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A comparative study of culture-independent, library-independent genotypic methods of fecal source tracking

Katharine G. Field, Eunice C. Chern, Linda K. Dick, Jed Fuhrman, John Griffith, Patricia A. Holden, Michael G. LaMontagne, Joann Le, Betty Olson and Michael T. Simonich

ABSTRACT

Culture-independent fecal source tracking methods have many potential advantages over library-dependent, isolate-culture methods, but they have been subjected to limited testing. The purpose of this study was to compare culture-independent, library-independent methods of fecal source tracking. Five laboratories analysed identical sets of aqueous samples that contained one or more of the following sources: sewage, human feces, dog feces, cattle feces and gull feces. Two investigators used methods based on PCR amplification of Bacteroidetes marker genes and both successfully discriminated between samples that did or did not contain human fecal material. One of these investigators was also able to identify the remaining sources, except for gull, with a low rate of false positives. A method based on *E. coli* toxin genes successfully identified samples containing sewage and cattle feces, but missed some samples with human feces because of low marker prevalence in individual human fecal samples. Researchers who used community terminal restriction fragment length polymorphism (T-RFLP) were limited by the amount of DNA recovered from samples, but they correctly identified human and cattle fecal contamination when sufficient DNA was obtained. Culture independent methods show considerable promise; further research is needed to develop markers for additional fecal sources and to understand the correlation of these source-tracking indicators to measures of human and environmental health.

Key words | Bacteroides, culture-independent, fecal source tracking, microbial source tracking

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INTRODUCTION

Fecal source tracking techniques have proved useful because they provide a mechanism for properly focusing water quality remediation efforts (Scott *et al.* 2002; Simpson *et al.* 2002*b*). Many fecal source tracking applications to date have been culture-based methods in which the occurrence of phenotypic or genotypic traits in fecal isolates from water is compared with occurrence in a library of isolates from fecal samples. New developments in the use of molecular methods to study uncultured microbes in natural populations have led to rapid and quantitative means of assessing natural microbial populations without the need for cultivation. In this study, our

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purpose was to investigate the performance of several culture-independent methods for fecal source tracking.

The available culture-independent fecal source tracking methods fall into two categories. The first involves fingerprinting the entire microbial community. A rationale for examining the entire bacterial community for host-specific markers is that several studies suggest that individual host species have unique time-invariant bacterial communities (Satokari *et al.* 2001; Simpson *et al.* 2002*a*; Tannock 2002; LaMontagne *et al.* 2003*b*) that can be detected in water (Cho & Kim 2000). Out of the range of community fingerprinting methods available, terminal restriction fragment length polymorphism analysis (T-RFLP; Liu *et al.* 1997) produces both graphical and numerical data and is particularly useful for generating comparisons of communities (LaMontagne & Holden 2003 (in press); LaMontagne *et al.* 2003*a* (in press)).

Other available culture-independent methods focus on detection of indicator genes. One is based on detecting small subunit ribosomal RNA genes (16S rDNA) from the Bacteroidetes group of fecal anaerobes (Bernhard & Field 2000*a*, *b*; Bernhard *et al.* 2003). Genetic diversity is high in this group, making it ideal for sequence comparison and primer design. Diagnostic sequences from uncultured Bacteroidetes have been identified using T-RFLP, sequence analyses or subtractive hybridization, and used to design PCR primers for fecal source tracking (Bernhard & Field 2000*a*, *b*; Dick *et al.* 2003 (submitted); Walters *et al.* unpublished). Another indicator gene approach is based on detection of toxin genes from diarrhoeic *E. coli*, an enterotoxin-producing pathogenic subset of *E. coli* associated with the gut in a variety of animals and humans. Unique sequences within one of the genes coding for the heat stable enterotoxin, STIb, are associated with human fecal waste and form the basis for PCR primers to detect human fecal pollution (Oshiro & Olson 1997). Similarly, bovine fecal waste is associated with the heat labile enterotoxin, LTIIa, the gene sequence of which was used to develop PCR primers to detect fecal pollution from cattle (Khatib *et al.* 2002). These markers have been shown to be highly specific and geographically stable, and to have a high enough prevalence for culture-independent detection as long as a large enough number of cells is screened (Khatib *et al.* 2002; Oshiro & Olson 1997).

Culture-independent methods have many potential advantages over cultured isolate-based methods. The first is that they do not require prior construction of a library of bacterial isolates. Library development is time-consuming, expensive and compromised by the presence of cosmopolitan or transient isolates that must be discarded to produce a good fit with library databases (Whitlock *et al.* 2002). Assessing the entire population via PCR amplification of a specific genetic sequence avoids sample size biases. Although culture-independent methods are based on prior knowledge of gene sequences for primer design, they do not depend on building a library of bacterial strains from a particular habitat or watershed.

Culture-independent methods are also potentially faster. Typical processing time for culturing isolates of indicator organisms is 24 h, which limits use of these methods in risk management decisions. Culture-independent data could be available on the same day that a beach closure decision needs to be made.

