

## Comparison of genotypic-based microbial source tracking methods requiring a host origin database

Samuel P. Myoda, C. Andrew Carson, Jeffrey J. Fuhrmann, Byoung-Kwon Hahm, Peter G. Hartel, Helen Yampara-Iquise, LeeAnn Johnson, Robin L. Kuntz, Cindy H. Nakatsu, Michael J. Sadowsky and Mansour Samadpour

### ABSTRACT

Microbial source tracking (MST) results, obtained using identical sample sets and pulsed field gel electrophoresis (PFGE), repetitive element PCR (rep-PCR) and ribotyping techniques were compared. These methods were performed by six investigators in analysis of duplicate, blind sets of water samples spiked with feces from five possible sources (sewage, human, dog, cow and seagull). Investigators were provided with samples of the fecal material used to inoculate the water samples for host origin database construction. All methods correctly identified the dominant source in the majority of the samples. Modifications of some of these methods correctly identified the dominant sources in over 90% of the samples; however, false positive rates were as high as 57%. The high false positive rates appeared to be indirectly proportional to the levels of stringency applied in pattern analysis. All the methods produced useful data but the results highlighted the need to modify and optimize these methods in order to minimize sources of error.

**Key words** | *Escherichia coli*, fecal enterococci, pulsed field gel electrophoresis, rep-PCR, ribotyping

**Samuel P. Myoda** (corresponding author)  
820 Silver Lake Blvd,  
Suite 220, Dover,  
DE 19904-2464, USA  
Tel: 302.739.4590  
Fax: 302.739.6140  
E-mail: [samuel.myoda@state.de.us](mailto:samuel.myoda@state.de.us)

**C. Andrew Carson**  
**Helen Yampara-Iquise**  
University of Missouri,  
Department of Veterinary Pathobiology,  
Columbia, MO 65211, USA

**Jeffrey J. Fuhrmann**  
University of Delaware,  
Department of Plant and Soil Sciences,  
Newark, DE 19717, USA

**Byoung-Kwon Hahm**  
**Cindy H. Nakatsu**  
Purdue University,  
Department of Agronomy, West Lafayette,  
IN 47907, USA

**Peter G. Hartel**  
**Robin L. Kuntz**  
Department of Crop and Soil Sciences,  
University of Georgia,  
Athens, GA 30602, USA

**LeeAnn Johnson**  
**Michael J. Sadowsky**  
University of Minnesota,  
Department of Soil, Water, and Climate,  
BioTechnology Institute,  
St Paul, MN 55108, USA

**Mansour Samadpour**  
Institute for Environmental Health,  
Seattle, WA 98155, USA

### INTRODUCTION

Many genome-based typing methods have been developed to identify species or subspecies of bacteria in an epidemiological or systematics context (Tenover *et al.* 1997; Luows *et al.* 1999; Olive & Bean 1999; van Belkum *et al.* 2001), and recently there has been considerable interest in using these methods in microbial source tracking (MST). These methods target the whole genome, particular genes,

or a specific DNA sequence. The application of these methods to MST is based on the hypotheses that subspecies of specific bacteria originating from a particular animal host species are more closely related than those from other animal species, and that by matching genotypes of bacterial isolates from the environment to bacterial isolates from various warm-blooded animals, the

host of origin for the environmental isolates can be determined. These methods can be categorized as being either host origin database dependent or host origin database independent. The three most common genotypic methods used for MST that are host origin database dependent are pulsed field gel electrophoresis (PFGE), repetitive element PCR (rep-PCR) and ribotyping.

These three MST methods have advantages and disadvantages (Farber 1996) as they differ in cost and rigour. Currently, only one method, ribotyping, is automated. However, all three methods produce a series of bands (a fingerprint) on agarose gels, and band patterns obtained from a host origin database of known host patterns are compared with patterns obtained from unknown environmental isolates.

PFGE has been used by the Centers for Disease Control and Prevention (CDC) to study outbreaks of *E. coli* O157:H7 (Olive & Bean 1999). Parveen *et al.* (2001) evaluated PFGE data using the restriction enzyme *Sfi*I and found no relationship between bacterial fingerprint patterns and host source. However, Simmons *et al.* (2000), using the restriction enzyme *Not*I, reported success in comparing unknowns with known source patterns.

rep-PCR has been used successfully to distinguish strains of *E. coli* (Lipman *et al.* 1995; Dombek *et al.* 2000; Carson *et al.* 2003), *Rhizobium meliloti* (de Bruijn 1992), *Bradyrhizobium japonicum* (Judd *et al.* 1993), *Streptomyces* spp. (Sadowsky *et al.* 1996), *Xanthomonas* spp. (Bouzar *et al.* 1999) and other bacteria (Versalovic *et al.* 1998; Sadowsky & Hur 1998). Dombek *et al.* (2000) reported that rep-PCR was useful for MST, and Carson *et al.* (2003) reported that the performance of rep-PCR was equal or superior to the performance of ribotyping using the restriction enzyme *Hind*III.

Ribotyping has been the mostly widely used fingerprinting protocol for MST (Samadpour & Chechowitz 1995; Parveen *et al.* 1999; Carson *et al.* 2001; Jenkins *et al.* 2003; Scott *et al.* 2003). The DNA is typically cut with the restriction enzyme *Hind*III alone (Parveen *et al.* 1999), or the enzymes *Eco*RI and *Pvu*II (Samadpour & Chechowitz 1995). With *E. coli*, superimposing the band patterns generated with *Eco*RI and *Pvu*II, or combining the enzymes as was done in this study, are considered superior to patterns generated with *Hind*III alone because the combi-

nation produces more informational bands. The single restriction enzyme *Pst*I, however, provides good discrimination between fecal enterococci (Kuntz *et al.* 2003).

While these methods may be useful in individual applications, no studies have simultaneously evaluated multiple methods and multiple laboratories using the same test samples. In this study, six investigators, each using one or two of the three genotypic methods, were compared with respect to their accuracy in predicting the source of fecal material in blind, spiked water samples.

## MATERIALS AND METHODS

### Overview

Fecal samples from seagulls, dogs, cows, sewage influent and humans and blind water samples (spiked with fecal material) created from the same source materials were prepared at a central laboratory and replicates sent to the study participants. Each investigator isolated 60 bacteria (*E. coli* or fecal enterococci) from the five fecal sources to create a host origin database consisting of 300 isolates. Each investigator also isolated 25 or 50 isolates of the indicator bacteria of their choice from the blind water samples for DNA pattern comparison against their host origin database.

