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Microbial Source Tracking: Library Based Methods

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Abstract

The Clean Water Act requires states to identify and list impaired waters which are waters that do not meet state-established water quality standards. Section 303(d) of this act requires states to prioritize impaired waters and establish Total Maximum Daily Loads (TMDLs). A TMDL estimates all significant sources of pollution and the reductions needed to bring an impaired water into compliance with state standards. Pathogen TMDL reduction requirements and associated management activities require knowledge of the sources of fecal pollution. Currently, the only technologies designed to provide this information are microbial source tracking (MST) methods. Among the available MST methods, the most commonly used ones are "library-based" methods in which a database, or "library," is constructed of specific characteristics of target microorganisms (typically *Escherichia coli* or enterococcus bacteria) from fecal samples from humans and various animals. The same characteristics of target microorganisms derived from environmental water samples are compared to the library to determine the likely host of origin of each microorganism and hence the likely pollution source. These methods are still in the developmental stage and potential users should be aware of their limitations.

Introduction

Fecal waste may contain disease-causing microorganisms. Human fecal waste contains, in addition to cross-species pathogens, pathogens that are human-specific and human-adapted (e.g., *Vibrio cholerae, Shigella* spp., and many viral pathogens). Thus, human fecal pollution in water is more hazardous to humans than fecal pollution from animals (AWWA 1999). In addition, there have been many documented human disease outbreaks in recent years due to pathogens from domestic animals but far fewer recorded outbreaks due to pathogens from indigenous (wild) animals (Craun et al. 2004). Hence, it is generally accepted that the comparative human health risk of these fecal sources is (high to low): human > domestic animal > indigenous (wild) animal. However, the amount of difference in disease risk between these groups is not known.

There are a number of ways human and domestic animal waste can contaminate water (AWWA 1999; Moe 1997) and all surface waters contain some fecal waste from indigenous animals. Human illness can occur if water is consumed that contains fecal wastes. Illness can also occur if shellfish, harvested from such waters, are consumed. This is because shellfish are filter-feeders; they concentrate waterborne microbes from the surrounding waters. Therefore it is important to detect and quantify fecal waste contamination and if possible determine the specific source(s).

Fecal contamination is usually detected by testing water for the presence of certain fecal-derived "indicator" bacteria, such as total coliforms, fecal coliforms, *Escherichia coli (E.coli)*, and enterococci (Leclerc et al. 1996; Leclerc et al. 2001; Toranzos and McFeters. 1997). Most bacteria that comprise the coliform group, including *E. coli* ("EC"), are typically derived from the intestines of humans and other warm-blooded animals. However, some coliform bacteria (e.g., *Klebsiella* spp. and *Enterobacter* spp.) may have environmental (non-fecal) sources as well. Such tests can determine if a waterbody contains fecal pollution, and how much pollution is present (depending on which test method is used), but they are not able to identify the source of the pollution.

Over the last decade new methods collectively called **microbial source tracking (MST)** tests, also known as bacterial source tracking (BST) tests, have been developed. These tests have demonstrated value for discriminating sources of fecal bacteria in waterbodies (Malakoff 2002; Scott et al. 2002; Simpson et al. 2002). These tests rely on the premise that humans and animals are hosts to some host-specific or host-adapted strains of EC (or other target)

bacteria (Kariuki et al. 1999; Souza et al. 1999). The methodologies further assume that the "clonal composition" of the strains found in the environment is the same as that of the fecal inputs (but see below as well as Horst et al. 1999).

A majority of the MST methods are called "**library-based**." This is because they rely on the construction of a database or "library" of specific characteristics of EC (or other target microbe) obtained from the feces of humans and targeted domestic and indigenous animals. The same characteristics of EC isolated from environmental waters are compared to those in the "fecal source library" to determine their likely source of origin as shown in the cartoon in Figure 1. Library-based MST methods include phenotype tests and genotype tests. Phenotype tests rely on microbial characteristics that can be observed following simple growth assays. Such characteristics include resistance to (growth in the presence of) antibiotics or the ability to metabolize one or more of a suite of biochemicals. Genotype tests rely on molecular techniques that isolate the DNA from the microbes and characterize differences in the nucleic acid sequence.