A third reason for investigating culture-independent methods is that uncultured microbes are numerically dominant in feces, and therefore may be easier to detect. Sequence-based studies of feces and the gut habitat routinely yield mostly unknown sequences (Wilson & Blitchington 1996; Pryde *et al.* 1999; Suau *et al.* 1999; Bernhard & Field 2000*b*; Daly *et al.* 2001; Favier *et al.* 2002; Holben *et al.* 2002; Hold *et al.* 2002; Leser *et al.* 2002; Zoetendal *et al.* 2002). The many new methods now used to monitor uncultured microbes in natural populations have opened up the possibility of using dominant, yet uncultured or difficult-to-culture, fecal bacteria as indicator organisms.

This paper presents an evaluation of culture-independent methods based on application to identical sets of blind samples containing known amounts of human, cattle, dog and gull fecal material or human sewage in an aqueous matrix. We report on results from these analyses, plus additional analyses carried out after the end of the blind testing. The work is part of a larger study in which multiple classes of methods were evaluated (Griffith *et al.* 2003), providing the opportunity not only for an unbiased evaluation of culture independent methods, but also for comparison with results produced by library-based methods.

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MATERIALS AND METHODS

Blind water samples and reference materials (approximately 1 g of fecal material or transport swabs from each of the scats used to create the water samples) were prepared and distributed to each laboratory as outlined in Griffith *et al.* (2003). Combined feces from 11 healthy adult humans, 12 dogs, 12 cattle (dairy and beef), four different flocks of gulls, or sewage influent from the Orange County Sanitation District primary wastewater stream were used to create the samples.

Community T-RFLP

Twelve samples suspended in distilled water and 12 mixed matrix (seawater matrix or humic acid amended distilled water) samples were sent to the University of California at Santa Barbara for community T-RFLP analysis. Fecal reference samples were washed (1 g 10 ml^{-1}) in phosphate saline buffer, treating each replicate sample independently. A fraction (2 ml) of the suspension was concentrated by centrifugation (10,000 × g, 2 min). The supernatant was aspirated away and the pellets were stored at −80°C. DNA was extracted from these pellets with a previously described bead-beating protocol (LaMontagne *et al.* 2002). Blind test samples (labelled A to X) were concentrated by centrifugation (7,000 × g, 10 min) and resuspended in 100 μl phosphate buffered saline solution. This concentrate was split. One section was transferred to a microcentrifuge tube, pelleted and archived at −80°C. The other section was mixed with an equal amount of 1.6% molten agar in a 1.5 ml tube. Agar blocks were digested overnight with Proteinase K and exchanged with TE buffer several times (Moreira 1998). DNA concentration in these blocks was determined by Picogreen™ fluorometry according to the manufacturer's instructions (Molecular Probes, Eugene, Oregon), after melting a section of the block in TE. For community analysis, a section of the agar block, equivalent to 50 ng DNA, was transferred to a thin-walled PCR tube. For comparison, DNA was also extracted from pellets of the archived samples by the bead-beating protocol described above.

PCR amplification of 16S rRNA genes from purified DNA samples was carried out using universal Bacterial primers 8F hex (fluorescently labelled forward primer; 5′AGAGTTTGATCCTGGCTCAG; Liu *et al.* 1997) and 1389R (5′ACGGGCGGTGTGTACAAG; Osborn *et al.* 2000) as described previously (LaMontagne & Holden 2003, in press). Each 100 μl PCR reaction contained 50 ng template, 3 units Taq polymerase (Qiagen, Valencia, California), 0.2 mM each dNTP, 50 pmol DNA primers, 1 × PCR buffer (Qiagen) and 2.5 mM $MgCl_2$ (including 1.5 mM from reaction buffer). Reaction mixtures were incubated at 94°C for 6 min and then held at 58°C while Taq and dNTPs were added. Reactions were cycled 28 times through three steps with a PCRSprint™ thermal cycler (Hybaid US, Franklin, Massachusetts): denaturing (94°C, 45 sec), annealing (58°C, 45 sec) and primer extension (72°C, 90 sec).

PCR products were purified with the Qiagen PCR purification kit (Qiagen, Valencia, California) and digested with *HhaI* (800 ng of purified PCR product with 1 unit restriction enzyme 16 h at 37°C). Restriction enzymes were inactivated by heating at 65°C for 10 min. Fluorescently labelled terminal fragments were separated by electrophoresis and fragment lengths determined with an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems Instruments, Foster City, California). T-RFLPs were aligned to determine the number of reproducible peaks. Fragment lengths were calculated with the Local Southern algorithm in Genescan (ABI). Terminal restriction fragments (TRFs) were aligned by hand to identify peaks shared between profiles generated from independent replicates. Fragments that differed by less than 1 bp were considered shared. Peaks that were unique to a profile, i.e. those that were not shared among replicate samples, were not analysed further.

Genetic markers based on uncultured fecal Bacteroidetes bacteria, assessed by T-RFLP

Two sets of 12 samples suspended in distilled water and 12 mixed matrix (seawater matrix or humic acid amended distilled water) samples were sent to the University of Southern California. Upon receipt, reference samples were stored at −80°C. Samples were subsequently thawed in their original containers and DNA

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extraction was performed using a FastDNA SPIN kit for soil (Q-Biogene, Carlsbad, California) following the manufacturer's protocol. DNAs from reference samples from humans and cattle were pooled and run as controls to verify proper performance of the instrument. DNA concentrations in extracts were quantified using Picogreen™ as described previously with a Versafluor fluorometer™ (BioRad, Hercules, California). DNA extracts were stored at −20°C.

