Evaluation of microbial source tracking methods using mixed fecal sources in aqueous test samples

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ABSTRACT - Microbiological source tracking methods are potentially powerful tools that are increasingly being used to identify sources of fecal contamination in surface waters, but these methods have been subjected to limited comparative testing. In this study, 22 researchers employing twelve different methods were provided sets of identically prepared blind water samples. Each sample contained one to three of five possible fecal sources (human, dog, cattle, seagull, or sewage). Researchers were also provided portions of the fecal material used to inoculate the blind water samples for their use as library material. No MST method tested predicted the source material in the blind samples perfectly. Host-specific PCR performed best at differentiating between human and non-human sources, but primers are not yet available for differentiating among the non-human sources. Virus and F+ coliphage methods reliably identified sewage, but were not able to identify fecal contamination from individual humans. Library-based isolate methods were able to identify the dominant source in most samples, but had difficulty with false positives, identifying the presence of fecal sources that were not in the samples. Among the library-based methods, genotypic methods generally performed better than phenotypic methods.

INTRODUCTION

Fecal contamination of surface waters can result from numerous sources of fecal pollution, including human sewage, manure from livestock operations, indigenous wildlife and urban runoff. Effective management requires identification of, and targeting mitigative action towards, the dominant source of fecal contamination in the watershed. Several microbiological source tracking (MST) methods have been developed to fill this need. MST methods are intended to discriminate between human and non-human sources of fecal contamination, and some methods are designed to differentiate among fecal contamination originating from individual animal species.

There are four basic types of MST methods (Scott *et al*. 2002, Simpson *et al*. 2002). The first is genotypic library-based methods, such as ribotyping, pulsed-field gel electrophoresis (PFGE) and box-PCR, which distinguish among sources of fecal contamination by identifying patterns in the genetic material of bacterial isolates and matching them with libraries from known sources. The second class is library-based phenotypic methods, such as antibiotic resistance analysis (ARA) or carbon source utilization (CSU), which are also library based, but rely instead on growth patterns produced when bacterial isolates are subjected to a suite of antibiotics or grown on differing carbon sources. The third class is non-library based genetic methods, which include host-specific PCR, t-RFLP and toxin-gene biomarkers, which differentiate between sources by identifying the presence of genetic markers unique to the fecal bacteria of the targeted host species. Library independent methods operate at the population rather than the isolate level. The fourth class is direct measurement of human or bacterial viruses. Methods in this class target viruses that occur in human fecal material, but not in that of other animals and include those that detect human enteroviruses and adenoviruses or F+ coliphage, a virus that infects *E. coli*.

These methods have been used successfully to meet management needs in at least limited applications. For instance, Hagedorn *et al*. (1999) used ARA of enterococci to determine that cattle were the main source of fecal contamination impacting

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streams in a rural Virginal watershed. Management actions instituted as a result of these findings led to a 94% reduction in levels of fecal coliforms. Boehm *et al*. (2003) combined measurements of fecal indicator bacteria with detection of human-specific markers for *Bacteroides/Prevotella* and enterovirus to identify human sewage as the main source of fecal pollution in Avalon Bay, California. Studies in Florida used ARA to correctly identify human fecal material as the dominant source in waters that were later found to be sewage contaminated (Harwood *et al*. 2000; Whitlock *et al*. 2002).

Despite some initial success using MST techniques to disentangle sources of fecal contamination, most of these methods are still experimental. They have been tested in a limited number of locations, often within a single watershed, and with a limited number of possible fecal sources. They have not been subjected to standardized comparative testing, and most have not been tested in marine waters. Public agencies are preparing to spend millions of dollars on MST applications with the hope of identifying sources of recreational water contamination. Without comparative studies, water quality managers do not have the necessary information to make logical, cost-effective choices regarding which source tracking method to use, nor will they know the extent to which they can rely on the results when the methods are employed.

As a first step to addressing this problem, the U.S. Environmental Protection Agency, Southern California Coastal Water Research Project, California State Water Resources Control Board, and National Water Research Institute sponsored a February 2002 workshop held in Irvine, California that brought together nationally recognized experts in environmental microbiology, molecular biology, and microbial detection methods for the purpose of summarizing existing knowledge about source tracking methods and to define the tests necessary to compare, evaluate, and validate a wide range of MST methods (Malakoff 2002). Workshop participants recommended a set of method evaluation criteria (Table 1) and a four-phased approach with increasing levels of complexity (Table 2) for implementing MST evaluation studies.

