

Evaluation of antibiotic resistance analysis and ribotyping for identification of faecal pollution sources in an urban watershed

D.F. Moore¹, V.J. Harwood², D.M. Ferguson¹, J. Lukasik³, P. Hannah¹, M. Getrich¹ and M. Brownell²

¹Orange County Public Health Laboratory, Santa Ana, CA, USA, ²Department of Biology, University of South Florida, Tampa, FL, USA, and ³Biological Consulting Service of North Florida Inc., Gainesville, FL, USA

2004/0935: received 12 August 2004, revised 30 December 2004 and accepted 20 January 2005

ABSTRACT

D.F. MOORE, V.J. HARWOOD, D.M. FERGUSON, J. LUKASIK, P. HANNAH, M. GETRICH AND M. BROWNELL. 2005.

Aims: The accuracy of ribotyping and antibiotic resistance analysis (ARA) for prediction of sources of faecal bacterial pollution in an urban southern California watershed was determined using blinded proficiency samples.

Methods and Results: Antibiotic resistance patterns and *Hind*III ribotypes of *Escherichia coli* ($n = 997$), and antibiotic resistance patterns of *Enterococcus* spp. ($n = 3657$) were used to construct libraries from sewage samples and from faeces of seagulls, dogs, cats, horses and humans within the watershed. The three libraries were analysed to determine the accuracy of host source prediction. The internal accuracy of the libraries (average rate of correct classification, ARCC) with six source categories was 44% for *E. coli* ARA, 69% for *E. coli* ribotyping and 48% for *Enterococcus* ARA. Each library's predictive ability towards isolates that were not part of the library was determined using a blinded proficiency panel of 97 *E. coli* and 99 *Enterococcus* isolates. Twenty-eight per cent (by ARA) and 27% (by ribotyping) of the *E. coli* proficiency isolates were assigned to the correct source category. Sixteen per cent were assigned to the same source category by both methods, and 6% were assigned to the correct category. Addition of 2480 *E. coli* isolates to the ARA library did not improve the ARCC or proficiency accuracy. In contrast, 45% of *Enterococcus* proficiency isolates were correctly identified by ARA.

Conclusions: None of the methods performed well enough on the proficiency panel to be judged ready for application to environmental samples.

Significance and Impact of the Study: Most microbial source tracking (MST) studies published have demonstrated library accuracy solely by the internal ARCC measurement. Low rates of correct classification for *E. coli* proficiency isolates compared with the ARCCs of the libraries indicate that testing of bacteria from samples that are not represented in the library, such as blinded proficiency samples, is necessary to accurately measure predictive ability. The library-based MST methods used in this study may not be suited for determination of the source(s) of faecal pollution in large, urban watersheds.

Keywords: *Enterococcus*, *Escherichia coli*, indicator organisms, source tracking, water quality faecal pollution.

INTRODUCTION

The levels of faecal indicator bacteria in recreational waters, surface runoff and sewage have been used for decades to measure faecal contamination and the suitability of the water for recreational uses or discharge. Previous studies have reported an increased risk of gastrointestinal symptoms and diseases correlated with high levels of faecal indicator bacteria, especially *Escherichia coli* and *Enterococcus* spp. (US Environmental Protection Agency 1984; Pruss 1998). Total coliforms, faecal coliforms, *E. coli* and *Enterococcus* spp. are faecal indicator bacteria commonly used to identify contaminated waters and normally reside in the gastrointestinal tracts of all warm-blooded and some cold-blooded animals (American Public Health Association 1995; Harwood *et al.* 1999). The enumeration and/or detection of bacterial indicators to measure water quality are widely used, and can be an effective strategy for public health protection. Unfortunately, these methods cannot identify the sources of bacteria as animal or human in origin, which causes difficulties for risk assessment and the design of effective remediation measures to improve water quality. An ideal water quality indicator would accurately predict not only animal *vs* human source, but would also discriminate among various animal sources.

Microbial source tracking (MST) techniques based on genotypic or phenotypic analysis of faecal indicator bacteria have been proposed as methods with potential usefulness for determining sources of faecal contamination. In published studies, the most frequently utilized genotypic subtyping method has been ribotyping of *E. coli*, which has been accomplished using one restriction enzyme (Parveen *et al.* 1999; Carson *et al.* 2001, 2003; Scott *et al.* 2003). Antibiotic resistance analysis (ARA), a phenotypic method that relies upon differences in the susceptibility of bacterial isolates to a battery of antibiotics at various concentrations, has also been utilized with faecal indicators that include faecal coliforms (Harwood *et al.* 2000; Whitlock *et al.* 2002), or faecal streptococci/*Enterococcus* spp. (Wiggins 1996; Hagedorn *et al.* 1999; Wiggins *et al.* 1999; Harwood *et al.* 2000; Graves *et al.* 2002). Other methods based on antibiotics include multiple antibiotic resistance (Parveen *et al.* 1997; Guan *et al.* 2002), in which only one concentration of each antibiotic is used.

Ribotyping and ARA are library-dependent MST methods, that is, the methods predict the source of strains by comparing the ribotype (RT) or antibiotic resistance profile of an environmental isolate with a database or library of patterns constructed using isolates from known faecal sources. Isolates from known sources are grouped by source categories using statistical grouping programs. The accuracy of these methods has previously been assessed by determin-

ing the internal accuracy of the library of bacterial isolates from known faecal sources, which is termed the average rate of correct classification (ARCC) (Wiggins 1996; Hagedorn *et al.* 1999; Harwood *et al.* 2000). Previous studies using various methods have reported ARCCs ranging from 34.7 to over 95% by ribotyping (Carson *et al.* 2001; Scott *et al.* 2003), and from 62.3 to over 90% using ARA (Wiggins 1996; Harwood *et al.* 2000; Graves *et al.* 2002) when the data analysis included more than two source categories. However, the use of the library as both the calibration dataset (the known-source isolates) and the test dataset (the unknowns) does not fully test the library's ability to accomplish its intended task, which is to identify environmental isolates whose profiles are not part of the known source library.

One study, which was published in a series of articles (Field *et al.* 2003; Griffith *et al.* 2003; Harwood *et al.* 2003; Myoda *et al.* 2003; Stewart *et al.* 2003; Weisberg *et al.* 2003), was designed to be a more rigorous test of MST libraries by including analysis of water samples seeded with faecal material whose source was unknown to the investigators. Although this study advanced the understanding of the limitations of current MST methods, the faecal samples used to seed the water samples were subsamples of the faecal material used to create the libraries. While the accuracy of correct source prediction was very high for some methods, including *E. coli* ribotyping (Myoda *et al.* 2003), the study design almost certainly contributed to the apparent accuracy of the methods.