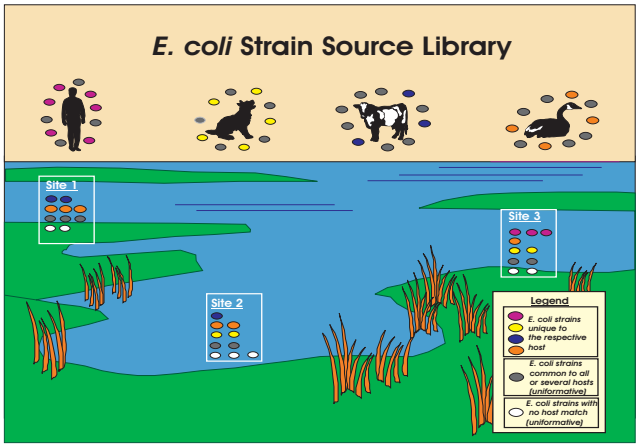

Figure 1. Cartoon illustrating "library-based" microbial source tracking methods. Characteristics of *E. coli* (ovals) isolated from environmental sites (#1, #2, and #3) are compared to the same characteristics of *E. coli* isolated from the feces of targeted animals to determine their likely host of origin. Some characteristics are unique to certain hosts (colors) while others (grey) are common to multiple hosts (host of origin cannot be determined). Some *E. coli* found in the environment have characteristics (white) that do not match those of any *E. coli* in the "fecal source library" (their origin is also unknown).

Phenotype Tests: Multiple Antibiotic Resistance

Description

Some commonly-used phenotypic library-based tests are called multiple antibiotic resistance (MAR) tests. These tests are also known as antibiotic resistance analysis (ARA) tests. There are a variety of such tests (Harwood et al. 2003). The tests rely on the premise that EC (or other target organisms) from the intestines of humans and domestic animals have different "antibiotic resistance (AR) profiles." The AR profiles supposedly differ because their hosts are given different antibiotics or differing amounts of the same or similar antibiotics. Wild animals will also have distinct AR profiles because they are not directly exposed to any antibiotics. However, it has been shown in dairy calves that antibiotic resistance in enteric EC is independent of exposure to antimicrobial drugs (Khachatryan et al. 2004). This means that the antibiotic resistance profiles of EC may not necessarily be related to their antibiotic exposure history.

EC bacteria isolated from feces or from a waterbody are tested against a suite of antibiotics: either a single concentration or multiple concentrations of each antibiotic, depending on the test. If an EC is resistant to an antibiotic, when cultured in the presence of that antibiotic, the cell will grow and multiply. If not resistant, it will not grow or multiply in the presence of the antibiotic. When each EC is cultured, separately, with many antibiotics or antibiotic concentrations, a growth/no-growth pattern or AR profile is obtained as shown in Figure 2. Some EC have AR profiles that are unique to a specific animal host or are more frequently observed in one host compared to other hosts. Other profiles are observed in EC derived from several types of animals (although this does not appear to be a major problem; Stoeckel et al. 2004). Some profiles from environmental EC do not match any profile in the fecal source library. This is because either not enough EC were characterized from the feces of the targeted animals, the EC is derived from the feces of an animal that was not targeted (not present in the library), or the EC is a non-fecal (environmental) isolate (McLellan 2004). Inability to match environmental EC to library EC appears to be a significant issue affecting the performance of MST tests (Stoeckel et al. 2004).