Following the workshop, 11 organizations agreed to cooperatively fund a comparative evaluation of MST methods that responded to recommendations from the workshop. The study involved twenty-two leading researchers in the field.

This paper describes the study design and provides a broad overview of the results. The remaining papers provide more detailed results organized according to method class.

Methods

The study focused on Phase two from the Irvine workshop recommendations, which involves evaluation of whether methods can accurately identify the source(s) of contamination in laboratory-created blind water samples. One to three of five possible fecal contamination sources (feces from human, dog, cattle, seagull and primary sewage influent) were added to these samples in various proportions, and these fecal sources were blind to the participants.

Twenty-two researchers performing 12 methods (Table 3) participated in the study. With the exception of PFGE, toxin-gene biomarkers, and adenovirus, each method was performed by at least two researchers. Each laboratory processed samples and conducted data analysis using its own operating procedures with no attempt made to standardize protocols within or across methods. Detailed methodologies are provided in the individual papers that follow this one.

Each researcher analyzed 12 blind test samples in a sterile freshwater matrix. A subset of eleven researchers also analyzed an additional 12 blind test samples of similar fecal composition suspended in 0.22 μm filtered seawater or freshwater amended with humic acids, to assess potential matrix interference effects.

Human fecal material for the study was obtained from twelve healthy adult volunteers residing in various locations throughout southern California. Canine fecal material was obtained from three dogs each at a dog park, a dog beach, and a humane shelter in Huntington Beach, CA. Three additional dog scat samples were obtained from personal pets in Garden Grove, CA. Cattle fecal material was obtained from three cows each at three dairies in Chino, CA and from three steers at the Beef Production Unit of the California State Polytechnic University in Pomona, CA. Fecal samples from individual gulls were not large enough to meet our needs and so composite guano samples were obtained from separate flocks of Western Gulls at Seal Beach, Bolsa Chica State Beach, Huntington State Beach, and Newport Beach, all located in Orange County, CA. Guano was obtained by placing

Table 1. Method evaluation criteria agreed on by Irvine MST Workshop participants. Criteria are divided into three tiers that reflect different aspects of performance.

Category of criteria	Specific evaluation criteria
Tier 1: Measurement reliability	--Reproducibility of results within and across laboratories
	--Accuracy of isolate classification into the correct group of sources (for library dependent methods)
	--Confidence that an identified indicator is from the presumed source (for library independent methods)
	--Level of resolution, or ability to discriminate among sources (i.e., human vs. non-human, livestock vs. wildlife, non-human species level, cattle from separate farms)
	–Matrix stability (in what matrices, e.g., saltwater, freshwater, turbid water, humic acid environments, is the method applicable?)
	--Geographical stability (over what area is the method applicable?)
	--Temporal stability (over what time frame is the method applicable?)
	--Confirmation by peer review
Tier 2: Management relevance	--Relationship to source(s) of contamination
	--Relationship to public health outcomes
	--Relationship to commonly used water quality indicators
	--Ease of communication to the public
	--Ease of communication to management audiences
Tier 3: Cost and logistics	–Equipment and laboratory facilities required
	--Training required
	--Library size required (for library dependent methods)
	--Library development effort per “unit” required (for library dependent methods)
	--Implementation time
	--Cost of ensuring results are legally defensible
	--Cost per sample, including all operations and maintenance overhead
	--Sample turnaround time

breadcrumbs on large polyethylene sheets placed on the beach and periodically scraping fecal material from the sheet. Primary sewage influent was collected from the Orange County Sanitation District primary wastewater stream. Samples from all sources were collected on October 8, 2002, stored on ice out of direct sunlight and transported to the laboratory in ice chests.

Many MST methods require a library of genotypic or phenotypic patterns from potential fecal sources and participants were provided fecal material from the above collections for this purpose. Participants using methods that rely on DNA extraction were provided a sample of each scat prepared using a sterile lab scoop to break off a portion of the scat (ca. 1g) and place it in a sterile plastic container. Participants using methods that rely on bacterial isolates were provided Culturette™ bacterial transport swabs containing 0.5 g modified Stuart’s transport medium (Becton-Dickinson, Sparks MD) that were inserted into the interior of each scat sample. Participants creating a source-specific library of bacterial isolates were asked to standardize the library size to 60 isolates per source (5 per swab for humans, cattle and dogs; 15 per swab for gulls).