The current study included a more rigorous approach for comparing MST techniques and the predictive accuracy of libraries for the source of environmental isolates for determination of faecal pollution in a watershed. All environmental sampling and bacterial isolation was performed by a local public health laboratory. Isolates were sent to one laboratory for ribotyping and to a second laboratory for ARA testing. The two testing laboratories were selected based on their successful use of these techniques in previous studies (Harwood *et al.* 2000; Scott *et al.* 2003). Ribotyping and ARA were compared using the same *E. coli* collection for library preparation. Two different bacterial indicator groups, *E. coli* and *Enterococcus* spp., isolated from the same faecal samples were compared by ARA typing. The accuracy of each technique was tested using a panel of *E. coli* and *Enterococcus* spp. proficiency isolates. The sources were known to the public health laboratory but blinded to the testing laboratories. Furthermore, the proficiency isolates were not obtained from the same faecal samples used to obtain the library isolates. This approach more accurately mimics the actual use of MST techniques for determining sources of unknown bacterial strains than simply determining the internal accuracy of a library.

Table 1 Sources of *Escherichia coli* and *Enterococcus* spp. isolates for assembling ARA and ribotyping libraries

No. faecal samples	No. faecal samples	No. isolates per faecal source (average no. isolates per sample)		
		ARA and ribotyping initial libraries		ARA (expanded library)‡
		<i>E. coli</i>	<i>E. coli</i>	<i>Enterococcus</i> spp.
Cat*	71	116 (1.6)	380 (5.3)	299 (4.2)
Dog*	89	124 (1.4)	423 (4.8)	434 (4.9)
Horse*	104	159 (1.5)	497 (4.8)	400 (3.8)
Seagull*	174	157 (0.9)	693 (4.0)	682 (3.9)
Human*	182	159 (0.87)	532 (2.9)	773 (4.2)
Sewage influent†	55	148 (2.7)	480 (8.7)	553 (10.1)
Sewage effluent†	51	134 (2.6)	472 (9.3)	516 (10.1)
Total	726	997	3477	3657

*One faecal sample was collected per individual.

†Individual samples were collected at different times.

‡Isolates for expanded libraries were obtained from the same samples as those in the smaller libraries.

MATERIALS AND METHODS

Sample collection and identification

Table 1 lists the number of samples and isolates from each faecal source used to construct the libraries. All samples were obtained within the 134-square mile San Juan Creek watershed in southern Orange County (CA, USA) and were collected over a period of 10 weeks. The same faecal and sewage samples were used to construct the *E. coli* ARA and ribotyping libraries and the *Enterococcus* ARA library. Each sample was cultured once and a maximum of five random isolates per faecal sample, and nine to 10 isolates per sewage grab sample were randomly selected and archived. One to two isolates per faecal sample and two to three isolates per sewage sample were used to make the smaller ($n = 997$) *E. coli* libraries, and the *E. coli* RT library and the *E. coli* ARA library were composed of the same 997 isolates. Isolates for the larger libraries (expanded *E. coli* ARA and *Enterococcus* ARA) were obtained from the same faecal samples. Human clinical samples were obtained from two hospitals, and faecal samples from cats and dogs were obtained from two veterinarian clinics. Cat and dog faecal samples were obtained from the animal cages/runs. Horse faecal samples were obtained from stables located near the study area. Seagull droppings were collected near the outlet of San Juan Creek, which empties into the Pacific Ocean at Doheny Beach. Sewage samples were obtained from a wastewater treatment plant located near San Juan Creek.

The samples used to obtain proficiency isolates were collected in parallel (at the same time) but separately (from different faecal samples) from samples used for library

isolates. The proficiency sample sets consisted of one isolate each of *E. coli* and *Enterococcus* spp. per faecal source sample and two isolates each per sewage sample. Ninety-seven *E. coli* and 99 *Enterococcus* isolates were included in the proficiency libraries which were nearly evenly distributed among the six source groups (Tables 2b, 3b and 4b).

Faecal samples were inoculated onto CHROMagar ECC (CECC; Hardy Diagnostics, Santa Maria, CA, USA) and Enterococcosel agar (BD Diagnostics Systems, Sparks, MD, USA), which are used for *E. coli* and *Enterococcus* spp. isolation from faeces, and incubated for 24 h at 44.5 and 41°C respectively (van Horn *et al.* 1996; Ohkusu 2000).

Sewage samples were processed using the membrane filtration technique following standard methods (APHA). Sample volumes (0.001-, 0.01-, 0.1- and 1.0-ml) were added to phosphate buffer solution and filtered through 47-mm diameter, 0.45- μ m pore size membranes (Millipore Corp., Bedford, MA, USA) to obtain 20–80 colonies per plate and transferred onto CECC for isolation of *E. coli* and mEI agar (BD Diagnostics) for isolation of *Enterococcus* spp. For sewage samples, mEI was used instead of Enterococcosel as mEI is recommended for recreational water quality assessment (US Environmental Protection Agency 1997) and used by local wastewater treatment plants. The CECC plates were incubated for 22–24 h at 44.5°C and the mEI plates were incubated for 22–24 h at 41°C as per USEPA Method 1600 (US Environmental Protection Agency 1997).

Presumptive *E. coli* and *Enterococcus* colonies were isolated based on colony morphology and pigmentation. Isolates were inoculated into 96-well microtitre plates containing

Table 2 Classification of *Escherichia coli* library and proficiency isolates by ARA