Multiple Antibiotic Resistance (MAR)

Patterns	% Total	MAR Index
Domestic Cats		
Pen-Van	71.2	0.17
Pen-Str-Van	28.8	0.25
Total isolates = 163		
Different isolates = 2		
Avg. MAR Index = 0.19		
Humans		
Amp-Amx-Nal-Otc-Pen-Sul-Van	41.7	0.583
Amp-Amx-Nal-Otc-Pen-Str-Sul-Van	12.5	0.667
Amp-Amx-Kan-Nal-Otc-Pen-Sul-Van	4.17	0.667
Amp-Amx-Kan-Nal-Otc-Pen-Sul-Tet-Van	1.39	0.75
Amp-Amx-Ctc-Nal-Otc-Pen-Str-Sul-Van	37.5	0.75
Amp-Amx-Ctc-Nal-Otc-Pen-Str-Sul-Tet-Van	1.39	0.833
Amp-Amx-Ctc-Kan-Nal-Otc-Pen-Str-Sul-Van	1.39	0.833
Amp-Amx-Ctc-Kan-Nal-Neo-Otc-Pen-Str-Sul-Tet-Van	1.39	1
Total isolates = 72		
Different isolates = 8		
Avg. MAR Index =0.67		
Total anitibiotics tested = 12.		

Figure 2. Antibiotic resistance profiles of *E. coli* isolates from domestic cats and humans in Monmouth County, NJ (Palladino and Tiedemann 2004). Resistance to an antibiotic is shown by a three-letter abbreviation for that antibiotic. Amp = ampicillin (40 µg/ml), Amx = amoxicillin (15 µg/ml), Ctc = chlortetracycline (25 µg/ml), Kan = kanamycin (25 µg/ml), Nal = naladixic acid (25 µg/ml), Neo = neomycin (50 µg/ml), Otc = oxytetracycline (25 µg/ml), Pen = penicillin (75 U/ml), Str = streptomycin sulfate (15 µg/ml), Sul = sulfathiazole (750 µg/ml), Tet = tetracycline (25 µg/ml), Van = vancomycin (10 µg/ml). MAR Index = number of antibiotics resistant / the number of antibiotics tested.

Advantages

1. In a number of published studies these methods have shown success in identifying fecal pollution sources. A few such studies include Parveen et al. (1997), Wiggins et al. (1999), Harwood et al. (2000) and Graves et al. (2002). In a phenotype test comparison study, the MAR method of Wiggins et al. (2003), which employs enterococci as target organisms and 3 or 4 concentrations each of 11 different antibiotics, performed slightly better than other MAR methods (Harwood et al. 2003).

2. In at least one case, it was shown that, after identifying pollution sources, the success of pollutionmitigation actions was demonstrated (Hagedorn et al. 1999).

3. Phenotypic methods are easier to perform, less expensive and, in most cases, faster than genotypic methods (Harwood et al. 2003).

Disadvantages

1. Genotypic methods generally perform better than phenotypic methods in accurately identifying fecal sources (Griffith et al. 2003; Stoeckel et al. 2004).

2. The environmental relevance of the "average rate of correct classification" (ARCC) is unknown.

   When a fecal source database is constructed it is "internally tested." Each microbial isolate in the fecal source database is treated as an "unknown" and compared to all of the other isolates in the database. The statistical pattern-matching algorithm (see below) "classifies" that isolate as to its probable source by comparing its AR profile to the rest of the profiles in the database. If a fecal source database consisted of just 4 sources, each EC library isolate would be classified correctly as to its host source 25% of the time by random chance alone. In this case the ARCC would be 25%. Published MAR methods often have high ARCC values for various fecal pollution sources, 80% to 90% or higher in many cases. However these high ARCC values come from comparing fecal source isolates with each other. The "clonal composition" of environmental isolates appears to be different than that of the fecal source isolates (see below), so method ARCC values may not be relevant with regard to environmental isolates (Harwood et al. 2003).

3. Antibiotic resistance genetic elements (e.g. plasmids, transposons) can be readily transferred among different EC or other groups of enteric microorganisms. AR resistance elements appear to be widespread in nature and not necessarily related to the antibiotic exposure history of the organism (Guardabassi and Dalsgaard 2004; Hoyle et al. 2004).