### rep-PCR analysis of *Escherichia coli*

#### Investigator A

Fecal swab samples were inoculated into lactose broth (Becton Dickinson, Sparks, Maryland) and incubated overnight at 37°C. Isolates were selected by growth on mFC, mENDO, MacConkey with MUG, and Luria-Bertani agar (all Becton Dickinson) at prescribed temperatures. Fecal *E. coli* isolates were confirmed by use of BBL Crystal Identification Systems Enteric/Nonfermenter System (Becton Dickinson) supported by indole and oxidase tests.

rep-PCR was performed using the BOXA1R primers and the protocol previously described by Dombek *et al.*

(2000). The protocol was slightly modified in that Lyse-n-Go PCR reagent (Pierce Chemical Co., Rockford, Illinois) was added to aid cell lysis (Carson *et al.* 2003). Following amplification, PCR products were separated by electrophoresis in 1.5% SeaKem agarose gel (Bio Whittaker, Rockland, Maine) at 100 V for 4 h at room temperature. Gel images were captured with an EDAS 290 system (Kodak Co., Rochester, New York).

### Investigator B

Fecal swabs were streaked directly onto the surface of EMB agar plates (Becton Dickinson) and incubated at 44.5°C overnight. Aqueous samples were diluted in sterile 0.85% saline, plated onto mFC plates, and incubated at 44.5°C overnight. Fecal coliform colonies that appeared blue on mFC plates were chosen randomly and transferred to EMB plates for purification. Isolates were subsequently cultivated on tryptic soy agar (TSA) plates overnight at 37°C and *E. coli* identification confirmed by growth on citrate slants.

rep-PCR was performed using the BOXA1R primers and the protocol previously described by Dombek *et al.* (2000). Following amplification, PCR products were separated by electrophoresis in a 1.5% agarose gel (0.5 × TBE) at 64 V for 18 h at room temperature. Gel images were captured using a Polaroid camera (Kodak) and digitized using an Epson Expression 386XL scanner (Epson America, Long Beach, California) and PhotoShop (Adobe Systems, San Jose, California).

### Investigator C

Fecal swabs were streaked onto mFC agar plates and incubated at 44.5°C for 24 h. Sewage influent and water samples were diluted 1:10 in 0.1% peptone water and plated onto mFC agar plates. *Escherichia coli* was isolated and confirmed as described in Dombek *et al.* (2000).

rep-PCR was performed using the BOXA1R primers and the protocol previously described by Dombek *et al.* (2000). Pattern analysis was enhanced by labelling one of the PCR primers with a fluorophore to enhance band identification, a procedure known as fluorophore-enhanced rep-PCR (FERP). Following amplification, PCR

products were separated by electrophoresis at 4°C at 70 V for 17–18 h with constant buffer recirculation (Rademaker & de Bruijn 1997). FERP images were captured using a Typhoon 8600 Variable Mode Imager (Molecular Dynamics, Amersham Biosciences, Sunnyvale, California) in the fluorescence acquisition mode. Separated gel images were processed using ImageQuant image analysis software (Molecular Dynamics) and converted to 256 greyscale TIFF images.

### Ribotyping *Escherichia coli*

#### Investigator D

Fecal swabs were streaked onto MacConkey agar plates and water samples were passed through a 0.45 µm filter. Colonies from the agar plates and membrane filters were restreaked onto MacConkey agar plates and incubated for 24 ± 2 h at 35 ± 0.5°C. Colonies were transferred to blood agar plates and incubated for 24 ± 2 h at 35 ± 0.5°C. Isolates were subsequently tested for indole production and citrate utilization. Colonies that were lactose-positive, indole-positive and citrate-negative were considered *E. coli*.

For DNA extraction, confluent growth from the blood agar plates was scraped with a sterile flat-headed toothpick and cell material was suspended in 800 µl 50 mM Tris and 50 mM EDTA (pH 8.0). Genomic DNA was extracted using the phenol/chloroform procedure described by Sambrook & Russel (2001). Restriction endonuclease digestion reactions were performed using *EcoRI* and *PvuII* (BoehringerMannheim GmbH, Germany) according to the manufacturer's protocol. DNA fragments were separated by electrophoresis at 33 V for 12–14 h in a 0.8% agarose gel in 1 × TBE (Tris-borate-EDTA). The DNA was transferred to a Nitran filter (Schleicher & Schuell, Keene, New Hampshire), baked at 80°C for 1 h, and probed with <sup>32</sup>P-labelled copies of *E. coli* rRNA produced using random hexanucleotide primers and Avian Myeloblastosis Virus reverse transcriptase (Stratagene, La Jolla, California) according to the manufacturer's protocol.

#### Investigator E

Fecal swabs were streaked onto mTEC agar plates (Difco) and then the plates were incubated at 44.5 ± 0.2°C for

24 h. Yellow colonies were streaked onto TSA and incubated at 35°C for 24 h. Isolates that did not hydrolyse urea after 18–24 h incubation were re-streaked onto mTEC agar and incubated at 35°C for 24 h. Yellow colonies were considered to be *E. coli* and were re-streaked onto TSA. Water samples were diluted and each passed through a 0.45 µm filter. Filters were placed on mTEC agar plates, incubated at 35°C for 2 h, and then at 44.5 ± 0.2°C for 22 h. Following incubation, filters were placed on an absorbent pad saturated with urea substrate. Urea-negative isolates were streaked onto TSA and incubated at 35°C overnight. Well-separated colonies were restreaked twice onto TSA to ensure their purity.

Genomic DNA isolation and restriction endonuclease digestion were performed as described above for Investigator D. DNA fragments were separated by electrophoresis at 33 V for 12–14 h in a 0.8% agarose gel in 1 × TBE and transferred to an Immobilon Ny+ membrane (Millipore, Bedford, Massachusetts). The filters were baked at 80°C for 1 h, and probed with chemiluminescent DIG-dUTP labelled copies of *E. coli* rRNA gene probes based on full-length sequences of *E. coli* 16S and 23S rRNA genes (accession numbers 16445223, 16127994 and J01695) obtained from the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Hybridization was performed in 5 × SSC at room temperature overnight as per the manufacturer's protocol (Roche, Indianapolis, Indiana).