Isolate source (<i>n</i>)	No. (%) of isolates classified to each source category					
	Cat	Dog	Horse	Seagull	Human	Sewage
(a) Library isolates						
Cat (116)	46 (39.7)	29 (25.0)	18 (15.5)	8 (6.9)	11 (9.5)	4 (3.4)
Dog (124)	17 (13.7)	60 (48.4)	9 (7.2)	18 (14.5)	14 (11.3)	6 (4.8)
Horse (159)	3 (1.9)	20 (12.6)	84 (52.8)	23 (14.5)	3 (1.9)	26 (16.4)
Seagull (157)	9 (5.7)	31 (19.7)	16 (10.2)	62 (39.5)	23 (14.6)	16 (10.2)
Human (159)	16 (10.1)	24 (15.1)	11 (6.9)	25 (15.7)	71 (44.6)	12 (7.5)
Sewage (282)	10 (3.5)	23 (8.2)	54 (19.1)	38 (13.5)	36 (12.8)	121 (42.9)
Total	101	187	192	174	158	185
RCP*	45.5%	32.1%	43.8%	35.6%	44.9%	65.4%
ARCC†	44.5% (444/997)					
(b) Proficiency isolates						
Cat (14)	4 (28.6)	6 (42.8)	1 (7.1)	2 (14.3)	0 (0.0)	1 (7.1)
Dog (14)	0 (0.0)	1 (7.1)	4 (28.6)	3 (21.4)	2 (14.3)	4 (28.6)
Horse (11)	0 (0.0)	2 (18.2)	7 (63.6)	2 (18.2)	0 (0.0)	0 (0.0)
Seagull (14)	0 (0.0)	3 (21.4)	3 (21.4)	6 (42.8)	0 (0.0)	2 (14.3)
Human (16)	0 (0.0)	2 (12.5)	6 (37.5)	5 (31.2)	1 (6.2)	2 (12.5)
Sewage (28)	2 (7.1)	2 (7.1)	11 (39.3)	4 (14.3)	1 (3.6)	8 (28.6)
Total	6	16	32	22	4	17
RCP*	66.7%	6.2%	21.9%	27.3%	25%	47.0%
ARCC†	27.8% (27/97)					

Bold values represent isolates characterized into the correct source category.

*RCP, rate of correct prediction (isolates correctly classified into category divided by total isolates classified into category).

†ARCC, average rate of correct classification (isolates correctly classified into all categories divided by total isolates).

Table 3 Classification of *Escherichia coli* library and proficiency isolates by ribotyping

Isolate source (<i>n</i>)	No. (%) maximum similarity jackknife analysis of <i>E. coli</i> ribotyping					
	Cat	Dog	Horse	Seagull	Human	Sewage
(a) Library isolates						
Cat (116)	80 (69.0)	16 (13.8)	1 (0.9)	3 (2.6)	11 (9.5)	5 (4.3)
Dog (124)	17 (13.7)	83 (66.9)	2 (1.6)	8 (6.4)	5 (4.0)	9 (7.2)
Horse (159)	2 (1.2)	2 (1.2)	131 (82.4)	4 (2.5)	0 (0.0)	20 (12.6)
Seagull (157)	4 (2.5)	10 (6.4)	7 (4.4)	108 (68.8)	13 (8.3)	15 (9.6)
Human (159)	12 (7.5)	8 (5.0)	1 (0.6)	11 (6.9)	120 (75.5)	7 (4.4)
Sewage (282)	8 (2.8)	29 (10.3)	38 (13.5)	24 (8.5)	20 (7.1)	163 (57.8)
Total	123	148	180	158	169	219
RCP*	65.0%	56.1%	72.8%	68.4%	71.0%	74.4%
ARCC†	68.7% (685/997)					
(b) Proficiency isolates						
Cat (14)	2 (14.3)	5 (35.7)	3 (21.4)	3 (21.4)	0 (0.0)	1 (7.1)
Dog (14)	3 (21.4)	5 (35.7)	0 (0.0)	4 (28.6)	0 (0.0)	2 (14.3)
Horse (11)	2 (18.2)	2 (18.2)	4 (36.4)	0 (0.0)	0 (0.0)	3 (27.3)
Seagull (14)	2 (14.3)	4 (28.6)	1 (7.1)	3 (21.4)	3 (21.4)	1 (7.1)
Human (16)	1 (6.2)	3 (18.8)	0 (0.0)	0 (0.0)	10 (62.5)	2 (12.5)
Sewage (28)	2 (7.1)	6 (21.4)	2 (7.1)	8 (28.6)	8 (28.6)	2 (7.1)
Total	12	25	10	18	21	11
RCP*	16.7%	20.0%	40.0%	16.7%	47.6%	18.2%
ARCC†	26.8% (26/97)					

Bold values represent isolates characterized into the correct source category.

*RCP, rate of correct prediction (isolates correctly classified into category divided by total isolates classified into category).

†ARCC, average rate of correct classification (isolates correctly classified into all categories divided by total isolates).

Table 4 Classification of *Enterococcus* library and proficiency isolates by ARA

Isolate source (<i>n</i>)	No. (%) of isolates classified to each source category					
	Cat	Dog	Horse	Seagull	Human	Sewage
(a) Library isolates						
Cat (299)	104 (34.8)	78 (26.1)	13 (4.3)	43 (14.4)	38 (12.7)	23 (7.7)
Dog (434)	75 (17.3)	168 (38.7)	16 (3.7)	90 (20.7)	38 (8.8)	47 (10.8)
Horse (400)	9 (2.2)	8 (2.0)	302 (75.5)	23 (5.8)	14 (3.5)	44 (11.0)
Seagull (682)	61 (8.9)	75 (11.0)	32 (4.7)	326 (47.8)	105 (15.4)	83 (12.2)
Human (773)	98 (12.7)	88 (11.4)	38 (4.9)	187 (24.2)	272 (35.2)	90 (11.6)
Sewage (1069)	58 (5.4)	60 (5.6)	181 (16.9)	135 (12.6)	61 (5.7)	574 (53.7)
Total	405	477	582	804	528	861
RCP*	25.7%	35.2%	51.9%	40.5%	51.5%	66.7%
ARCC†	47.7% (1746/3657)					
(b) Proficiency isolates						
Cat (13)	3 (23.1)	3 (23.1)	1 (7.7)	0 (0.0)	2 (15.4)	4 (30.8)
Dog (14)	1 (7.1)	5 (35.7)	0 (0.0)	1 (7.1)	3 (21.4)	4 (28.6)
Horse (14)	1 (7.1)	0 (0.0)	11 (78.6)	0 (0.0)	1 (7.1)	1 (7.1)
Seagull (14)	0 (0.0)	1 (7.1)	1 (7.1)	2 (14.3)	2 (14.3)	8 (57.1)
Human (16)	7 (43.8)	1 (6.2)	3 (18.8)	1 (6.2)	0 (0.0)	4 (25.0)
Sewage (28)	0 (0.0)	0 (0.0)	1 (3.6)	2 (7.1)	1 (3.6)	24 (85.7)
Total	12	10	17	6	9	45
RCP*	25.0%	50.0%	64.7%	33.3%	0%	53.3%
ARCC†	45.4% (45/99)					

Bold values represent isolates characterized into the correct source category.

*RCP, rate of correct prediction (isolates correctly classified into category divided by total isolates classified into category).

†ARCC, average rate of correct classification (isolates correctly classified into all categories divided by total isolates).