Genotype tests

Description

There are a variety of genotypic library-based test methods (Olive and Bean 1999; Myoda et al. 2003). They are also referred to as DNA fingerprinting tests. These methods target the DNA of the entire organism [genome], particular genes, or a specific DNA sequence. The three most common library-based methods are pulsed field gel electrophoresis ("PFGE"; the entire genome is analyzed), repetitive extragenic palindromic elements – polymerase chain reaction ("rep-PCR"; the DNA between adjacent repetitive non-gene DNA sequences is targeted) and "ribotyping" (the DNA of the seven sequences that code for ribosomal RNA [rRNA operons] are targeted). DNA is isolated from individual EC and further processed and analyzed according to the particular method used. Each method results in a series of bands of DNA on agarose gels. The DNA bands form a visual pattern or "genotype,"(the locations of the bands in the gel are based on size and/or charge differences of DNA pieces) as shown in Figure 3.

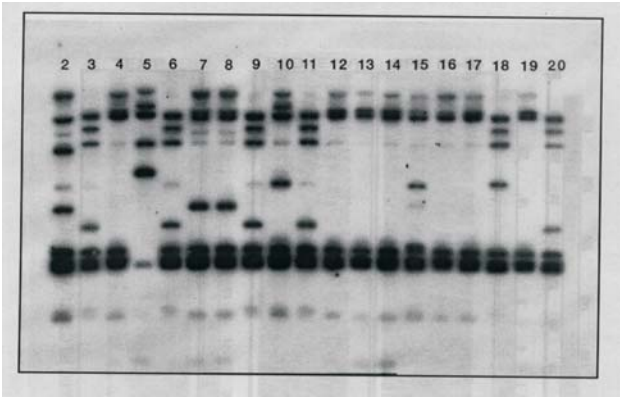

Figure 3. Photograph of 19 *E. coli* DNA "ribotypes" (from Barsotti et al. 1999)

Like the phenotype methods, these methods rely on the premise that the DNA sequences of subspecies of EC (or other target bacteria) from one animal type are more similar than those from other types of animals. By matching the genotypes of EC isolated from the environment to EC genotypes in the fecal source library, the host of origin can be determined. Some genotypes are unique to or more commonly found in humans or certain animals while others are common to multiple animal groups. As with AR profiles, some genotypes from environmental EC do not match any patterns in the library.

Advantages

1. As with the phenotypic methods, in a number of published studies the genotypic methods have shown success in identifying fecal pollution sources. A few such studies include Simmons and Herbein (1995), Parveen et al. (1999), Bernhard and Field (2000); Dombek et al. (2000), Carson et al. (2001), Guan et al. (2002), and Simmons et al. (2002).

   Some studies have shown rep-PCR methods to be easier to perform, compared to other genotyping methods, as well as comparable to PFGE and superior to ribotyping in accuracy (Olive and Bean 1999; Carson et al. 2003). Myoda et al. (2003) showed that no single test performed significantly better than any other and any differences in performance were investigator-dependent rather than test-dependent. A more recent, rigorous comparison study showed PFGE and a ribotyping method that used two restriction enzymes (*Eco*RI and *Pvu*II) to be the most accurate methods, but with the caveat that most environmental EC isolates were not unclassifiable (Stoeckel et al. 2004).

2. At least some genotypic methods perform better than phenotypic methods in accurately identifying pollution sources (Quednau et al. 1999; Griffith et al. 2003; Stoeckel et al. 2004).

Disadvantages

Most of the genotype tests are more expensive (including expensive equipment), complex, time-consuming, labor intensive, and require more expertise than the phenotypic methods (Oliver and Bean 1999). Of the three method types discussed above, the PFGE procedure is the most time-consuming.

All library-based MST tests

Advantages

1. These tests can identify pollution sources or at least dominant sources of pollution in a waterbody (Griffith et al. 2003).

   To a limited extent the tests can also quantify the respective pollution source inputs. Such information is vital to the success of TMDL reduction efforts. No other technology is capable of directly providing this type of information. There are a number of non library-based methods that can detect human pollution sources (see below) and some of these may be superior to library-based methods, but they typically provide presence/absence rather than quantitative information (Griffith et al. 2003). However, the accuracy of the quantitative information generated using library-based MST methods should be carefully evaluated (see below).