## Ribotyping fecal enterococci

### Investigator F

Samples were processed with the Enterolert™ system (IDEXX Laboratories, Westbrook, Maine) according to the manufacturer's protocol. To obtain isolates of fecal enterococci from each well, the back of a Quanti-tray was surface-disinfected with 70% ethanol and the well was slit open with a sterile scalpel. A 10-µl portion was removed from the well with a sterile plastic loop and streaked onto a 5-cm plate of Enterococcosel agar (Becton Dickinson). Plates were incubated in Ziploc bags (Dow Brands, Indianapolis, Indiana) at 37°C for 48 h. One black isolate (indicating esculin hydrolysis) was randomly picked from each Enterococcosel agar plate with a sterile plastic stab.

The isolates were confirmed as enterococci according to standard methods (Standard Methods 1998).

Bacterial isolates were processed on a RiboPrinter following the manufacturer's protocol (DuPont Qualicon, Wilmington, Delaware), except that each DNA sample was digested with the restriction enzyme *Pst*I. The DNA fragments were size-separated by electrophoresis on pre-cast agarose gels and transferred to nylon membranes. The membranes were exposed to a series of proprietary chemical and enzymatic treatments to produce chemiluminescent DNA fragments. The banding pattern image created was captured by a CCD camera and stored as a TXT file.

## PFGE of *Escherichia coli*

### Investigator D

Isolates used for ribotyping were also used for PFGE analysis by this investigator (Ribotyping, Investigator D). DNA preparation and pulsed field gel electrophoresis after restriction endonuclease digestion with *Xba*I (American Allied Biochemicals, Denver, Colorado) was performed as described by Renter *et al.* (2003) using a Hoffer Hula Gel system (Hoffer Scientific, San Francisco, California).

## Fingerprint analysis

Five of the six investigators (A, B, C, E and F) analysed fingerprint patterns using the BioNumerics software program (Version 3.0, Applied Maths, Sint-Martens-Latem, Belgium; Table 1). Identification of unknown bacteria was performed using a host origin database containing information from 60 isolates from each of the five possible sources. Investigators A and B analysed only bands between 300 and 10,000 bp. Investigator C normalized FERP gel lanes with a Genescan-2500 ROX internal lane standard. Both Investigators A and C calculated fingerprint similarities with a Pearson product moment correlation coefficient and 1% optimization. Only lanes with BioNumerics quality factors A–C (quantitative measures of the quality of match between an unknown and a member of the host origin database) were considered. A correct

**Table 1** | Band analysis, qualitative tolerance thresholds

Method	rep-PCR				Ribotyping				PFGE	
	A	B	C	D	D	E	F	F	D	D
Initial isolation media	mFC ( <i>E. coli</i> )	EMB ( <i>E. coli</i> )	mFC ( <i>E. coli</i> )	MacConkey ( <i>E. coli</i> )	mTEC ( <i>E. coli</i> )	Idexx Enterolert <sup>®</sup> (fecal enterococci)	MacConkey ( <i>E. coli</i> )	MacConkey ( <i>E. coli</i> )	Indole and citrate tests	Indole and citrate tests
Isolate confirmation	BBL crystal identification systems	Citrate test	Indole and citrate tests	Indole and citrate tests	Urea test	Standard methods (1998)	Indole and citrate tests	Urea test	Indole and citrate tests	Indole and citrate tests
Statistical program	BioNumerics	BioNumerics	BioNumerics	None	BioNumerics	BioNumerics	BioNumerics	BioNumerics	None	None
Band analysis	Pearson, quality factor A-C, $\geq 80\%$ similarity	Pearson, no minimum similarity	Pearson, quality factor A-C, $\geq 80\%$ similarity	Manual, 1:1 match	Jaccard, no minimum similarity	Dice, 100% similarity	Manual, 1:1 match	Jaccard, no minimum similarity	Manual, 1:1 match	Manual, 1:1 match



match was assumed when a fingerprint had  $\geq 80\%$  match to a pattern contained in the host origin database. Investigator B also used a Pearson product moment correlation coefficient, but set no minimum similarity for a match. Data for Investigator C were also corrected for rounding errors ( $1.6 \pm 1.7\%$ ) included in the initial data submission. Investigators E and F used a band matching program, Jaccard or Dice (Dice 1945), instead of the curve-fitting Pearson's coefficient, to analyse their data. Investigators B and E had no minimum similarity set for each match. In contrast, Investigator F accepted only matches at a 100% similarity index.

Investigator D analysed band patterns by visual inspection of autoradiograms produced from ribotyping or PFGE gels. Bands that were  $>3$  mm apart were scored as singles, while bands that were  $\leq 3$  mm of each other were counted as doublets or triplets. Each unique pattern was deemed a ribotype and assigned a numeric value. A letter designation was added to differentiate two isolates with the same numeric value but different banding patterns. Each individual ribotype was entered into a Microsoft Access database. Only exact matches (1:1 matching) were accepted when comparing ribotypes from blind samples for source identification.