Brucella broth (BD Diagnostics) with 15% glycerol, and incubated at 35°C overnight. A 48-pin replicator was used to transfer isolates to microtitre plates containing trypticase soya agar (BD Diagnostics), and the plates were incubated overnight as before. The microtitre plates were sealed and shipped on ice overnight to the testing laboratories for ARA and ribotyping analysis. The plates containing broth cultures were stored at -65°C.

Antibiotic resistance analysis

Isolates for ARA testing were sent to the Department of Biology (University of South Florida), as agar cultures in microtitre plates. A replica plating device was used to transfer the isolates to broth cultures in microtitre plates. *Escherichia coli* isolates were grown overnight in EC broth (Difco, Detroit, MI, USA) amended with 50 µg ml⁻¹ 4-methylumbelliferyl-β-D-glucuronide (MUG), and were incubated at 37°C. *Enterococcus* isolates were cultured in the same manner in Enterococcosel broth. The amount of overnight growth needed to inoculate Mueller-Hinton (MH) broth with antibiotics for this method was determined by direct plate counts and found to be consistent among repeated measures. Cultured cells from each indicator isolate were diluted separately in sterile water using 1.2-ml

microdilution tubes (USA Scientific, Ocala, FL, USA) to achieve a final concentration of *c.* 2 × 10⁷ CFU ml⁻¹. In most cases, *E. coli* cultures were diluted 1 : 30, while enterococci were diluted 1 : 10.

Escherichia coli and *Enterococcus* isolates were tested for their susceptibility to various antibiotics. Twelve antibiotics per bacterial indicator (at three concentrations each) were selected for ARA. *Escherichia coli* were tested in media containing amoxicillin (4, 20 and 128 µg ml⁻¹), cephalothin (8, 32 and 128 µg ml⁻¹), chloramphenicol (0.8, 8 and 32 µg ml⁻¹), chlortetracycline (4, 64 and 256 µg ml⁻¹), doxycycline (4, 64 and 128 µg ml⁻¹), moxalactam (0.2, 2 and 8 µg ml⁻¹), oxytetracycline (25, 100, 200 µg ml⁻¹), penicillin G (20, 200 and 500 µg ml⁻¹), polymixin B (0.1, 1 and 10 µg ml⁻¹), tetracycline (4, 64, 256 µg ml⁻¹), trimethoprim (0.25, 1 and 10 µg ml⁻¹) and trimethoprim/sulfamethoxazole in a 1 : 19 ratio (0.5, 5 and 50 µg ml⁻¹). *Enterococcus* spp. were tested in media containing amoxicillin (0.5, 2 and 20 µg ml⁻¹), bacitracin (10, 25 and 100 µg ml⁻¹), cephalothin (2, 10, 40 µg ml⁻¹), erythromycin (0.05, 0.5, 10 µg ml⁻¹), nitrofurazone (8, 24 and 80 µg ml⁻¹), penicillin G (0.5, 3 and 20 µg ml⁻¹), streptomycin (1, 20 and 100 µg ml⁻¹), tetracycline (0.4, 20 and 100 µg ml⁻¹), trimethoprim (0.5, 5 and 10 µg ml⁻¹), trimethoprim/sulfamethoxazole in a 1 : 19 ratio (5, 50 and 200 µg ml⁻¹),

tylosin (0.05, 5 and 100 $\mu\text{g ml}^{-1}$) and vancomycin (0.4, 4 and 8 $\mu\text{g ml}^{-1}$).

Antibiotic-amended MH broth was prepared in 100-ml volumes, and 100 μl media were added to each well of the microtitre plates with a multi-channel pipette. The media-containing plates were frozen at -20°C until use. Isolates were processed in rows A–G of the microtitre plates. These wells were filled with 100 μl of antibiotic-containing media with the exception of wells that served as positive (growth) controls; these contained MH broth without antibiotics. Row H (the bottom row) was used as a negative control to detect cross-contamination and was not inoculated. Five μl of inoculum from each culture were transferred to each microtitre well (except Row H) using a multi-channel micropipette (Rainin Instruments, Woburn, MA, USA). Following incubation for 24 h at 37°C , plates were shaken and absorbance for each well was recorded at 630 nm using an EL800 spectrophotometer (Bio-Tek, Winooski, VT, USA). Optical density readings for each well were electronically compared with a previously determined cutoff, which was established by reading A_{630} for the same concentration of cells as were used for the inoculum. Wells were scored positive for growth if absorbance exceeded that value, and negative if absorbance was equal to or less than that value. *Escherichia coli* and *Enterococcus* strains with known antibiotic susceptibility profiles were analysed on each processing day to ensure the consistency of culture and media preparation.

Ribotyping analysis

Escherichia coli isolates were shipped on ice to Biological Consulting Services (Gainesville, FL, USA) for ribotyping testing. Isolates were stored at 4°C until reisolated. Ribotyping was carried out as previously published (Parveen *et al.* 1999) with *Hind*III as the sole restriction enzyme.

Statistical analysis

Antibiotic resistance pattern data were converted to binary information and analysed by discriminant analysis with the SAS 8.0 program (SAS institute Cary, NC, USA). The known source isolates used as the classification rules in this study were self-crossed by jackknife (leave one out) and the percentages of correctly classified and misclassified isolates were determined. To confirm the significance of discrimination, an *F* ratio was calculated for all possible pairs of source groups (*k*) using the formula:

$$F = \frac{n_1 n_2 (n_1 + n_2 - p - 1)}{n_1 + n_2 (n_1 - n_2 - 2)p} \times D_2,$$

and was then compared with the appropriate critical value for $df_1 = p$, and $df_2 = n_1 + n_2 - p - 1$. Fisher's exact test was used to determine significant differences in proportions,

i.e. in rates of correct classification between different methods.

RT banding profiles were scanned digitally and converted to TIFF images. The images were then imported into a digital library and analysed by Bionumerics Software (Applied Maths, Austin, TX, USA). Banding patterns were grouped by cluster analysis (Dice coefficient) and compared by maximum similarity in a jackknife (pull each isolate out, then replace) analysis. The accuracy of the library-dependent methods was assessed using parameters reported previously (ARCC or rate of correct classification, RCC) (Wiggins 1996), which expresses the percentage of isolates from a given source that are correctly grouped into the corresponding source category. This is analogous to the sensitivity of a test (Motulsky 1995). The rate of correct prediction (RCP) was also assessed. RCP was calculated by dividing the number of isolates correctly classified into each source category by the total isolates classified (correctly and incorrectly) into the category, thus providing an indication of the confidence of assignment of isolates to a particular source category. This is analogous to positive predictive value of a test method (Motulsky 1995; Harwood *et al.* 2003).