Water samples were vacuum filtered in small aliquots (*c.* 10 ml) through 47 mm 0.22 micron GVWP filters (Millipore, New Bedford, Massachusetts) to near dryness. The second set of samples was filtered through 47 mm type A/E glass fibre filters (Pall, Ann Arbor, Michigan). In many cases, the filters quickly clogged and several filters were required to complete the filtration. Filters were placed in Whirlpak™ bags (Nasco, Modesto, California) and stored at −80°C until extraction. An initial DNA extraction was performed on the GVWP filters, with the glass fibre filters held in reserve. Later, DNA extraction was performed on a subset of the glass fibre filters to substitute for GVWP filters that yielded no measurable DNA. Filters were extracted using a FastDNA™ SPIN kit for soil (Q-Biogene, Carlsbad, California) according to the manufacturer's protocol, except that for the GVWP filters, the bead-beating step was omitted. Instead, frozen GVWP filters were placed directly into sterile 15 ml polypropylene culture tubes containing 3 ml of extraction buffer, vortexed vigorously for 90 sec, immersed in a boiling water bath for 5 min, and centrifuged at 2000 × g for 5 min in a clinical centrifuge to reduce foaming and remove the largest particles. Supernatant was transferred directly to spin columns in 400 μl aliquots. Extracts from multiple filters from the same sample were combined using an additional spin column. DNA concentrations in extracts were quantified using Picogreen™ as before. Following quantitation, aliquots of each DNA extract were split, transferred to anonymously labelled tubes by a third party, and stored at −20°C.

Approximately 5 ng of extracted sample DNA was used for the initial PCR amplification step of T-RFLP analysis. PCR was performed following a modified version of the protocol and using primers previously described (Bernhard and Field 2000*a*). Each 50 μl reaction consisted of 1 × *Taq* polymerase buffer, each general Bacteroidetes primer (Bac32f-D4 and Bac708r) at a concentration of 1 μM, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.25 U of *Taq* polymerase (Promega, Madison, Wisconsin), 0.64 μg μl^{-1} BSA and 1.5 mM $MgCl_2$. A negative control containing sterile water in place of sample DNA was run with each reaction to check for contamination. Amplification conditions were: 2 min hot start at 94°C; 35 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 30 sec; followed by a 6-min extension at 72°C in a BioRad thermal cycler (Hercules, California). PCR products were visualized by electrophoresis in a 2% LE agarose gel (SeaKem, San Diego California) stained with 1 × SYBR gold (Molecular Probes, Eugene, Oregon) and compared with a 100 bp DNA ladder (Promega, Madison, Wisconsin).

Bands were excised from gels and DNA recovered using a Zymo Gel Extraction kit (Zymo Research Products, Anaheim, California). DNA was quantified as above. 150ng of PCR products were combined with 10 units of *Hae*III restriction endonuclease (New England BioLabs, Beverly, Massachusetts) and incubated overnight at 37°C. Digested DNA was ethanol precipitated, resuspended in formamide and separated by electrophoresis on a Beckman CEQ™ 2000 DNA sequencer (Beckman, Fullerton, California) using the following conditions: a capillary temp of 45°C, a sample denature step of 90°C for 2 min, an injection of 2 kV for 10 sec and a separation of 3 kV for 160 min. Analysis was performed using the fragment analysis module integrated into the Beckman CEQ 2000 software package. Presence of a 119 bp fragment or a 222 bp fragment was scored as positive for human or cattle fecal contamination, respectively.

Genetic markers based on uncultured fecal Bacteroidetes bacteria, assessed by PCR (OSU)

Twelve samples suspended in distilled water and 12 mixed matrix (seawater matrix or humic acid amended distilled water) samples were sent to Oregon State University.

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Samples arrived at room temperature two days after shipping. The entire container was immediately placed at −10°C until DNA extraction began four days later.

Reference samples were thawed in their original containers. Approximately 10 ml of 5 M guanidine isothiocyanate (GITC) buffer was added to each and the samples were stirred into a slurry that could be pipetted with a wide bore transfer pipette. A 250–300 μl aliquot of each reference sample was extracted with the Fast DNA kit (Q Biogene) as above. A 25 μl aliquot of each reference DNA was placed in the same plate as the unknown sample DNAs and the well coordinates of all samples were recorded. A second plate with identical sample arrangement was made except each sample was diluted 10-fold in pure water. The diluted samples functioned as indicators of PCR inhibition.

The unknown water samples were 100 ml suspensions that ranged from visibly clear to opaque. Only one unknown (G) could be filter-concentrated through a 47 mm, 0.22 μm nylon filter. Unknowns B, E and R appeared filterable, but quickly clogged filters. The unfiltered portion from each clogged filter was returned to its original container. Filters were placed in 500 μl 5 M GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA, pH 8, 0.5% Sarkosyl), coated with the buffer by vortexing, and stored at −80°C until DNA extraction. The remaining suspensions were concentrated via successive loadings in 50 ml clean and sterile Oakridge centrifuge tubes and spinning at 18,000 × g at 4°C for 30 min per loading. The supernatant was removed by aspiration and the concentrated samples were resuspended with 5 ml of GITC buffer for cell lysis. The GITC resuspensions were transferred to fresh 15 ml polypropylene Falcon tubes and stored at −80°C until DNA extraction. Three tubes with GITC (extraction blanks) were added to the sample set as controls for cross-contamination during the DNA extraction.