### Evaluation criteria

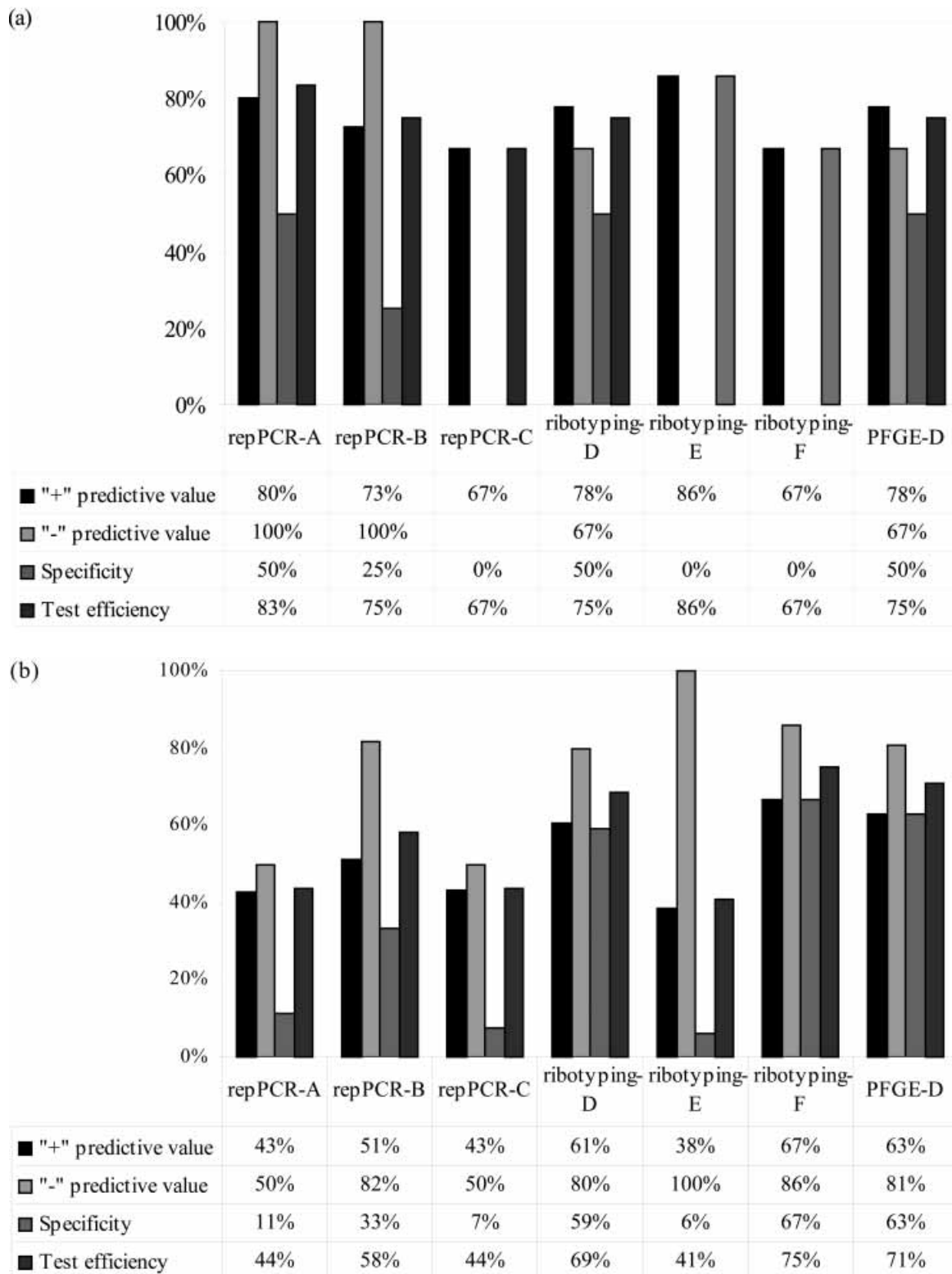
After each investigator submitted their prediction of the host origin of the feces and their respective contributions in the blind samples, their results were compared with the actual amount of spiked material according to the following evaluation criteria:

1. Ability to predict certain values:
  - (a) Positive predictive value (the percentage of reported positives that are true positives):  $TP/(TP + FP)$ , where TP is true positives and FP is false positives.
  - (b) Negative predictive value (the percentage of reported negatives that are true negatives):  $TN/(FN + TN)$ , where TN is true negatives and FN is false negatives.
  - (c) Sensitivity (the percentage of all samples with source 'n' present that are reported to contain source 'n'):  $TP/(TP + FN)$ .
  - (d) Specificity (the percentage of all samples without source 'n' present that are reported not containing source 'n'):  $TN/(FP + TN)$ .
  - (e) Test efficiency (percentage of the times that the test gives the correct answer compared with the total number of tests):  $(TP+TN)/(TP+TN+FP+FN)$ .
  - (f) False positive rate (the percentage of all samples incorrectly reported to contain source 'n'):  $FP/(TP + TN + FP + FN)$ .
2. Ability to accurately identify human and sewage influent sources of fecal material contained in a sample.
3. Ability to identify the dominant source of fecal material contained in a sample.
4. Ability to accurately identify all sources of fecal material contained in a sample.

### RESULTS

There was a wide range in the positive predictive value of the methods examined. This range was greatest among ribotyping methods, which exhibited both the highest (67%) and the lowest (38%) overall positive predictive values (Figure 1a and b). The positive predictive values were higher when considering identifying samples containing only sewage influent and/or human fecal sources. The positive predictive value for the human/sewage source ranged from 67 to 86% for ribotyping methods, 67 to 80% for rep-PCR methods and 78% for the PFGE method. Positive predictive values were inversely related to the rate of false positive results for each method; methods with the lowest positive predictive value had the highest number of false positive results and vice versa. With the exception of Investigators A and C (50%), negative predictive values were excellent and ranged from 80 to 100%, reflecting the rarity of false negative results among the data.

All methods exhibited excellent sensitivity in correctly identifying samples containing sewage influent and/or human fecal contamination. The three rep-PCR methods and two of the ribotyping methods



**Figure 1** | (a) Predictive value, specificity and test efficiency for samples containing a human/sewage source of fecal contamination. (b) Overall predictive value, specificity and test efficiency for samples containing fecal contamination from all sources.

(Investigators E and F) achieved sensitivities of 100%. The other ribotyping method (Investigator D) achieved a sensitivity of 89%. The sensitivity for the PFGE method was also 89%, identical to that of the ribotyping method performed at the same lab. The non-human/sewage source sensitivity was also excellent, ranging from 77 to 100%; one ribotyping method achieved 100%, two rep-PCR methods achieved 85% and the balance of the methods achieved 77%.

Conversely, many of these methods exhibited poor specificity. The non-human/sewage source specificity ranged from 4 to 78% and the human/sewage source specificity ranged from 0 to 50%. The non-human/sewage false positive rate (FP/(total number of samples  $\times$  sources)) for one ribotyping method (Investigator E) was 71%. The greatest variability in false positive rates was among ribotyping methods, ranging from 14 to 71%, but variability among rep-PCR methods was also high, ranging from 42 to 61%. The false positive rate for the PFGE method was 22%, slightly lower than that of the ribotyping method performed at the same lab. The human/sewage source false positive rates were much lower, ranging from 14 to 33% (Figure 2a), lowering the overall false positive rates to 19 to 57% (Figure 2b).

Test efficiency was also higher when considering samples containing sewage influent and/or human fecal contamination only. The test efficiency for the human/sewage source ranged from 67 to 86%, while the non-human/sewage source ranged from 25 to 78%. The overall test efficiencies for all methods were similar and ranged from 44 to 58% for rep-PCR, 41 to 75% for ribotyping and 71% for PFGE.

With the relatively small host origin database size used for these studies, none of the methods was able to correctly identify the dominant source of contamination in all samples. One rep-PCR method performed best, correctly identifying the dominant source in 83% of the samples (Investigator C), but the ribotyping and PFGE methods used by Investigator D performed almost as well, identifying 75% of samples. Variability in results for identifying the dominant source of contamination in samples among the rep-PCR and ribotyping methods ranged from 50 to 83% and 50 to 75%, respectively (Figure 3).