Reproducibility testing

A subset of the proficiency isolates (20 *E. coli*, 19 *Enterococcus*) was repeatedly subtyped by ARA and ribotyping. Subtyping of each isolate by ARA was replicated nine times on a given day on three different days, and ribotyping of each isolate was replicated three times on a given day on three different days. Each trial involved true (rather than pseudo) replication, i.e. the isolates typed on the same day were grown as discreet cultures, and were not repeatedly subsampled from one culture. Reproducibility was assessed by determining the percentage of trials in which each isolate was assigned to the same source category by discriminant analysis.

RESULTS

Internal accuracy of libraries

Ribotyping and ARA are library-dependent MST methods that require an initial library-building phase. The first test of the library is to determine internal accuracy, in which the patterns in the library are used as both the 'standard' (calibration data set) and the patterns to be categorized (test data set). Faecal material was obtained from six source categories: cat, dog, horse, seagull and human faeces, and sewage (Table 1). The probability that the profile of a given isolate would be classified into one of six categories by chance alone is 0.167 (16.7%). The rate of assignment to the correct source category was greater than would be expected

by chance for all source categories and all methods (by *F*-test). For the *E. coli* ARA library, the RCC for each source species ranged from 39.5% for gull isolates to 52.8% for horse isolates (Table 2a). The RCP ranged from 32.1% for the dog source category to 65.4% for the sewage category and the ARCC for 997 isolates was 44.5% (Table 2a).

The internal accuracy of the *E. coli* ribotyping library is presented in Table 3a as the result of maximum similarity/jackknife analysis of *E. coli* RTs. The RCCs ranged from 57.8% for sewage isolates to 82.4% for horse isolates. The RCPs ranged from 56.1% for the dog source category to 74.4% for the sewage category, and the ARCC was 68.7% (Table 3a). The rate of assignment to the correct category was better than that by chance for all categories. The rate of assignment to the correct category was greater than would be expected by chance for all categories.

For ARA of *Enterococcus* spp., the RCC ranged from 34.8% for cat isolates to 75.5% for horse isolates (Table 4a). The RCP ranged from 25.7% for the cat source category to 66.7% for the sewage source category, and the ARCC for 3657 isolates was 47.7%.

Accuracy of classification of proficiency isolates

To determine the potential accuracy of each library in predicting the source of environmental bacterial isolates, a proficiency panel of 97 *E. coli* strains was analysed by ribotyping and ARA, and 99 *Enterococcus* strains were analysed by ARA. The strains were collected independently from library isolates. Host sources for the proficiency panel were blinded to the laboratories conducting ARA and ribotyping. By ARA, the RCC of the *E. coli* proficiency isolates from various sources ranged from 6.2% for human isolates to 63.6% for horse isolates (Table 2b). The RCP ranged from 6.2% for the dog category to 66.7% for the cat category. The ARCC for *E. coli* proficiency isolates categorized by ARA was 27.8%, far <44.5% for the library (Table 2a). Ribotyping RCC for *E. coli* proficiency isolates ranged from 7.1% for sewage isolates to 62.5% for human isolates (Table 3b). The RCP ranged from 16.7% for the cat and seagull categories to 47.6% for the human category. The ARCC was 26.8%, far <68.7% for the library (Table 3a). The RCC for proficiency *Enterococcus* isolates by ARA ranged from 0% for human isolates to 85.7% for sewage isolates (Table 4b). The RCP ranged from 0% for the human category to 64.7% for the horse category. The ARCC for *Enterococcus* proficiency isolates by ARA was 45.4%, which was similar to the 47.7% ARCC for the library isolates (Table 4b).

As the same set of *E. coli* proficiency isolates was analysed by ARA and ribotyping, the source assignment of each isolate could be directly compared. Eighty-one (83.5%) of the 97 proficiency isolates were classified into different

source categories by the two methods. Fifty (51.5%) of these isolates were incorrectly classified by both methods. Sixteen of the proficiency isolates (16.5%) were classified into the same source groups by both methods, of which six (6.2%) were correct. Agreement on source assignment per host species category ranged from 0% for seagull isolates to 45% for horse isolates. Of the five isolates classified to the horse category by both methods, three were correct. Twenty-seven (27.8%) and 26 (26.8%) of 97 *E. coli* isolates were correctly classified by ARA and ribotyping respectively (Tables 2b and 3b). The RCC of the proficiency isolates by ARA vs ribotyping were not significantly different (Fisher's exact test; $P = 0.8728$).

Isolates from sewage could conceivably be from sources other than human, and might therefore appear to be misclassified when, in fact, the isolates were assigned to the correct source. The methods were therefore also compared without including proficiency isolates from sewage, but there was no significant difference in the accuracy of source prediction (data not shown).

The source categories of the proficiency isolates were collapsed into animal and human (human and sewage) groups to determine the accuracy of human vs nonhuman grouping. In this case, the probability of a given pattern being assigned to any one source category by chance is 50%. The data were regrouped to determine the accuracy of predicting human vs animal sources. The ARCC was 56.7% for *E. coli* ARA, 67.0% for *E. coli* ribotyping and 59.6% for *Enterococcus* ARA. The percentage of proficiency isolates correctly classified (ARCC) was not significantly different (Fisher's exact test) for any pairwise comparison of methods.

Performance evaluation using a larger *E. coli* ARA library

To determine the effect of increased library size on performance of *E. coli* ARA, 2480 isolates were added to the library. These isolates were from the same faecal samples used for the original library, and a maximum of five isolates per faecal sample was utilized in the expanded library. The increase in library size from 997 to 3477 did not improve the library's accuracy (RCP, ARCC) or the accuracy of the proficiency analysis (data not shown).

Method reproducibility

A subset of the proficiency isolates, including 20 *E. coli* strains and 19 *Enterococcus* strains, were repeatedly analysed in independent assays to assess the reproducibility of ARA and ribotyping. The ability of the methods to assign isolates to the same source category was tested with nine replicate trials on three different days ($n = 27$) for ARA, and three replicate trials on three different days

($n = 9$) for ribotyping. Ribotyping was found to be more reproducible in terms of source prediction than ARA of *E. coli* or *Enterococcus*. Thirteen *E. coli* isolates (65%) were assigned to the same source for all of the trials by ribotyping, and five additional isolates (25%) were assigned to the same source for at least 67% of the trials (data not shown). Three *E. coli* isolates (15%) were assigned by ARA to the same source for all trials, while 10 additional isolates (50%) had the same results for 67% of trials. The reproducibility of *Enterococcus* isolates by ARA was similar to that of *E. coli* ARA; three isolates (15.8%) were assigned to the same source for all trials while eight (42.1%) were reproducible for at least 67% of trials.