The blind test samples were created from the scat samples by first creating source-specific stock solutions prepared by dissolving equal portions (by mass) of each scat into 2 liters of sterile water and stirring to create a homogenous concentrate. Source-specific fecal concentrates were then diluted with sterile water, 0.22 um filtered seawater, or sterile water amended with 0.01% w/v humic acids (Sigma-Aldrich, St. Louis MO) to produce source/matrix-specific stock solutions containing an estimated 10^4 *E. coli*/100 mL. The amount of dilution necessary to attain that density was estimated using published fecal bacterial concentration data (Geldreich 1978,

Table 2. Phased study approach identified by the Irvine workshop participants. Each phase reflects an ascending level of complexity.

Phase	Question Addressed
Phase 1: Repeatability	--Can an individual laboratory produce repeatable results in multiple runs of the same sample? --Can investigators in different laboratories produce consistent results for the same samples?
Phase 2: Accuracy	--Can methods accurately identify mixed bacterial sources in laboratory created aqueous matrix samples?
Phase 3: Field Accuracy	--Can methods accurately detect the primary source(s) in samples from dominant-source watersheds?
Phase 4: Field Repeatability	--Do different methods produce comparable results in samples from complex natural systems that contain multiple unknown sources?

Table 3. Methods used by study participants.

Method	Target Organism	Resolution	Source Discrimination
Antibiotic Resistance Analysis[1]	*E. coli* or Enterococci	quantitative	human, cattle, dog, gull
Carbon Source[2] Utilization	*E. coli* or Enterococci	quantitative	human, cattle, dog, gull
Ribotyping[3]	*E. coli*	quantitative	human, cattle, dog, gull
Pulsed Field Gel Electrophoresis[4]	*E. coli*	quantitative	human, cattle, dog, gull
Box-PCR[5]	*E. coli*	quantitative	human, cattle, dog, gull
Host-specific PCR[6]	*Bacteroides/Prevotella* group	non-quantitative	human, cattle, dog
Toxin-gene Biomarkers	*E. coli*	non-quantitative	human
Terminal-Restriction[7] Fragment Polymorphism	*Bacteroides/Prevotella* group	non-quantitative	human, cattle
Community Terminal-Restriction Fragment Polymorphism[8]	Eubacteria	quantitative	human, cattle, dog, gull
F+ Phage Typing[9]	F+ coliphage	non-quantitative	human
Enterovirus[10]	human enterovirus	non-quantitative	human
Adenovirus[11]	human adenovirus	non-quantitative	human

[1]Wiggins 1996, Hagedorn et al. 1999, Harwood et al. 2000, Whitlock et al. 2002, Wiggins 2003.
[2]Holmes et al. 1994, Wallis and Taylor 2003.
[3]Parveen et.al. 1999, Carson et al. 2001, Carson et al. 2003. Scott et al. 2003.
[4]Simmons et al. 1995, Macdonald and Kalmakoff 1995.
[5]Dombeck et al. 2000, Carson et al. 2003.
[6]Bernhard and Field 2000b.
[7]Bernhard and Field 2000a.
[8]Liu et al. 1997.
[9]Grabow and Coubrough 1986, Hsu et al. 1995.
[10]Tsai et al. 1993, Noble and Fuhrman 2001.
[11]Allard et al. 1992, Castingnolles et al. 1998, Jiang et al. 2001.

Alderisio 1999). Stock solutions were then combined volumetrically to produce water samples containing predetermined proportions from each source in either a freshwater, saltwater, or 0.001% w/v humic acid in freshwater matrix. Once combined, samples were stored overnight at 4 °C prior to packing and shipping the morning of October 9. All samples were shipped overnight in insulated containers on ice. When all shipments arrived on October 10, participants were given the OK to begin processing samples. The simultaneous starting time was intended to minimize differences in bacterial composition of the samples among participants due to die-off during shipping.