Filters in GITC obtained from unknowns B, E, R and G were extracted using DNeasy spin columns (Qiagen, Valencia, California) according to the manufacturer's protocol, with the following changes: we omitted the proteinase K digestion and added two more column washes with buffer AW2. Filter extracts were eluted from the spin columns with 100 μl of buffer EB and heating for 5 min at 60°C prior to centrifugation. Eluted DNAs from the filters were stored in low-retention siliconized microcentrifuge tubes (Axygen, Union City, California) at −20°C. Extracts from B, E and R filters were later pooled with the DNA from the unfilterable portion of the original suspension. The GITC-stored concentrates from the non-filterable suspensions were thawed, vortexed, and a 250–300 μl aliquot of each was extracted using the Fast DNA kit from Q Biogene (Carlsbad, California) according to the manufacturer's protocol, with the addition of a second SEWS-M buffer wash. We eluted DNA with 200 μl Buffer EB (Qiagen) and heating at 60°C for 5 min. Eluted DNAs were stored in low retention siliconized microcentrifuge tubes at −20°C. For easier PCR reaction set up, 25 μl aliquots of each unknown DNA sample were arranged in a low retention 96-well plate (Axygen), also stored at −20°C.

PCR analysis was performed for human, ruminant and dog Bacteroidetes 16S rDNA markers using source specific forward primers paired with Bac708R, a general Bacteroidetes reverse primer. Human and ruminant specific primers and general Bacteroidetes primer were as previously described (Bernhard & Field 2000*b*). The dog specific primer was recently developed (Dick *et al.* 2003 (submitted)). Each 25 μl PCR reaction contained 1 × TaKaRa ExTaq PCR buffer (Panvera, Madison, Wisconsin), 200 μM each dNTP, 0.2 μM forward primer and 0.2 μM Bac708R, 0.06% BSA, 0.63 U TaKaRa ExTaq and 1 μl sample DNA 1 × or 0.1 × eluate. A positive control template of human, cattle or dog fecal DNA was included with the appropriate marker PCR as verification of the PCR performance. PCR conditions were: a 94°C denaturation for 3 min, followed by 35 (40 for dog marker) cycles of 94°C 1 min, anneal 1 min, 72°C extend 1.5 min, a final extension step at 72°C for 7 min and a 4°C hold in a PCR Express Thermal Cycler (Thermo Hybaid, Middlesex, UK). Annealing temperatures were 63°C for the human markers, 62°C for the ruminant markers and 64°C for the dog marker. Completed PCR reactions were scored for presence/absence of the correct size product for each marker on 96-well Ready-to-Run 2.2% agarose-TBE gels containing ethidium bromide (Amersham Biosciences, Piscataway, New Jersey). Five μl of PCR reaction was mixed with 5 μl of 1 × TBE buffer in each gel lane according to the manufacturer's protocol. Samples were

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separated by electrophoresis at 120 V for 7 min. Gels were photographed with a CCD imager.

Genetic markers based on uncultured fecal Bacteroidetes bacteria, assessed by PCR (USC)

DNA extracts from 12 samples suspended in distilled water and 12 mixed matrix (seawater matrix or humic acid amended distilled water) samples were analysed for host specific markers of human and ruminant Bacteroidetes at the University of Southern California. DNA extracts were aliquots from the same extracts used for T-RFLP identification of human and ruminant genetic markers based on uncultured fecal Bacteroidetes bacteria as described previously.

Approximately 2–15 ng of extracted sample was PCR amplified using a modified version of the protocol and the PCR primers previously described (Bernhard & Field 2000*b*). Negative controls containing all reagents and sterile water in place of DNA extract were included in each set of reactions to check for cross-contamination. Positive controls contained DNA extracted from human or cattle feces as described previously. PCR took place in a Barnstead thermal cycler (Barnstead/Thermolyne, Dubuque, Iowa) using Bacteroidetes specific primers Hf183F (human) or Cf128F (cattle) paired with a common reverse primer Bac708R (Bernhard & Field 2000*b*). Each 50 μl reaction consisted of 1 × *Taq* polymerase buffer, each primer at a concentration of 1 μM, each dNTP at a concentration of 200 μM, 1.25 U of *Taq* polymerase (Promega, Madison, Wisconsin), 0.64 μg of bovine serum albumin per μl and 1.5 mM $MgCl_2$ run under the following conditions: 2 min denaturation at 94°C, then 25 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, followed by a 6-min extension at 72°C.

Following the initial amplification, 1 μl of each PCR product was re-amplified using the same conditions as above with fresh reagents for an additional 25 cycles. PCR products were visualized by electrophoresis in a 2% LE agarose gel (SeaKem, San Diego, California) stained with 1 × SYBR gold (Molecular Probes, Eugene, Oregon) and compared with a 100 bp DNA ladder (Promega, Madison, Wisconsin). Positive results for human feces produced a 525 bp product while positive results for cattle produced a 580 bp product. To verify negative results for human sources and check for PCR inhibition, each sample that produced a negative result was re-run with the addition of 10 pg of DNA extracted from human stool. A positive result was considered evidence of a 'true negative' for a human source, while a negative result was deemed 'inconclusive' because an unknown substance in the sample, and not lack of the proper DNA template, may have interfered with the amplification reaction.