DISCUSSION

This study was designed to closely mimic an environmental source tracking study by using established libraries to predict the source of isolates collected in the same area, but from different faecal samples. A local laboratory collected all samples and isolated bacterial strains that were then sent to two testing laboratories experienced in ARA and ribotyping testing. Diverse populations among host categories were deliberately sampled to obtain maximal microbial diversity. The testing laboratories used the collected strains to construct libraries of *E. coli* and *Enterococcus* isolates from five host species (human, cat, dog, gull and horse), and sewage representative of the bacterial populations within a single large watershed. The internal accuracy of these libraries was determined and compared with predictive accuracy for a set of proficiency isolates not included in the library. Finally, the accuracy of ARA and ribotyping for the same panel of *E. coli* proficiency isolates was determined. The use of proficiency isolates, whose source is known to the submitting laboratory but blinded to the testing laboratories, is a new approach for predicting accuracy of source tracking technique that allows a more realistic determination of testing performance using unknown isolates. Most MST studies have tested the predictive accuracy of library-based methods by calculating the internal classification accuracy; very few studies have used any type of external validation beyond environmental water samples thought to be contaminated by a particular source (Hagedorn *et al.* 1999; Harwood *et al.* 2000; Hagedorn *et al.* 2001; Whitlock *et al.* 2002; Hagedorn *et al.* 2003; Scott *et al.* 2003). Library performance was validated by blind proficiency samples in only two other studies published to date. In the first, (Harwood *et al.* 2003; Myoda *et al.* 2003), the same faecal samples that were used to make the library were also used to make the proficiency samples, reducing its effectiveness to determine accuracy for unknown isolates not contained in the library. In the second (Stoekel *et al.* 2004), the source

of *E. coli* proficiency isolates was not accurately predicted by most methods, with ARCCs for *E. coli* ARA and *HindIII* ribotyping comparable with those observed in this study.

The internal accuracy (ARCC) of the ARA libraries generated in this study was lower than those reported for most ARA libraries (Wiggins 1996; Hagedorn *et al.* 1999; Harwood *et al.* 2000; Graves *et al.* 2002; Choi *et al.* 2003) and ribotyping libraries (Parveen *et al.* 1999; Carson *et al.* 2001; Myoda *et al.* 2003; Scott *et al.* 2003). Some previous studies generated *E. coli* RT libraries from <500 isolates (Wiggins 1996; Parveen *et al.* 1999; Carson *et al.* 2001, 2003; Myoda *et al.* 2003), and these generally had ARCCs of 80% or higher. Although the hypothesis that larger libraries will be characterized by higher ARCCs is intuitively appealing, some studies have found that larger libraries tend to have lower ARCCs (Harwood *et al.* 2000; Wiggins *et al.* 2003). Although large (>1500 isolates), diverse libraries may tend towards lower internal accuracy because of sharing of certain subtypes between host species, a major benefit of such libraries is that they are more likely to be representative of microbial diversity in the faeces of host populations (Wiggins *et al.* 2003; Johnson *et al.* 2004), which is a requirement for accurate identification of the source of isolates from proficiency or water samples (Hagedorn *et al.* 1999).

Library ARCCs are influenced by many factors including the number of host species sampled, the number of source categories into which the host species are grouped, the number of individual faecal samples analysed, and the number of isolates analysed from each sample. Perhaps more than any other factor, the ARCC is affected by the microbial diversity in the faeces of host animals, and the extent to which the strains (subtypes) are host specific. The urban nature of the watershed sampled here may have contributed to low ARCCs in this study, as animals that have more contact with humans and with each other, sharing habitat and food, may tend to have more overlaps in their faecal flora. Many cases support this hypothesis in which *E. coli* and *Enterococcus* ARA libraries constructed in urban areas (Harwood *et al.* 2000; Whitlock *et al.* 2002; Choi *et al.* 2003; Harwood *et al.* 2003) have lower ARCCs than ARA libraries from rural areas (Wiggins 1996; Hagedorn *et al.* 1999; Wiggins *et al.* 1999; Graves *et al.* 2002). In addition, there has been little evidence presented that supports the hypothesis underpinning these techniques; that there are source-specific strains of indicator bacteria (Gordon 2001). An interesting observation was that few *E. coli* or *Enterococcus* sewage isolates were grouped into the human category, when a large overlap was expected. Many factors may have contributed to this observation, including both low accuracy rates of the libraries and differences in population structure between the secondary environment (sewage) compared with the host population, as was suggested for *E. coli* (Gordon 2002). As a result of this

finding, sewage was kept as a separate source category for both library and proficiency isolate calculations.

The proficiency isolates were designed to be a rigorous test of the predictive accuracy of the libraries, but <50% of these isolates were assigned to the correct source category by all methods in this study. The ARCC for the proficiency isolates was substantially lower than the ARCC of the source libraries for *E. coli* ARA (28% vs 46%) and ribotyping (27% vs 69%). In contrast, for *Enterococcus* ARA, the proficiency ARCC was very similar to the source library ARCC (45% vs 48%). The methods agreed on a source for 16.5% of 97 isolates, of which 6% of source assignments were correct, indicating that (i) agreement on source assignment between the two different methods was not achieved, and (ii) combining the results from two methods would not improve overall accuracy in this case.

Two measures that are analogous to diagnostic sensitivity (accuracy) of a test have been the standard measure of accuracy of previous studies: the RCC and the ARCC, both of which measure the proportion of isolates from each or all source categories correctly classified. However, a measure of the confidence in source assignment to categories is also useful: given the placement of isolate 'x' into source category 'Y', how confident can one be in that assignment? For this measure, which is analogous to the positive predictive value of a test, the RCP was calculated (Harwood *et al.* 2003). As RCP accounts for isolates incorrectly classified into a category as well as those correctly classified, it is a more useful measure of the accuracy of a prediction than RCC alone. While the overall ARCC of the *Enterococcus* ARA proficiency panel was 45%, the RCP for each predicted source category ranged from 0% for humans to 65% for horse. The RCP for each source category varied by testing technique, and no one source category had a consistently higher RCP than others.