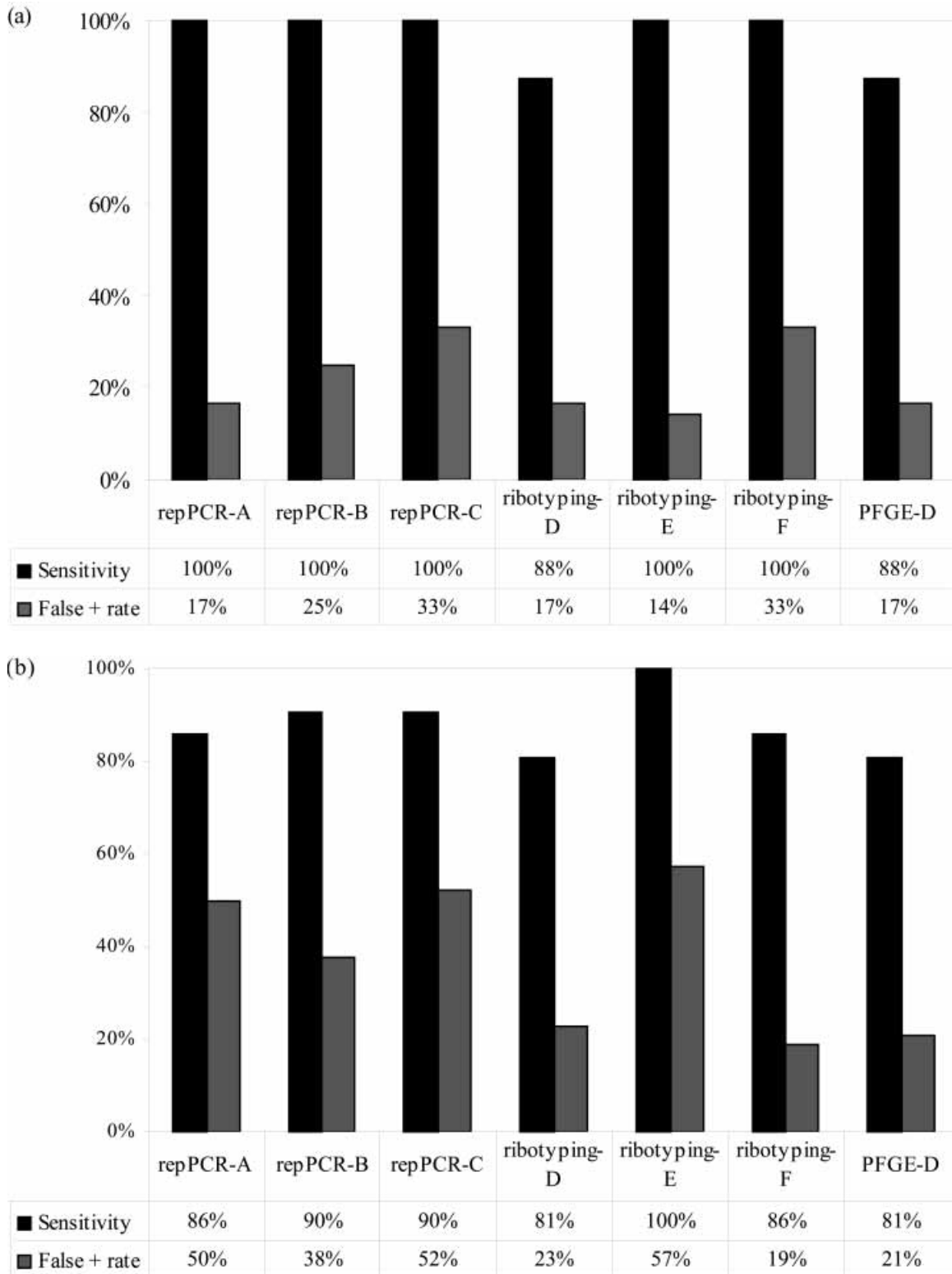
Every method performed poorly in identifying all sources of contamination in each sample, a result consistently attributable to identifying source materials that were not present in the sample (Table 2). The PFGE and ribotyping methods performed by Investigator D did best, correctly identifying all sources in 42% of the samples. Ribotyping using fecal enterococci identified 33% of the samples correctly and one rep-PCR method identified 17% (Figure 4). None of the other investigators was able to correctly identify all sources in any samples.

## DISCUSSION

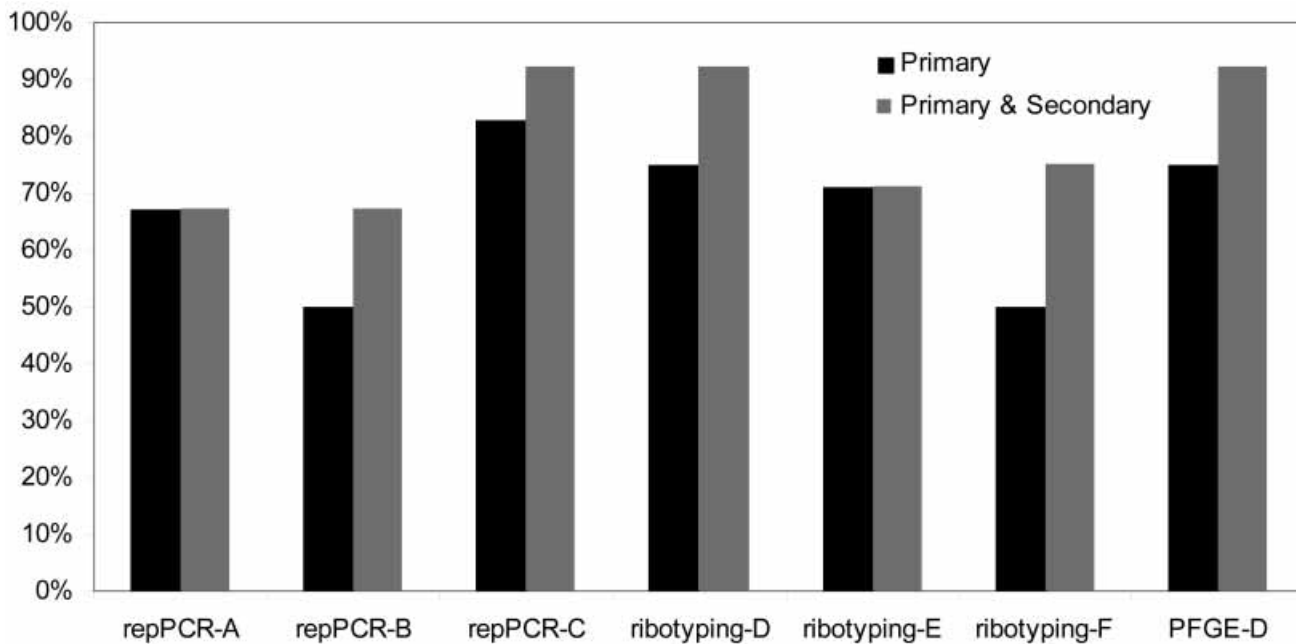
The principal goal in most applications of MST technology is to identify the dominant fecal source in a waterway. On average, investigators were successful in identifying the dominant source in more than two-thirds of the blind water samples in this study. This, however, is likely to be an underestimation because of several limiting factors imposed by the sample design. For instance, the number of isolates examined from the blind samples was standardized at 50 to allow for comparison across methods. However, the average *E. coli* concentration in the blind samples was  $>1.5 \times 10^5$  CFU 100 ml<sup>-1</sup> and thus 50 isolates only represents  $<0.03\%$  of the sample population. In a real world application, a larger number of isolates would probably have been obtained and quantified.