Bacterial concentrations in the stock solutions were analyzed in the originating laboratory in California on October 9 and for each of the following three days. Concentrations for both *E. coli* and enterococci were measured using the IDEXX defined substrate method (Colilert® and Enterolert®). Bacterial concentrations measured on the day samples were received by study participants were used to estimate relative amounts of fecal bacteria from each source present in the blind water samples (Table 4). Stock solutions were prepared based on anticipated *E. coli* concentrations and the percentages differed for each sample between *E. coli* and enterococci due to their unequal density in the source material.

Results provided by the participants for the blind water samples were assessed using five criteria:

- Ability to correctly identify the presence of human fecal material.
- Ability to correctly identify the absence of human fecal material.
- Ability to correctly identify the dominant source of fecal material contained in a sample.
- Ability to accurately identify all sources of fecal material contained in a sample.
- Stability of response across the three matrices.

Some of the methods in the study only provide a presence/absence response for human fecal material and their evaluation was limited to criteria 1 and 2. Analysis for all questions was conducted after pooling results across participants within method; variation in results among individual researchers within a class of methods is presented in subsequent papers within this volume.

For all assessments, sewage and human fecal material was treated as a single source because sewage is predominantly human material and most methods are unable to discriminate between these sources. For the third assessment, the dominant source was defined based on the target bacterial species used by that participant. For the fourth assessment, a sample was scored as correct if all sources contained in the sample were correctly identified (regardless of the percentage contribution) and no false positive results were reported. To assess the effects of the saltwater and humic acid amended matrices, the difference in response between two freshwater matrix samples containing the identical source material (samples A and K) were compared with the difference in response between replicate source material placed in different matrices.

Results

Most methods were able to correctly identify samples containing human fecal contamination. Host-specific PCR and CSU identified 100% of these samples. ARA and ribotyping also performed well in this regard, identifying an average of greater that 90%. Methods targeting human viruses or coliphage correctly identified less than 50% of the samples containing fecal material from individual humans, but were able to identify most samples containing sewage influent (Figure 1).

In contrast, many methods were unsuccessful in identifying samples that did not contain human fecal contamination (Figure 2). False positive rates for the two phenotypic methods approached 100%. There was greater variability in false positive rates among genotypic library-based methods, with incorrect classification ranging from 25-75%. Only host-specific PCR, human virus, and coliphage methods had false positive rates at or near zero.

None of the methods were able to identify the dominant source in all samples. The three library-based genotypic methods (box-PCR, PFGE, ribotyping) correctly identified the dominant source in about 75% of the samples, while library-based phenotypic methods (ARA and CSU) correctly classified the dominant source in only about 50% percent of the samples (Figure 3).

Every method performed poorly in identifying all sources of contamination in the sample, a result consistently attributable to identifying source materials that were not present in the sample. PFGE and host-specific PCR did best, correctly identifying all sources in about half of the samples. Community

Table 4. Percent contribution of E. coli or enterococci from each fecal source in the blind water samples.

Sample ID	Sewage		Human		Dog		Cattle		Gull	
	E. coli	Ent.	E. coli	Ent.	E. coli	Ent.	E. coli	Ent.	E. coli	Ent.
A	100	100								
C			96	18					4	82
E					86	54	14	46		
F			58	1					42	99
G									100	100
I			100	100						
J	1	1			1	13	98	86		
K	100	100								
L			35	7	3	12	62	81		
N	58	4			42	96				
P							99	77	1	23
U							100	100		

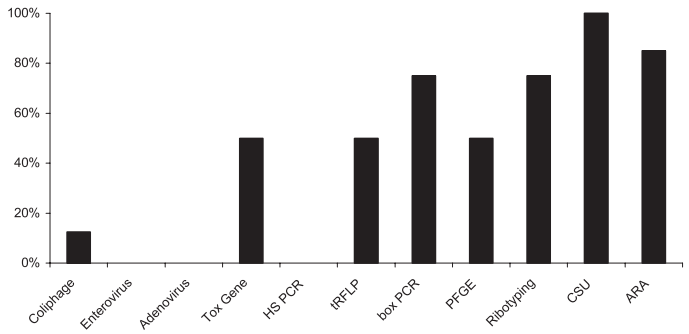

Figure 1. The percent of samples containing human fecal material that were not identified as containing a human source (false negatives).

t-RFLP, BOX-PCR and CSU were not able to correctly identify all sources in any samples.