PCR primers based on *E. coli* toxin genes

Twelve samples suspended in distilled water were sent to the University of California at Irvine for toxin gene analysis. 10 ml of each water sample and fecal material from each transport swab resuspended in water were filtered through a 0.45 mm, 47 mm porous nylon membrane filter (Osmonics/MSI, Minnetonka, Minnesota). Filters were placed on mTEC agar (Difco) and incubated at 35°C for 1.5 h, followed by 44.5°C for 20 ± 2 h. Plates were flooded with 1 ml 1 × phosphate-buffered saline, colonies were resuspended in the buffer and then pipetted into 1.5 ml eppendorf tubes. Tubes were centrifuged for 10 min at 12,000 × g, the supernatant discarded and cells kept for DNA extraction.

A modified phenol/chloroform extraction method (Tsai & Olson 1991) was used to extract bacterial DNA. The addition of Tris-HCl-saturated phenol to the DNA after the freeze–thaw cycles was omitted and an additional wash step was added after the isopropanol precipitation. DNA was resuspended in 200 ml of 75% ethanol (reagent grade) at −20°C for 1 h and processed as described in the method. RNAase was also omitted from the protocol and the DNA without further purification was stored at −50°C until PCR analysis.

To increase sensitivity, a second set of primers for both toxin genes was developed for nested PCR (*STIb*: 5′-TGTATTGTCTTTTTCACC and 5′-GCTTGTACCGGG TGCTATTAA and *LTIIa*: 5′-GCATGGAGAAAGAGATG AGC and 5′-CTTACCACATAGATCCCACG). These primers were tested for cross-reactivity by screening all sequences contained in GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST (Altschul *et al.* 1990).

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Each 50 μl PCR reaction contained 5 mM Tris-HCl (pH 8.3), 25 mM KCl, 1.0 mM $MgCl_2$, 100 mM of each dNTP, 0.2 mM of each primer, 2.5 U of AmpliTaq DNA polymerase (Promega, Madison, Wisconsin) and 10 μl of DNA sample extract. PCR run conditions were: a 94°C denaturation step for 1 min, followed by 30 cycles of 94°C for 30 sec, 45°C (57°C for nested PCR) for 30 sec for *STIb* and 61°C (56°C for nested PCR for *LTIIa*) and 72°C for 30 sec with a final extension at 72°C for 6 min in a Perkin Elmer (model 9600, version 1.05) DNA thermal cycler (Wellesley, Massachusetts).

PCR products (187 bp band using single PCR and 167 bp band using nested PCR for *STIb*; 204 bp band using single PCR and 358 bp band using nested PCR for *LTIIa*) were separated by electrophoresis on a 2% agarose gel containing 5 mg ml^{-1} of ethidium bromide using the Alpha Innotech (Alpha Innotech Corp., San Leandro, California, model 2.1.3) system, and documented using Alpha Imager 3300 software (version 3.1.1). A 50 bp molecular weight marker (Promega, Madison, Wisconsin) was used to determine the size of the fragments.

Results were confirmed by Southern hybridization following dot blot onto positively charged 0.45 mm nylon filters (MSI, Minnetonka, Minnesota). A 26 bp probe and a 38 bp probe were used for the STIb and LTIIa toxin genes, respectively. The probes were 5′ end labelled with [α-^{32}P] ATP using T4 kinase in an exchange reaction as suggested by the manufacturer (Gibco Life Technologies, Carlsbad, California). Filters were exposed to X-OMAT film (Eastman Kodak, Rochester, New York) at −80°C for 6 to 18 h depending on the activity of the probe. Restriction analysis was used to further confirm results. *STIb* positive samples produced 3 fragments (24 bp, 27 bp and 115 bp) when cut with *Hinf*I and 2 fragments (50 bp and 110 bp) when cut with *Bst*API. *LTIIa* PCR positive samples produced bands of 245 bp and 113 bp when cut with *Pst*I. For addition confirmation, excised bands from gels from samples positive for the *STIb* trait were sent to the UC Davis DBS DNA Sequencing Facility for sequencing.

RESULTS

Results from analysis of blind water samples were assessed using four criteria:

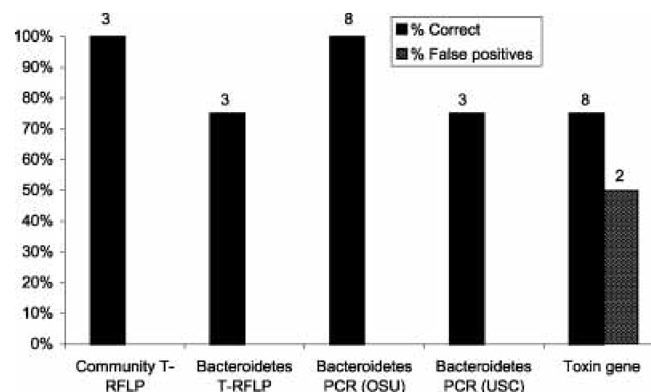

Figure 1 | True positive and false positive results for human fecal contamination in distilled water by investigator.

1. Ability to identify presence of human fecal material in distilled water samples.
2. Ability to identify absence of human fecal material in distilled water samples.
3. Ability to detect multiple sources of fecal contamination in distilled water samples.
4. Stability of response across three matrices: distilled water, salt water and humic acids.