Further contributing to underestimation of the dominant source was uncertainty about the percentage of sources actually added into the blind test samples and the small host origin database size used. Several investigators measured bacterial indicator densities when the samples arrived in their labs and these densities routinely varied by two orders of magnitude. This difference suggests that there was either differential die-off of bacteria during transport or heterogeneity in sample preparation, both of which could lead to misrepresentation of the true dominant source in the samples. This supposition is further suggested by the consistency of some of the predictions made among the investigators (Table 2). For example, six of eight investigators reported cattle as the dominant source in Sample E, even though cattle feces were supposedly the secondary source in this sample.





**Figure 2** | (a) Percentage of samples correctly (sensitivity) and incorrectly (false positive rate) identified as containing a human/sewage source of fecal contamination. (b) Overall percentage of samples correctly (sensitivity) and incorrectly (false positive rate) identified as containing fecal contamination from all sources.



**Figure 3** | Percentage of samples in which the dominant (primary) or one of the top two dominant (primary and secondary) sources of contamination were correctly identified.

To allow for the possible uncertainty in the true dominant source due to these factors, results were reanalysed to determine whether the dominant source identified by the investigator was one of the top two fecal sources added to the sample. When reanalysed, the results from three of the investigators achieved more than 90% correct classification (Figure 3). This type of analysis may even be more appropriate than the original analysis because managers are not typically interested in defining a single dominant source; they are more interested in defining all major contributing sources, both controllable and uncontrollable, so that sensible pollution control strategies can be developed.

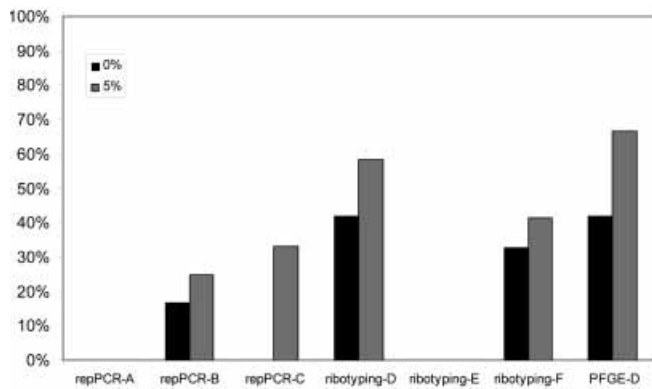
A second important MST application goal is to assess the presence of human feces in a sample, even if it is not the dominant source, since managers will potentially make different health risk decisions based on presence of human fecal material. The study documented virtually no false negatives on presence of human fecal material, but a high percentage of false positives for all sources, including humans, was found.

The high rate of false positives appears to reflect inadequacies in the algorithms used to match banding

patterns between the host origin database materials and the blind samples. The algorithms to match unknowns with reference patterns are not currently standardized and were not in this study. The algorithms ranged from assigning all isolates from the blind samples to the closest matched source (Investigators B and E), to assigning only those isolates that matched perfectly with a source sample (Investigators D and F). Methods that had the lowest rate of false positives were those that used algorithms with high qualitative tolerance thresholds. After composition of the blind samples was revealed, several investigators examined how their results would have changed if they had used alternative algorithms with higher qualitative thresholds; in all cases, the percentage of false positives declined. However, high qualitative tolerance thresholds generally led to the exclusion of a large subset of isolates. Discarding too many isolates leads to concerns about obtaining an accurate answer, as the data obtained are from a subset of bacteria that may no longer be representative of the population in the original sample. This concern would not manifest itself as strongly in a controlled study where there were a limited number of possible

**Table 2** | Blind data submission: true positives, true negatives, false positives and false negatives ('H', sewage influent and/or human; 'D', dog; 'C', cow; 'G', seagull; '+', true positive; '-', true negative; 'FP', false positive; 'FN', false negative; 'nr', not reported)

Source	Key	rep-PCR												Ribotyping												PFGE															
		A				B				C				D				E				F				D				H				C				G			
		H	D	C	G	H	D	C	G	H	D	C	G	H	D	C	G	H	D	C	G	H	D	C	G	H	D	C	G	H	D	C	G	H	D	C	G				
A	+	-	-	-	+	F	F	F	+	F	F	F	+	F	F	F	+	F	F	+	F	F	F	+	F	F	F	+	F	F	F	+	F	F	F						
C	+	-	-	+	+	F	F	F	+	-	F	F	+	+	+	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
E	+	+	-	-	+	F	F	F	+	-	F	F	+	-	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr						
F	+	-	-	+	+	F	F	F	+	+	F	F	+	+	F	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
G	-	-	+	+	+	F	F	F	+	+	F	F	+	+	F	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
I	+	-	-	-	+	F	F	F	+	+	F	F	+	+	F	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
J	+	+	+	-	+	F	F	F	+	+	F	F	+	+	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr						
K	+	-	-	-	+	F	F	F	+	+	F	F	+	+	F	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
L	+	+	+	-	+	F	F	F	+	+	F	F	+	+	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr						
N	+	+	-	-	+	F	F	F	+	+	F	F	+	+	+	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
P	-	-	+	+	-	F	F	F	+	+	F	F	+	+	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr						
U	-	-	+	-	+	F	F	F	+	+	F	F	+	+	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr						
False negatives	0	0	1	2	0	1	0	1	0	2	0	0	1	2	0	1	0	0	0	0	0	0	0	1	1	1	1	1	1	2	0	1	1	2	0	1					
False positives	2	8	7	7	3	7	6	2	4	7	7	7	2	3	4	2	1	5	7	3	4	0	5	2	3	3	2	3	2	3	3	2	3	3	2	2					