The saltwater and humic acid amended matrices had minimal effect on the results (Figure 4). The difference in response between freshwater matrix replicates and identical source material in different matrices was particularly small for ARA. Researchers performing amplification of genetic material by polymerase chain-reaction reported some difficulty in obtaining results from samples containing humic acids, which was evident in a slightly larger difference from results obtained in the freshwater replicate (Figure 4).

Discussion

No method performed consistently well across all evaluation criteria. Library-independent methods outperformed library-based methods in their ability to identify or exclude samples with respect to human fecal contamination, but they were unable to resolve more than the human source or produced only a presence/absence result. Library-based methods produced quantitative results for all sources of fecal contamination, but exhibited a high rate of false positives, often assigning a large percentage of contamination to sources not present in a sample.

Host-specific PCR was the most accurate library-independent method, correctly classifying all samples in terms of presence/absence of human fecal contamination. Human virus and F+ coliphage methods were adept at excluding samples which did not contain human contamination, but their ability to detect human material was limited to those samples containing sewage. This is not unexpected, as the target organisms for these methods are rare or occur infrequently in healthy individuals and suggests that these methods are best suited to detection of sanitary sewer leaks, for which the population of individuals contributing to the source of contamination provides sufficient signal for them to be effective.

Among the library-based methods, our findings were more negative than previous method evaluations (Parveen *et al*. 1999, Hagedorn *et al*. 1999, Harwood *et al*. 2000, Dombek *et al*. 2000, Carson *et al*. 2003, Wiggins *et al*. 2003). Most of this difference is probably due to the more difficult type of challenge involved in this study. Previous evaluations were primarily based on assessing repeatability of isolate identification within and between laborato-

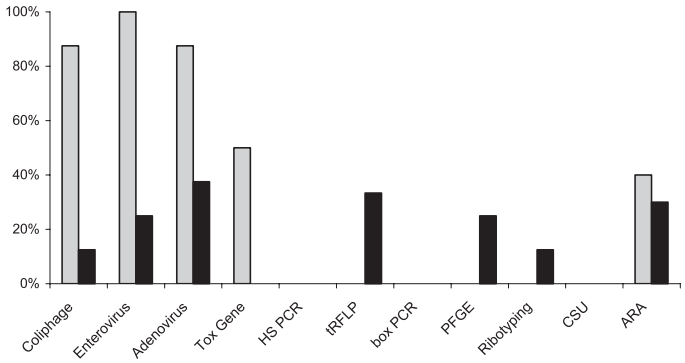

Figure 2. The percent of samples not containing human fecal material that were incorrectly identified as containing a human source (false positives).

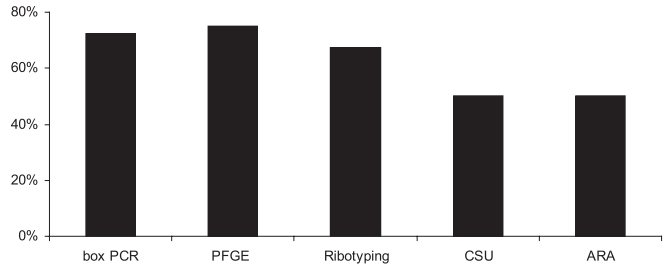

Figure 3. The percent of samples in which the dominant fecal source of contamination was correctly identified.

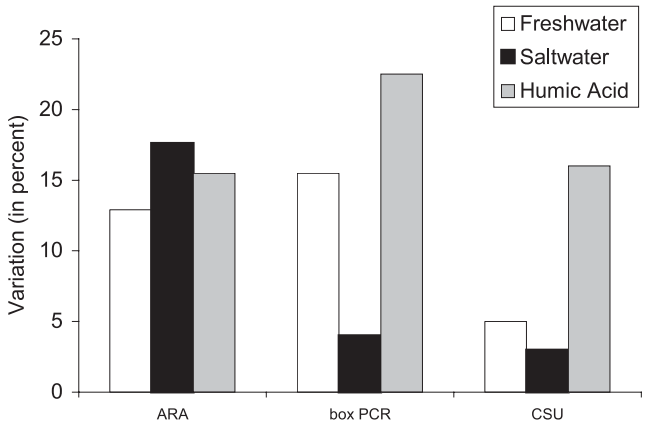

Figure 4. Comparison of percent variation in results between replicate samples in freshwater matrix and replicates in either saltwater or humic acid amended matrices.

ries. This is the first study to attempt quantification of mixed sources in an aqueous matrix.