Calculations were based on samples for which a result was reported. Several investigators encountered technical difficulties, in obtaining sufficient DNA for analysis, in amplifying DNA or in completing the analysis of blind samples in the time allocated.

All of the methods correctly identified a majority of the distilled water samples containing sources of human fecal contamination. No method identified less than 75% of samples for which results were obtained (Figure 1). Community T-RFLP and the OSU host specific PCR methods correctly identified human contamination in all samples for which results were reported. The toxin gene biomarker method correctly identified 75% of samples containing a human source of contamination, including correct identification of all samples containing sewage (Table 1).

Bacteroidetes T-RFLP and host specific PCR methods performed best at identifying samples that did not contain a human source of contamination. The toxin gene method incorrectly identified 50% of these samples.

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Table 1 | Sources identified in each sample by each investigator (H=human, C=cattle, D=dog, G=gull, S=sewage, N=no result); * indicates sources that were identified in supplemental analyses conducted after the sample was unblinded

Sample	Community T-RFLP	Bacteroidetes T-RFLP	Bacteroidetes PCR (OSU)	Bacteroidetes PCR (USC)	Toxin gene	Actual fecal sources present
(a) distilled water matrix						
A	N	S	S	S	S	S
C	N	N	H,C,D	N		H,G
E	C,D	C	C,D	C	H,C*	C,D
F	H,C,D	N	H,D	N		H,G
G	N	N	N		N	G
I	N	N	H	N	H	H
J	C,D,G,H	C	C,D,S	C	C*,S	C,D,S
K	N	S	D,S	S	S	S
L	H,C,D	H,C	H,C,D	H,C	H,C*	H,C,D
N	N	N	D,S	N	S	D,S
P	N	C	C	C	H,C*	C,G
U	N	C	C	C	C*	C
(b) saltwater or humic acid matrix						
B	N	N	N	C		H,G
D	H,C,D	C	C	C		C
H	H,C,G,S	N	C	C		C,G
M	N	N	H	N		H
O	H,C,D	N	C	N		C
Q	C,D,H	C	C,D,S	C		C,D,S
R	N	N	N	S		S
S	N	N		N		G
T	N	H	H	H		H,G
V	H,C,D	H	H	H		H,G
W	N	N	S	S		S
X	N	N	H	N		H,G

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Most of the methods are not yet capable of identifying non-human sources. The OSU host-specific PCR method is capable of identifying all sources except gull feces. The only non-human source identifiable by the USC host-specific PCR method and the Bacteroidetes T-RFLP methods is cattle and both methods correctly identified cattle in every sample in which it occurred. During the blind portion of the study, only results for human sources were reported using the toxin gene method, but samples were subsequently screened for presence of cattle fecal material using the LTIIa biomarker. The LTIIa assay correctly detected 100% of samples containing cattle fecal contamination (Table 1).

Only the community T-RFLP method is currently capable of detecting all sources, but it produced many false positives. Cattle feces were incorrectly identified as occurring in every quantifiable sample. Peaks identified as specific to dogs and humans also yielded several false positives. However, there were some promising aspects of the results for this method. For example, the host-specific peaks comprised reasonably large fractions of the total T-RFLP signal for individual sources.

The saltwater matrix had little effect on the stability of the methods tested (Table 1), as all methods obtained results similar to those obtained when the same sources were suspended in distilled water (Table 1). Samples containing humic acids were more problematic and caused difficulty with DNA extraction or PCR amplification in some cases.

Several investigators had difficulty in obtaining sufficient DNA to perform their analyses (Table 1). The problem was most severe for the two investigators performing T-RFLP, who were unable to analyse more than half the samples, but all investigators had problems gathering sufficient DNA for at least one sample. Two investigators quantified the amount of DNA recovered from each sample. There was good agreement between them as to which samples had low DNA content.

Community T-RFLP

DNA yield varied greatly between samples. The sample of sewage plant influent had the highest concentration (59 ng μl^{-1}). Most of the samples (14 of 23) yielded insufficient DNA for community analysis (<5 ng μl^{-1}). Sufficient DNA for community analysis was recovered from all of the mixtures that contained feces from cattle except sample U. Only two samples that did not contain feces from cattle, F and V, amplified well with Bacterial primers. Thus, the presence of cattle feces was almost always necessary to recover nucleic acids for PCR amplification with the combination of centrifugation and commercial kit used. This is consistent with the actual composition of these mixtures. Because of the relatively low culturable bacterial counts in cattle feces (Griffith *et al.* 2003), to obtain similar contributions of *E. coli*, several orders of magnitude more grams of feces from cattle were added than other sources. While this yielded relatively equivalent contributions of these culturable bacteria (Griffith *et al.* 2003), the mass of nucleic acids from the sources was not equivalent.

T-RFLPs generated from cattle, dogs and sewage differed from one another and shared more peaks in within-source comparisons than between-source comparisons (LaMontagne *et al.* 2003*b*). The higher number of shared peaks within sources suggests that bacterial ribotypes differ significantly between fecal sources. To determine if T-RFLPs could determine the relative contribution of feces from different species in a mixture, we identified source-specific peaks for each of the five sources. These peaks were rare or relatively small in T-RFLPs from other sources.