The deficiencies in predictive accuracy of the methods probably have multiple contributing factors. Low reproducibility in source classification was particularly problematic for ARA. Detailed studies of the reproducibility of antibiotic resistance patterns (as opposed to source classification) have shown reproducibility of 85% for ARA by the broth method utilized here (Harwood *et al.* 2002); however, small differences in antibiotic resistance pattern can lead to classification in different source categories. In this study ribotyping was more reproducible than ARA; however, neither technique was able to achieve a desired level of at least 90% reproducibility. Unlike antimicrobial susceptibility testing in clinical microbiology laboratories which are rigorously standardized utilizing NCCLS standard procedures and control strains for quality control (NCCLS 2003a,b, 2004), the methods used by ARA testing laboratories are not carried out utilizing these standards. This is due, in part, to selection of antimicrobial concentrations that give the most

differentiation between strains, not for correlation with *in vivo* susceptibility of organisms to antimicrobial therapy.

Predictive inaccuracy of the source of proficiency isolates could also be due to genetic diversity of the organisms utilized. If the *E. coli* libraries were not representative of genetic diversity in the host population sampled, the proficiency isolates may have been quite dissimilar from those in the library, as the proficiency isolates were obtained from different animals and samples than the library isolates. The inaccurate classification of *E. coli* proficiency isolates suggests that the initial *E. coli* libraries ($n = 997$) were not representative of *E. coli* diversity in the watershed. Neither the internal nor the external measures of accuracy improved when 2480 isolates were added to the *E. coli* ARA library ($n = 3477$), but these isolates were derived from the same samples as the isolates in the initial library, therefore a similar under-sampling issue may contribute to the nonrepresentative nature of both *E. coli* libraries. A recent publication examining rep-PCR fingerprints of *E. coli* from various hosts concluded that genetic diversity had not been adequately sampled by 1535 unique fingerprints (Johnson *et al.* 2004), indicating the great genetic diversity of this bacterium and the difficult task of constructing representative libraries.

The ARCC of the *Enterococcus* ARA library (48%) was very close to the ARCC of the proficiency isolates (45%), suggesting that the *Enterococcus* library of 3657 isolates was representative of *Enterococcus* diversity in the host populations sampled within the watershed. Misclassification of library and proficiency *Enterococcus* isolates by discriminant analysis can therefore be attributed to incomplete group separation, i.e. the antibiotic resistance patterns of some isolates from different sources were so similar that groups could not be discriminated (Choi *et al.* 2003). Inadequate group separation also occurred in the *E. coli* libraries, as evidenced by misclassification of known source isolates in the library.

The results of this study suggest that current library-based methods for prediction of the sources of faecal pollution do not have sufficient accuracy to identify bacterial pollution sources in large, urban watersheds. Of the three methods studied, the *Enterococcus* ARA method was most accurate, correctly classifying the sources for 45% of the proficiency isolates. Significant improvement in source prediction is necessary to achieve useful predictive accuracy for determining the source of faecal pollution in environmental studies. Further studies are needed to address such factors as validation of method accuracy and reproducibility, number and selection of sources to build the library, indicator organism(s) used, and variability due to geographic and host distribution of bacterial strains. The usefulness of currently published methodologies may be restricted to water bodies that are impacted by a very limited number (e.g. two to three) of potential

sources in a limited geographical area over a short temporal period. We recommend that all future source tracking studies include testing proficiency samples comprised of bacterial strains from known sources that are not part of the original database to determine the actual accuracy of the method before unknown environmental samples are analysed.

ACKNOWLEDGEMENTS

This study was funded in part by the California State Water Resources Control Board. We are grateful to South Orange County Wastewater Authority, Mission Hospital Regional Medical Center, San Clemente Hospital and Medical Center, Golden Lantern Animal Hospital, Avery Animal Clinic and Sycamore Stable for providing sewage and faecal samples, and to Matthew Anderson, John E. Whitlock, Brian Mochon, Mariam Zhouwandai and Frank Duarte for technical assistance and data analysis.