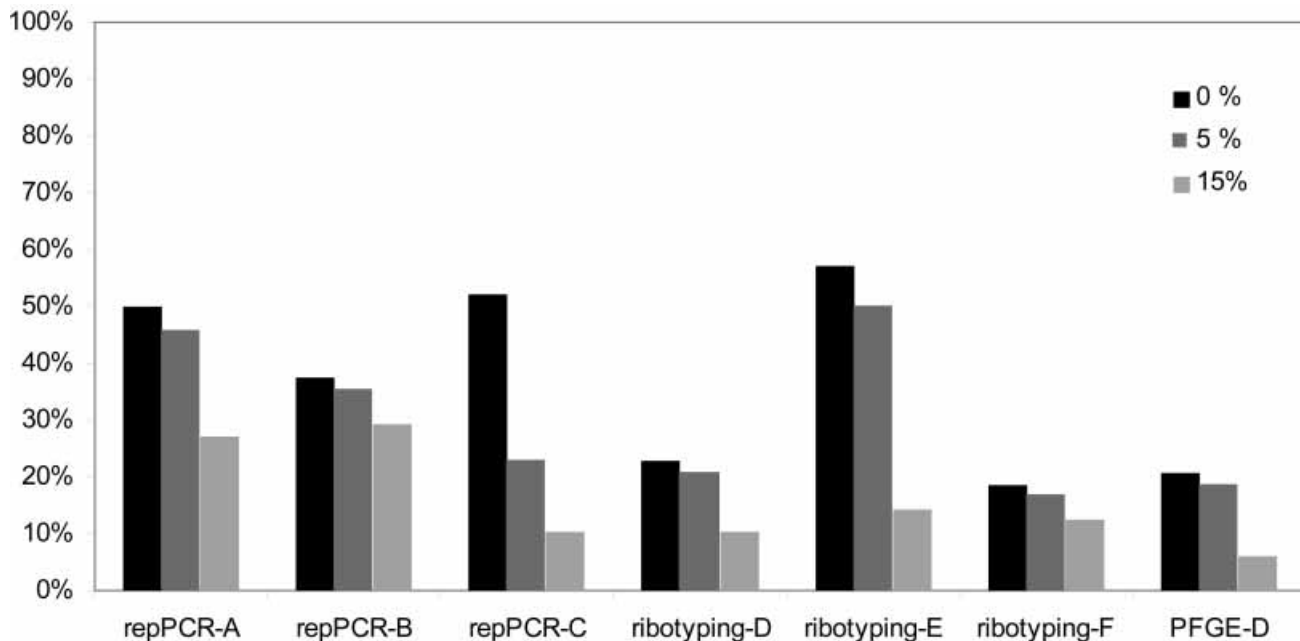
**Figure 4** | Percentage of samples in which all sources were correctly identified with no reporting threshold and a 5% reporting threshold (no false positives or false negatives).

sources and investigators had access to all of the source material, as it would in an actual application where the number of sources is potentially much greater.

An alternative way to reduce the number of false positives is to establish a quantitative threshold below which managers are advised to ignore the source identi-

cation. Applying a 5% reporting threshold to the results from this study decreased the number of false positives only slightly, although the effect was investigator dependent (Figure 5). Increasing the reporting threshold to 15%, though, led to a dramatic improvement, with false positives falling below 10% for most investigators.

It is important to note, however, that application of more restrictive algorithms or qualitative tolerances will only solve a portion of the problem. Previous studies suggest that the bacterial subspecies in an environmental sample occur in multiple species. Often referred to as transient or cosmopolitan, these subspecies provide limited diagnostic information. Our understanding of population genetics for these bacteria is still rudimentary and none of the host origin database-dependent MST methods directly (or knowingly) targets a specific trait that is unique to a bacterial group originating from a specific host species. The patterns produced by the different PCR primers or restriction enzymes may be random with respect to the genetic differences that make these groups unique. More research is needed to understand the specificity of selected strains to a source, and to select



**Figure 5** | Influence of qualitative reporting threshold (0 to 15%) on overall false positive rate.

which primers and bacterial species provide the greatest specificity.

Because data analysis varied among investigators, it is difficult to determine which genotypic method was the most efficient. Investigator D applied two methods, PFGE and ribotyping, to an identical set of isolates and, using the same algorithm for analyses, found that the two methods produced comparable results. This agreement suggests that given equal information obtained from the method chosen, analysis strategy is more important than the underlying method used to create the banding patterns for the isolates. If this is the case, then selection of the most appropriate method may depend on the application. For instance, PFGE produces more unique banding patterns than ribotyping, which would be advantageous in applications with a limited number of possible sources that need to be well differentiated. In contrast, PFGE may be disadvantageous when applied to a more complex situation because the larger number of unique isolates requires a larger host origin database than for ribotyping, adding substantially to study cost. Therefore, when choosing which MST method to use for a particular application, factors such as laboratory accuracy, ability to acquire source samples, throughput, financial constraints, study area and study goals must all be considered to design an effective study.

## CONCLUSIONS

Although all the methods were able to identify the dominant source in most of the blind samples, the methods also had high false positive rates. This problem seemed to be largely affected by the algorithm used to match banding patterns between isolates in the host origin database and blind samples; algorithms requiring a closer match for source assignment have a smaller rate of false positives. None of the MST methods stood out as being superior to the others at correctly identifying fecal sources in blind samples. The majority of differences were investigator dependent, indicating the need to modify and optimize these methods, particularly the analytical algorithms used, to minimize sources of error.