Although the fecal reference samples had distinctive source-specific peaks that appeared diagnostic, we were unable to accurately quantify the fecal sources in the unknown samples, most likely because of the disproportionate representation of cattle fecal DNA in the samples. Since for the most part we only obtained PCR-amplifiable DNA from samples with cattle feces, this result is not surprising. The nine samples that we tested all scored positive for cattle feces. A relatively high abundance of cattle-specific peaks obtained from mixtures F and V may represent contamination. Peaks designated as specific for dogs and humans yielded several false positives. In the case of the human-specific peaks, false positives may result from the overlap between T-RFLPs generated from cattle and human feces. In addition, because relatively

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little fecal matter from dogs and humans was present in the mixtures, in terms of grams wet weight, the false positives we observed might be a function of the detection limit of this protocol. A second possibility is that when sources are mixed together there is an interaction between the templates that could create bias (amplification is not quantitative). Our ongoing research is addressing this issue.

Bacteroidetes markers (OSU)

The human assays correctly identified reference and unknown samples containing human feces or sewage with no false positives. The cattle assays generated one false positive, C. Since neither cattle assay showed any cross-reactivity with the human and gull reference samples that were used to prepare Sample C, the most likely explanation for this false positive is contamination, which could have occurred during sample preparation, DNA extraction or PCR. The dog assay generated three false positives; all contained gull feces. In order to increase signal, the unknown samples were amplified with the dog primer for 40 cycles instead of the correct 30 cycles for which the primer was optimized. At 40 cycles, the dog primer cross-reacted with one of the gull reference samples, but it did not cross-react at 30 cycles. When the samples were reanalysed with 30 cycles of PCR with the dog primer, none of the gull samples cross-reacted.

Interference from heterologous DNAs did not affect these assays. For example, the human-specific primers accurately detected the presence of human fecal contamination in samples containing only human feces (A, I and K), human and one other type of feces (C, F and N), and human and two other types of feces (J and L).

These assays were not sensitive to salt water; however, two out of four of the samples containing humic acids did not amplify.

Bacteroidetes markers by T-RFLP (USC)

No measurable DNA was recovered from nine samples. DNA concentrations were consistently highest in extracts from samples containing cattle feces. Measurable DNA was recovered from only one of four of the samples amended with humic acid, but no PCR product was produced, perhaps due to inhibition of the PCR reaction by the humic acid. Concentrations of DNA in reference sample extracts varied widely from sample to sample and source to source. The highest concentrations were observed in cattle and the lowest in gulls.

Bacteroidetes markers by PCR (USC)

An additional extraction from glass fibre filters originally intended for human enterovirus analysis produced DNA for three additional samples for which the previous method had not obtained DNA. One false positive result for cattle was reported for sample A, which contained only human and gull feces. This may have been caused by contamination, as no other false positives in samples containing these sources were observed.

E. coli toxin genes

STIb was present in one of four composite gull fecal samples, and one of 12 dog fecal swabs. All individual *E. coli* isolates were negative for the trait. To our knowledge, *STIb* has never previously been observed in other than a human host. This result could be explained by sample contamination, but it is consistent with false positives obtained in unknown samples as well. The method identified two samples, E (containing dog feces) and P (containing gull feces), as containing human contamination. Two *STIb* gene sequences, obtained from the positive gull fecal sample, and from unknown P (containing gull feces), were more similar to each other than either was to *STIb* sequences from sewage (results not shown). If *STIb* occurs in some gulls and dogs, there would be an expected (but low) rate of false positives for samples contaminated from either of these sources. Surveys of gull and dog populations could accurately quantify this rate. Because dilution analyses indicated a higher concentration of *STIb* in the positive gull reference sample than in the positive dog reference sample (results not shown), we expected more false positives in samples contaminated with gull feces than we observed. The fewer than expected

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false positives containing gull feces, and more than expected false positives containing dog feces, may be due to incomplete fecal mixing in the preparation of mixed fecal inocula, or may reflect the stochastic distribution of genes when they are diluted to very low copy numbers.

Although the *STIb* assay correctly detected the presence of human contamination in all unknown samples containing primary sewage influent, it only identified two of the four unknown samples containing human feces. Only one out of 11 of the human fecal samples used to create the unknown samples was positive for *STIb*. For unknowns containing human feces, this assay would score samples as negative if the amount added of the single positive human fecal sample was low. The limit of detection of this method is about 10 copies of the gene; we maximized sensitivity by performing nested PCRs. Because of the limits of detection and because not all humans carry the *STIb* gene in their feces, assaying for *STIb* is effective in detecting contamination from sewage, but inappropriate for detection of single human fecal sources.

DISCUSSION

Criteria to assess methods include the incidence of false positives and false negatives, or missed samples. A false negative occurs when a particular source is present, but the method does not detect it. In this study there were at least three causes for false negatives: operator error; insufficient sensitivity (if the source, or the trait being assayed, was below the limits of detection); or interference, for example from humic acids that inhibit the PCR.

A false positive occurs when a particular source is not present, but the method reports it as present. In this study there were at least three different kinds of false positives: first, a particular source was not present, but the method detected it, due to insufficient specificity of the assay; second, the 'wrong' host has the marker, so that although a particular source was not present, the method detected it due to the presence of another source which has the marker; third, operator error, such as sample contamination.

For any method of fecal source tracking, there will be an associated rate of false positives. As long as the rate is relatively low, and measurable, it does not negate the usefulness of the method. Careful studies could establish the expected rate of false positives for each of these methods.