2. These tests can eliminate as well as identify a suspected pollution source(s).

Disadvantages

There are important limitations of which potential users should be aware (Simpson et al. 2002; Griffith et al. 2003; Stewart et al. 2003; Harwood et al. 2003; Myoda et al. 2003). A recent, rigorous comparison of seven MST tests showed that the performance of all was substantially lower than anticipated on the basis of values reported in the literature (Stoeckel et al. 2004).

1. All library-based MST methods are laborious, expensive, and time-consuming. Ascertaining sources in a single watershed can take weeks to months and can cost thousands of dollars. "DNA fingerprinting may not be a cost-effective methodology to identify and quantify fecal pollution sources given the extensive diversity and undercharacterized genetic structure of the natural *E.coli* population" (McLellan, 2004).

2. Almost all MST methods, and in particular MAR methods, are subject to a significant amount of false positives, indicating that a pollution source is present when in fact it is not present (Griffith et al. 2003; Stoeckel et al. 2004).

   In addition, the tests do not always identify sources when present (false-negatives). In a study comparing test methods using blinded test samples with known fecal source inputs, no MST method predicted the source material in the samples perfectly (Griffith et al. 2003). When sources are apportioned, there are no error bars or confidence intervals provided to assist the reader in determining the uncertainty in the values. The level of uncertainty can be considerable. The financial implications of incorrectly identifying the presence of fecal sources and taking management actions in response can be serious.

   One study showed that a source has to contribute more than 25% of the total amount to be considered a real source and not a possible misclassification error (Wiggins et al. 2003).

3. A large library is required.

It takes considerable time and money to compile libraries. There is a very high level of diversity of EC isolates from some, if not all, fecal sources (Aslam et al. 2003; Jenkins et al. 2003; Johnson et al. 2004; McLellan 2004). The magnitude of the minimum size for a "representative" library of AR profiles to encompass this diversity has been estimated to be at least 2,300 microbial isolates (Wiggins et al. 2003), but logistical constraints, and cost typically factor in final library size (Stoeckel et al. 2004). Even so, the library that is constructed, typically, is derived from only a small percentage of animals in a watershed. Inadequate library size appears to be a significant problem affecting MST test performance (Stoeckel et al. 2004)

Time and resource limitations often result in individual pollution sources being "pooled" to obtain a sufficiently large database. For example, rather than separate libraries for cows, pigs, cats, dogs, geese, gulls, ducks, rats, mice, etc., there might be just two categories, domestic animals and indigenous animals.

Source pooling can increase discriminatory power (Johnson et al. 2004). Pooling does not necessarily compromise TMDL objectives, but regulators and others should be aware that pooling is a fairly common practice.

4. The EC population from fecal hosts may change following environmental exposure.

It is likely that only a subset of EC from a host survive in the environment. The diversity of environmental EC isolates is lower than that from hosts (McLellan 2004). These survivors may constitute the majority of the strains isolated from contaminated waters but might be a minority in the host animal (Gordon 2002; McLellan 2004).

5. The genetic sequence of individual EC may change over time while in the environment.

An experiment that employed PFGE genotyping found that the genetic sequence of one of three EC isolates changed (a change in one of the restriction enzyme sites occurred) near the end of an eight week period (Lu et al. 2004). During this period, the organisms were kept at room temperature, in the dark, in the presence of cell-free irrigation canal water (the original source of the isolates). It was hypothesized that the genetic change may have been due to mutation or recombination that occurred during the prolonged survival period.

6. It is not clear how "stable" a host source library is over time.

The composition of EC clones in a host changes rapidly over time (Whittam 1989; Horst et al. 1999; Aslam et al. 2003; Jenkins et al. 2003). This means that the composition of a host-based EC library should also change over time by continually adding new source isolates. EC clones that disappear from hosts over time are not typically removed from fecal source libraries. One study showed that a library was "stable" for at least a year (Wiggins et al. 2003), but the length of time that the composition of a non-updated library reflects the composition of EC or other target organisms in a given watershed is not known. Temporal variability may be a significant problem affecting MST test performance (Stoeckel et al. 2004).