REFERENCES

- American Public Health Association (1995) *In Standard Methods for the Examination of Water and Wastewater*. Washington, DC: American Public Health Association, Inc.
- Carson, C.A., Shear, B.L., Ellersieck, M.R. and Asfaw, A. (2001) Identification of fecal *Escherichia coli* from humans and animals by ribotyping. *Appl Environ Microbiol* **67**, 1503–1507.
- Carson, C.A., Shear, B.L., Ellersieck, M.R. and Schnell, J.D. (2003) Comparison of ribotyping and repetitive extragenic palindromic-PCR for identification of fecal *Escherichia coli* from humans and animals. *Appl Environ Microbiol* **69**, 1836–1839.
- Choi, S., Chu, W., Brown, J., Becker, S.J., Harwood, V.J. and Jiang, S.C. (2003) Application of enterococci antibiotic resistance patterns for contamination source identification at Huntington Beach, California. *Mar Pollut Bull* **46**, 748–755.
- Field, K.G., Chern, E.C., Dick, L.K., Fuhrmann, J., Griffith, J., Holden, P., LaMontagne, M.G., Le, J. *et al.* (2003) A comparative study of culture-independent, library-independent genotypic methods of fecal source tracking. *J Water Health* **01**, 181–194.
- Gordon, D.M. (2001) Geographical structure and host specificity in bacteria and the implications for tracing the source of coliform contamination. *Microbiology* **147**, 1079–1085.
- Gordon, D.M. (2002) The genetic structure of *Escherichia coli* populations in primary and secondary habitats. *Microbiology* **148**, 1513–1522.
- Graves, A.K., Hagedorn, C., Teetor, A., Mahal, M., Booth, A.M. and Reneau, R.B. Jr. (2002) Antibiotic resistance profiles to determine sources of fecal contamination in a rural Virginia watershed. *J Environ Qual* **31**, 1300–1308.
- Griffith, J.F., Weisberg, S.B. and McGee, C.D. (2003) Evaluation of microbial source tracking methods using mixed fecal sources in aqueous test samples. *J Water Health* **01**, 141–151.
- Guan, S., Xu, R., Chen, S., Odumeru, J. and Gyles, C. (2002) Development of a procedure for discriminating among *Escherichia coli* isolates from animal and human sources. *Appl Environ Microbiol* **68**, 2690–2698.
- Hagedorn, C., Robinson, S.L., Filtz, J.R., Grubbs, S.M., Angier, T.A. and Reneau, R.B. Jr. (1999) Determining sources of fecal pollution in a rural Virginia watershed with antibiotic resistance patterns in fecal streptococci. *Appl Environ Microbiol* **65**, 5522–5531.
- Hagedorn, C., Bowman, A.M., Graves, A.L. and Reneau Jr, R.B. (2001) *Developing Methods for Determining Sources of Fecal Pollution in Water*. Charlotte, NC: American Society of Agronomy Annual Meeting.
- Hagedorn, C., Crozier, J.B., Mentz, K.A., Booth, A.M., Graves, A.K., Nelson, N.J. and Reneau, R.B. Jr. (2003) Carbon source utilization profiles as a method to identify sources of faecal pollution in water. *J Appl Microbiol* **94**, 792–799.
- Harwood, V.J., Butler, J., Parrish, D. and Wagner, V. (1999) Isolation of fecal coliform bacteria from the diamondback terrapin (*Malaclemys terrapin centrata*). *Appl Environ Microbiol* **65**, 865–867.
- Harwood, V.J., Whitlock, J. and Withington, V. (2000) Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in predicting the source of fecal contamination in subtropical waters. *Appl Environ Microbiol* **66**, 3698–3704.
- Harwood, V.J., Moore, D.F., Brownell, M., Hannah, P., Whitlock, J.E. and Anderson, M. (2002) Bacterial source tracking by antibiotic resistance analysis in two California watersheds: method comparison and sampling considerations. In *American Society for Microbiology General Meeting Abstracts*. Salt Lake City, UT.
- Harwood, V.J., Wiggins, B., Hagedorn, C., Ellender, R.D., Gooch, J., Kern, J., Samadpour, M., Chapman, A.C.H. *et al.* (2003) Phenotypic library-based microbial source tracking methods: efficacy in the California collaborative study. *J Water Health* **01**, 153–166.
- van Horn, K.G., Gedris, C.A. and Rodney, K.M. (1996) Selective isolation of vancomycin-resistant enterococci. *J Clin Microbiol* **34**, 924–927.
- Johnson, L.K., Brown, M.B., Carruthers, E.A., Ferguson, J.A., Dombek, P.E. and Sadowsky, M.J. (2004) Sample size, library composition, and genotypic diversity influence accuracy of determining sources of fecal pollution among natural populations of *Escherichia coli* from different animals. *Appl Environ Microbiol* **70**, 4478–4485.
- Motulsky, H. (1995) *Intuitive Biostatistics*. New York: Oxford University Press.
- Myoda, S.P., Carson, C.A., Fuhrmann, J.J., Hahn, B.-K., Hartel, P.G., Yampara-Iquise, H., Johnson, L., Kuntz, R.L. *et al.* (2003) Comparison of genotypic-based microbial source tracking methods requiring a host origin database. *J Water Health* **01**, 167–180.
- NCCLS (2003a) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, Approved standard, 6th edn. NCCLS document M7-A6. Wayne, PA: NCCLS.
- NCCLS (2003b) *Performance Standards for Antimicrobial Disk Susceptibility Tests*, Approved standard, 8th edn. NCCLS document M2-A8. Wayne, PA, 19087–1898: NCCLS.
- NCCLS (2004) *Performance Standards for Antimicrobial Susceptibility Testing: Fourteenth Informational Supplement*, NCCLS document M100-S14. 2004. Wayne, PA: NCCLS.
- Ohkusu, K. (2000) Cost-effective and rapid presumptive identification of gram-negative bacilli in routine urine, pus, and stool cultures: evaluation of the use of CHROMagar orientation medium in

- conjunction with simple biochemical tests. *J Clin Microbiol* **38**, 4586–4592.
- Parveen, S., Murphree, R.L., Edmiston, L., Kaspar, C.W., Portier, K.M. and Tamplin, M.L. (1997) Association of multiple-antibiotic-resistance profiles with point and nonpoint sources of *Escherichia coli* in Apalachicola Bay. *Appl Environ Microbiol* **63**, 2607–2612.
- Parveen, S., Portier, K.M., Robinson, K., Edmiston, L. and Tamplin, M.L. (1999) Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human and nonhuman sources of fecal pollution. *Appl Environ Microbiol* **65**, 3142–3147.
- Pruss, A. (1998) Review of epidemiological studies on health effects from exposure to recreational water. *Int J Epidemiol* **27**, 1–9.
- Scott, T.M., Parveen, S., Portier, K.M., Rose, J.B., Tamplin, M.L., Farrah, S.R., Koo, A. and Lukasik, J. (2003) Geographical variation in ribotype profiles of *Escherichia coli* isolates from humans, swine, poultry, beef, and dairy cattle in Florida. *Appl Environ Microbiol* **69**, 1089–1092.
- Stewart, J.R., Ellender, R.D., Gooch, J.A., Jiang, S., Myoda, S.P. and Weisberg, S.B. (2003) Recommendations for microbial source tracking: lessons from a methods comparison study. *J Water Health* **01**, 225–231.
- Stoeckel, D.M., Mathes, M.V., Hyer, K.E., Hagedorn, C., Kator, H., Lukasik, J., O'Brien, T.L., Fenger, T.W. et al. (2004) Comparison of seven protocols to identify fecal contamination sources using *Escherichia coli*. *Environ Sci Technol* **38**, 6109–6117.
- US Environmental Protection Agency (1984) *Health Effects Criteria for Fresh Recreational Waters*. Washington, DC: US Environmental Protection Agency.
- US Environmental Protection Agency (1997) *Method 1600: Membrane Filter Test Methods for Enterococci in Water*. Washington, DC: US Environmental Protection Agency.
- Weisberg, S.B., Griffith, J.F. and McGee, C.D. (2003) Evaluation of microbial source tracking methods using mixed fecal sources in aqueous test samples. *Int J Water Health* **1**, 141–151.
- Whitlock, J.E., Jones, D.T. and Harwood, V.J. (2002) Identification of the sources of fecal coliforms in an urban watershed using antibiotic resistance analysis. *Water Res* **36**, 4273–4282.
- Wiggins, B.A. (1996) Discriminant analysis of antibiotic resistance patterns in fecal streptococci, a method to differentiate human and animal sources of fecal pollution in natural waters. *Appl Environ Microbiol* **62**, 3997–4002.
- Wiggins, B.A., Andrews, R.W., Conway, R.A., Corr, C.L., Dobratz, E.J., Dougherty, D.P., Eppard, J.R., Knupp, S.R. et al. (1999) Use of antibiotic resistance analysis to identify nonpoint sources of fecal pollution. *Appl Environ Microbiol* **65**, 3483–3486.
- Wiggins, B.A., Cash, P.W., Creamer, W.S., Dart, S.E., Garcia, P.P., Gerecke, T.M., Han, J., Henry, B.L. et al. (2003) Use of antibiotic resistance analysis for representativeness testing of multiwatershed libraries. *Appl Environ Microbiol* **69**, 3399–3405.