Still, there were some aspects of our study that may have led to an understatement of method efficiency. For instance, we limited participants to 60 isolates per fecal source in library creation and 50 isolates per blind sample to ensure that differences among methods were not attributable to differences in number of isolates processed. While the number was selected based on frequent practices, many researchers quantify more isolates on a routine basis and it is reasonable to expect some improvement if more isolates were analyzed (Wiggins *et al*. 2003). Later papers in this volume address that issue with further analysis that was conducted after the samples were unblinded.

Another factor leading to understatement of method efficiency was the absolute manner in which we judged false positives. Some participants counsel managers to ignore source material that is identified as present in low concentrations because they are aware that there are transient isolates which occur in multiple animal species and are easily misclassified as to source. Other researchers attempt to minimize this problem by classifying only those isolates that have high fidelity to a source group. If we had used a threshold percentage in our evaluation, the false positive problem would have been less severe. However, for many participants, particularly those using phenotypic methods, the threshold below which managers would need to ignore a source would have to be 30% or more to minimize the false positive problem. This issue is discussed more comprehensively by Harwood *et al*. (2003) and Myoda *et al*. (2003) later in this volume.

We also merged data across researchers performing similar methods, masking the results of individual researchers who performed better than their cohorts. For instance, one researcher used enterococci as the target species in ARA and did appreciably better than those who used *E. coli*, even approaching the efficiency of the genotypic methods. The results portrayed in this paper provide an overall assessment of the state of a particular method, but the differences among researchers points out the opportunity for optimization within technique. Individual researcher differences and the opportunities for method optimization are explored more thoroughly in the subsequent papers in this volume.

A confounding factor in the study was our inclusion of sewage influent as one of the sources of

human fecal contamination. Sewage was included because it is the source of greatest interest to managers and also because we wanted to evaluate MST methods that rely on measuring a scarce target, such as a pathogen, phage, or rare gene sequence, that typically occurs only in a sample from a large population of humans. Sewage, though is not a purely human source, containing pet fecal material that is flushed or wildlife feces that infiltrates through leaks in the system. All of the quantitative methods identified a high percentage (greater than 50% in some cases) of non-human material in the blind samples containing only sewage. The high percentage is most likely a prediction error, but if sewage truly contains a high percentage of non-human material, this presents an even greater challenge for MST methods, as the confounded source signature would make identification of a sewage leak using MST more difficult.

One of the factors that had little effect on the outcome of the results was the inclusion of complex matrices (Figure 4). Saltwater had little or no effect on any of the methods. Humic acids did, as expected, seem to interfere with PCR based methodologies, but the concentration amendments used in this study were higher than those found in natural samples (Abbaszadegan *et al*. 1993, Tebbe and Vahjen 1993, Queiroz *et al*. 2001). Even so, participants using PCR based methods were still able to obtain credible results for the humic acid laden samples.

The study also included some factors that simplify the evaluation in comparison to real applications, leading to some overstatement of method efficiency. The greatest simplification was that all fecal material used to construct the test samples was available to the investigators as library material, whereas in a typical application the library must be extrapolated from a small percentage of animals in the watershed. The effect of this extrapolation will need to be evaluated in future studies.

While our findings were not as positive about MST methods as previous studies, there were several positive aspects to the results. Non-library based methods performed well in differentiating between human and non-human sources of fecal contamination. Host-specific PCR performed best in this regard, but human virus and F+ coliphage methods were reliable for detecting human sewage. Quantitative methods did not fare well in identifying all sources, but were generally able to identify the dominant source of contamination in a sample. Each method used in the study appears to have a different set of positive attributes, ranging through cost, quantification capability, range of detectable sources and accuracy. In order to utilize the available MST methods to their best advantage, managers will need to accurately define the question they hope to address with their particular application, including weighing their tolerance for an incorrect answer, when selecting the most appropriate method(s) from the available toolbox.

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