## REFERENCES

- Bouzar, H., Jones, J. B., Stall, R. E., Louws, F. J., Schneider, M., Rademaker J. L.W., de Bruijn, F. J. & Jackson, L. E. 1999 Multiphasic analysis of xanthomonads causing bacterial spot disease on tomato and pepper in the Caribbean and Central America: Evidence for common lineages within and between countries. *Phytopathol.* **89**, 328–335.
- Carson, C. A., Shear, B. L., Ellersieck, M. R. & Asfaw, A. 2001 Identification of fecal *E. coli* from humans and animals by ribotyping. *Appl. Environ. Microbiol.* **67**, 1503–1507.
- Carson, C. A., Shear, B. L., Ellersieck, M. R. & Schnell, J. D. 2003 Comparison of ribotyping and repetitive extragenic palindromic-PCR for identification of fecal *E. coli* from humans and animals. *Appl. Environ. Microbiol.* **69**, 1836–1839.
- de Bruijn, F. J. 1992 Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl. Environ. Microbiol.* **58**, 2180–2187.
- Dice, L. R. 1945 Measures of the amount of ecologic association between species. *Ecol.* **26**, 297–302.
- Dombek, P. E., Johnson, L. K., Zimmerley, S. T. & Sadowsky, M. J. 2000 Use of repetitive DNA sequences and the PCR to differentiate *E. coli* isolates from human and animal sources. *Appl. Environ. Microbiol.* **66**, 2572–2577.
- Farber, J. M. 1996. An introduction to the hows and whys of molecular typing. *J. Food Protect.* **59**, 1091–1101.
- Jenkins, M. B., Hartel, P. G., Olexa, T. J. & Stuedemann, J. A. 2003 Putative temporal variability of *Escherichia coli* ribotypes from yearling steers. *J. Environ. Qual.* **32**, 305–309.
- Judd, A. K., Schneider, M., Sadowsky, M. J. & de Bruijn, F. J. 1993 Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. *Appl. Environ. Microbiol.* **59**, 1702–1708.
- Kuntz, R. L., Hartel, P. G., Godfrey, D. G., McDonald, J. L., Gates, K. W. & Segars, W. I. 2003 Targeted sampling protocol as prelude to bacterial source tracking with *Enterococcus faecalis*. *J. Environ. Qual.* **32**.
- Lipman, L. J. A., de Nijs, A., Lam, T. J. G. M. & Gastra, W. 1995 Identification of *E. coli* strains from cows with clinical mastitis by serotyping and DNA polymorphism patterns with REP and ERIC primers. *Vet. Microbiol.* **43**, 13–19.
- Luows, F. J., Rademaker, J. L. W. & de Bruijn, F. J. 1999 The three Ds of PCR-based genomic analysis of phyto-bacteria: Diversity, detection, and disease diagnosis. *Ann. Rev. Phytopath.* **37**, 81–125.
- Olive, D. M. & Bean, P. 1999 Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* **37**, 1661–1669.
- Parveen, S., Portier, K. M., Robinson, K., Edmiston, L. & Tamplin, M. L. 1999 Discriminant analysis of ribotype profiles of *E. coli* for differentiating human and nonhuman sources of fecal pollution. *Appl. Environ. Microbiol.* **65**, 3142–3147.

- Parveen, S., Hodge, N. C., Stall, R. E., Farrah, S. R. & Tamplin, M. L. 2001 Phenotypic and genotypic characterization of human and nonhuman *Escherichia coli*. *Wat. Res.* **35**, 379–386.
- Rademaker, J. L. W. & de Bruijn, F. J. 1997 Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. In *DNA Markers: Protocols, Applications, and Overviews* (ed. G. Caetano-Anollés & P. M. Gresshoff), pp. 151–171. J. Wiley and Sons, New York.
- Renter, D. G., Sargeant, J. M., Oberst, R. D. & Samadpour, M. 2003 Diversity, frequency, and persistence of *Escherichia coli* O157 strains from range cattle environments. *Appl. Environ. Microbiol.* **69**, 542–547.
- Sadowsky, M. J. & Hur, H. G. 1998 Use of endogenous repeated sequences to fingerprint bacterial genomic DNA, pp. 399–413. In *Bacterial Genomes: Physical Structure and Analysis* (ed. F. J. de Bruijn, J. R. Lupski & G. M. Weinstock), pp. 394–411. Chapman and Hall, New York.
- Sadowsky, M. J., Kinkel, L. L., Bowers, J. H. & Schottel, J. L. 1996 Use of repetitive intergenic DNA sequences to classify pathogenic and disease-suppressive *Streptomyces* strains. *Appl. Environ. Microbiol.* **62**, 3489–3493.
- Samadpour, M. & Chechowitz, N. 1995 *Little Soos Creek Microbial Source Tracking: A Survey*. University of Washington Department of Environmental Health, Seattle, Washington.
- Sambrook, J. & Russel, D. 2001 Preparation of plasmid DNA by lysis with SDS. In *Molecular Cloning*, 3rd edition, pp. 1.55–1.58. Coldspring Harbor Laboratory Press, New York.
- Scott, T. M., Parveen, S., Portier, K. M., Rose, J. B., Tamplin, M. L., Farrah, S. R., Koo, A. & Lukasik, J. 2003 Geographical variation in ribotype profiles of *E. coli* isolates from humans, swine, poultry, beef, and dairy cattle in Florida. *Appl. Environ. Microbiol.* **69**, 1089–1092.
- Simmons, G. M., Wayne, D. F., Herbein, S., Myers, S. & Walker, E. 2000 Estimating nonpoint fecal coliform sources in Northern Virginia's Four Mile Run watershed. In *AMSTracts, Virginia Water Research Symposium 2000, VWRRC Special Report SR-19-2000, Blacksburg, Virginia* (ed. T. Younos & J. Poff), pp. 248–267.
- Standard Methods for the Examination of Water and Wastewater* 1998 20th edition, American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC.
- Tenover, F. C., Arbeit, R. D. & Goering, R. V. 1997 How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: A review for healthcare epidemiologists. *Infect. Cont. Hosp. Epidemiol.* **18**, 426–439.
- van Belkum, A., Struelens, M., de Visser, A., Verbrugh, H. & Tibayrenc, M. 2001 Role of genomic typing in taxonomy, evolutionary genetics, and microbial epidemiology. *Clin. Microbiol. Rev.* **14**, 547–560.
- Versalovic, J., de Bruijn, F. J. & Lupski, J. R. 1998 Repetitive sequence based PCR (rep-PCR) DNA fingerprinting of bacterial genomes. In *Bacterial Genomes: Physical Structure and Analysis* (ed. F. J. de Bruijn, J. R. Lupski & G. M. Weinstock), pp. 437–454. Chapman and Hall, New York.