for classifying isolates from multiple sources	Labor-intensive; reference database required; may be geographically specific; variations in methodology exist
Bacteroides-Prevotella molecular marker	Does not require culturing of organism; PCR method is rapid, easy to perform	Little is known about survival and distribution in water systems; currently not applicable to all animals
Caffeine	Useful for assessing impact from human sewage	Minute quantities in the environment make sensitivity an issue; requires expensive analyses
Fecal sterols and/or stanols	Some sterols/stanols have greater specificity for humans and/or animals	Present naturally in sediments; requires expensive analyses; Low prevalence makes sensitivity an issue

It is excreted in the urine of individuals who have ingested the substance, and subsequently, it has been suggested that the presence of caffeine in the environment would indicate the presence of human sewage (9). Levels of caffeine in domestic wastewater have been measured to be between 20 and 300 μg/liter (68). Levels in receiving waters are much lower due to significant dilution, and little is known about the fate of caffeine in the environment once it has been deposited (76).

Coprostanol. Coprostanol is a fecal stanol that is formed during catabolism of cholesterol by indigenous bacteria present in the gut of humans and higher animals and is the primary stanol detected in domestic wastewater (50). For this reason, it has been proposed as a chemical indicator of human fecal pollution (13, 20, 44, 45, 47, 81). Leeming et al. (47) characterized fecal samples from numerous animals and found that coprostanol constituted ~60% of the total stanols in human feces. Feces from pigs and cats were also found to contain coprostanol, but at levels that were 10-fold lower. Additional fecal stanols, such as 24-ethyl-coprostanol, were found to be predominant in herbivores, such as cows, horses, and sheep, suggesting potential use of this chemical as an indicator of fecal pollution from these sources.

In addition to caffeine and fecal sterols, chemicals found in laundry detergents such as fluorescent whitening agents, sodium tripolyphosphate, and linear alkyl benzenes have been used to predict human impact; however, these chemicals cannot reliably be traced to sewage or fecal pollution and can only be attributed to general human or industrial sources. Therefore, the use of these chemicals for fecal source tracking is omitted from this review but has been reviewed elsewhere (72).

While initial results seem promising, overall, the methodologies used for the detection of human-specific chemical substances in water are tedious and lack the desired sensitivity to be considered as universal indicators of human fecal pollution. Furthermore, to date, no direct relationships have been made between the presence of these chemical indicators and pathogenic organisms or to the subsequent risk to public health.

CONCLUSIONS AND FUTURE DIRECTIONS

A summary of the methods currently used for microbial source tracking as well as some advantages and disadvantages of each is presented in Table 1. Overall, there is no single method that is capable of identifying specific sources of fecal pollution in the environment with absolute certainty. Research is continuing at a rapid pace, and new techniques are sure to be developed. Future research should address issues such as relationships between the survival characteristics of indicator organisms with regard to those of the pathogens they are designed to predict. Furthermore, epidemiological studies should be undertaken that implement multiple source tracking methods so that assessments of risk can be more closely asso-

ciated with the results produced by a given technique. In addition, one important aspect that will surely require additional investigation is the relative stability of the genotypic and phenotypic profiles obtained using these procedures after organisms have been subjected to various environmental stressors.

Recent advances in gene chip, microarray, and biosensor technologies will allow increased sensitivity and specificity of detection as well as enable detection of multiple organisms or molecular markers with a single assay. Real-time techniques will also greatly improve the ability to remedy existing contamination problems as well as prevent future events, and most will circumvent the need for cultivation of the organisms in question, thus eliminating bias introduced by culturing techniques. For techniques that require a reference database (ribotyping, MAR, REP-PCR), the construction of national databases has been suggested by several investigators (51). In addition, the advantages of using host-specific organisms such as *B. fragilis* bacteriophage and the F+ RNA coliphage can be increased by research designed to further characterize these organisms so new groups or subgroups can be identified that may have uses in tracing sources of fecal pollution.

Overall, all current methods and those in progress require additional investigation; however, all have merit and are important constituents of the constantly expanding microbial source tracking toolbox. With Environmental Protection Agency-mandated total maximum daily loads being calculated throughout the United States, the need for current and future source tracking technologies is increasing, and these methods are certain to play a pivotal role in identifying point and nonpoint sources of fecal pollution in our nation's impaired water systems.

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