7. It is uncertain how "stable" a host source library is spatially.

The composition of a library from fecal sources in Watershed "A" may not be the same as a similar library from Watershed "B". The farther away Watershed "B" is from Watershed "A," the more dissimilar will be their respective libraries (Wiggins et al. 2003; Hartel et al. 2002). Scott et al. (2003) showed that human EC ribotypes were more geographically stable than animal EC ribotypes while Escobar-Paramo et al. (2004) showed geographic differences in EC populations from humans living in tropical versus temperate climates. Does a separate library have to be constructed for every watershed studied? How many libraries need to be constructed in New Jersey to "cover" the entire state? The answers to these questions are unknown.

8. Access to fecal samples from some wild animals can be problematic.

9. There is currently no standard or consensus pattern-matching procedure.

Regardless of whether phenotype or genotype tests are used, pattern matching (of either AR profiles or DNA band patterns) is done using pattern-matching algorithms in commercial statistical software (Ritter et al. 2003; Albert et al. 2003). A variety of statistical methods are employed such as discriminant analysis, nearest neighbor analysis, maximum similarity, average similarity, and other measures of distance or similarity. The specific algorithm used affects the

false-positive rate (see below). Algorithms that require a closer match for source assignation have a smaller rate of false positives (Stoeckel et al. 2004). In a test designed to correctly identify fecal sources in blind samples, no one MST statistical method appeared to be superior to any other (Ritter et al. 2003).

10. Some non library-based MST methods have been shown to be superior to library-based methods in their ability to differentiate between human and non-human sources (Jiang et al. 2001; Field et al. 2003; Noble et al. 2003; Stackelberg 2003; Maluquer de Motes et al.2004; Vinje et al. 2004). However, these methods do not provide quantitative information (Griffith et al. 2003).

Conclusions and Recommendations

1. All MST methods require further refinements (e.g., Johnson et al. 2004; Stoeckel et al. 2004).

   One of the biggest impediments to such development is the poor current understanding of microbial population genetics and host specificity (Stewart et al. 2003; Nielsen et al. 2004). Also, none of the methods take into account the change in composition of the microbial population from the intestinal to the environmental habitat. The amount of change is unknown but is likely to be considerable (Whittam 1989; Horst et al. 1999; Gordon et al. 2002). This change needs to be characterized. It is noteworthy that Stoeckel et al (2004) summarize their recent MST test comparison study by stating "the results…indicate that current protocols for isolate subtyping may be insufficient to accomplish many goals of MST…"

2. The goals and scope of MST studies should be carefully defined.

   Identifying pollution sources is not the same as locating them. Sources of bacteria can vary substantially within a single watershed (Whitlock et al. 2002). In large watersheds, identifying pollution sources may not be enough to initiate pollution reduction activities. Study area(s) should be small enough that identification and location are determined at the same time (Simpson et al. 2002).

3. Pollution source assignation might be improved in future studies by employing both phenotype and genotype tests simultaneously.

4. Until MST methods become standardized and validated, and because of the cost and time considerations involved in their use, it would be prudent to use MST methods sparingly and with an understanding that the results need to be interpreted carefully. The use of MST methods may be warranted when a waterbody is shown to contain an excessive amount of fecal pollution and when thorough sanitary surveys and spatially-intensive monitoring of that waterbody using standard quantitative fecal indicator tests reveal no obvious or likely pollution source(s).

5. Together with the MST methods, chemical markers of human pollution, such as the presence of caffeine (Buerge et al. 2003) or detergent and pharmaceutical compounds (e.g., Ricking et al. 2003; Stackeberg et al, 2003), should be investigated as potential source-tracking tools. Genotyping of bacterial viruses (Vinje et al. 2004) should also be investigated as a potential human pollution source-tracking tool.

Like MST methods, chemical marker methods also have disadvantages. Many chemical techniques employ mass spectrometry analysis that is expensive and time-consuming. In addition, test sensitivity may be an issue. For one chemical indicator of fecal pollution (coprostanol in the Raritan River), the chemical tracer was less sensitive than microbial ones (Kwak and Rosen 2002).

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