With the exception of community T-RFLP, results for the culture-independent methods were characterized by a low rate of false positives and a high rate of false negatives. This is in sharp contrast to results from the library-dependent methods that were evaluated as part of the larger study, which rarely missed a source but frequently identified multiple sources that were not present in the sample (Griffith *et al.* 2003). Library-dependent methods are prone to false positive results because they examine many individual isolates, providing multiple opportunities for mistakes, particularly if some isolates are cosmopolitan. Culture-independent methods are less prone to this type of error because they look only for the presence of a specific signal. In addition, library-dependent methods may assay genotypic or phenotypic traits that show considerable overlap among hosts. Most culture-independent methods are based on traits with little or no overlap among hosts.

Many results we have called 'false negatives' were actually 'no data due to no assay available', and reflect the limited number of primers that have been developed to date. Culture-independent fecal source tracking methods are still relatively new and primer development is limited by the number of specific genetic sequences available that are unique to a particular host. Of the methods included in this study, the host-specific PCR method for Bacteriodetes is furthest along in terms of the number of source-specific primer sets available, but other *E. coli* toxin genes have also been shown to have host-specific distributions (Provence & Curtiss 1994; Adams *et al.* 1997). *E. coli* markers for pig have already been proposed (Khatib *et al.* 2003). The success of culture-independent methods in this study should provide additional incentive for investment in further primer development research.

Another factor that contributed to false negatives was an inability to extract adequate DNA from some samples, a critical component of these methods. This finding highlights the need to standardize methods between laboratories. There were variations in yield due to

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differences in sample centrifugation, varied methods of lysis, purification and recovery. Each of the extraction methods used has been successfully applied in previous studies, yet some methods did not work well for these samples. The problem was most severe for the community T-RFLP, which obtained insufficient DNA from most samples. This method may require more DNA, and thus larger volume or more concentrated samples, than the other methods. The toxin gene method bypassed the problems associated with direct DNA extraction from the blind samples by employing a growth step prior to DNA extraction. This strategy effectively increased the DNA yield, but introduced the potential for culture bias.

A third factor, causing false negatives in the enterotoxin gene method, is the rare nature of the signal. Like human pathogenic viruses, enterotoxigenic bacteria are relatively rare in the human population, requiring a large sample size of humans to obtain a signal. Much like enteric viruses and coliphage (Noble *et al.* 2003), the enterotoxin gene method correctly identified all samples containing human sewage, but missed some of those containing fecal material from individual humans.

Assessment of these methods' ability to correctly identify the 'dominant' source of fecal material in samples was complicated because there could be several ways to assign a dominant source to samples. The fecal sources in the unknown samples were added according to estimates of their *E. coli* concentrations; vastly different amounts of feces from the different sources were added to facilitate recovery of culturable *E. coli*, resulting in variable amounts of DNA recovered from each fecal source. In addition, the dominant sources estimated by *E. coli* concentrations were often not the same as the dominant sources estimated by concentrations of enterococci. The enterotoxic *E. coli* method is the only method that specifically detects *E. coli*. For the other methods, some other measure of fecal concentration, such as fecal mass, might have been more appropriate.

Fecal indicators are used to predict human and environmental health risks. Thus over the long term it is more important to understand the correlation of these source-tracking indicators to measures of human and environmental health than their correlation with coliforms or enterococci. However, since management decisions are largely controlled by legislation specifying permissible levels of either *E. coli* or enterococci, in the short term, indicators are needed that correlate to these public health markers.

A shortcoming of the culture-independent methods is that most are not quantitative. Community T-RFLP was the only method that attempted to estimate percentage contribution from different fecal sources in each sample; the other methods reported presence or absence of fecal sources. For management purposes, it is desirable to be able to quantify the relative contribution of each source of fecal contamination. There are several promising technologies that may provide the means for accomplishing this. For instance, quantitative real-time PCR (Q-PCR) can accurately measure the number of copies of a marker gene in a template. These techniques would be applicable to any presence/absence PCR-based assays described here. A Q-PCR assay for Bacteroidetes genes was recently described that is well correlated with counts of *E. coli* and enterococci in sewage (Dick & Field 2003 (submitted)). However, interpreting results of Q-PCR for source-specific markers will require further research to assess stability of marker dosage over time and space.

The issue of quantitation is complicated by the related issue of survival of fecal markers in water. An older method of fecal source tracking, comparing the ratio of fecal coliforms to fecal streptococci, has been largely abandoned because it has been shown that the two groups survive differently, causing the ratio to change over time (Pourcher *et al.* 1991; Sinton *et al.* 1993). The relative survival of the fecal indicators being used for source discrimination has not been investigated. This is a major shortcoming of all source-tracking technologies, not just culture-independent methods.

CONCLUSIONS

The strengths of culture-independent fecal source tracking methods lie in their rapid identification of presence/absence of certain fecal sources, without the need for culturing bacteria, and without the need for libraries. However, they are currently limited by the number of

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species for which there are published markers. There is a crucial need for both marker development and information on survival of markers, especially compared with pathogens and standard public health indicators.

We predict that a productive approach in future will involve initial application of one of these culture-independent methods. In many situations, presence/absence data will be sufficient to allow mitigation efforts to be properly focused. If further information is needed, the presence/absence data will allow the cost-effective, focused application of library-based methods in specific areas only.

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