Bacteroidales terminal restriction fragment length polymorphism (TRFLP) for fecal source differentiation in comparison to and in combination with universal bacteria TRFLP

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Abstract

Terminal restriction fragment length polymorphism (TRFLP) is an attractive community analysis method for microbial source tracking (MST) because it is accessible, relatively inexpensive, and can discern multiple fecal sources simultaneously. A new Bacteroidales TRFLP (Bac-TRFLP) method was developed and its source identification performance was evaluated by itself, in comparison to, and in combination with an existing universal bacterial TRFLP method in two laboratories. Sixty-four blind samples from 12 fecal sources (sewage, septage, human, dog, horse, cow, deer, pig, chicken, goose, pigeon, and gull) were used for evaluation. Bac- and Univ- TRFLP exhibited similarly high overall correct identification (>88 and >89%, respectively), excellent specificity regardless of fecal sources, variable sensitivity depending on the source, and stable performance across two laboratories. Compared to Univ-TRFLP, Bac-TRFLP had better sensitivity and specificity with horse, cow, and pig fecal sources but was not suited for certain avian sources such as goose, gull, and pigeon. Combining the general and more targeted TRFLP methods (Univ&Bac-TRFLP) achieved higher overall correct identification (>92%), higher sensitivity and specificity metrics, and higher reproducibility between laboratories. Our results suggest that the Bac-TRFLP and Univ&Bac-TRFLP methods are promising additions to the MST toolbox and warrant further evaluation and utilization in field MST applications.

Introduction

Culture-independent microbial community analyses have been widely used in microbial source tracking (MST; Cao *et al.* 2011b) for characterization of fecal sources (McLellan *et al.* 2010, Shanks *et al.* 2011) and development of source-specific single indicator qPCR (quantitative polymerase chain reaction) assays (Bernhard and Field 2000, Lu *et al.* 2009, Ryu *et al.* 2012). These methods are particularly appealing for source identification because they can discern multiple fecal sources simultaneously, including those for which source-specific single-marker genes have not been identified (Cao *et al.* 2011b, Cao *et al.* 2013) and have demonstrated their efficacy for

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source identification in the field (Unno *et al.* 2010, Cao *et al.* 2011a, Dubinsky *et al.* 2012).

Among community-based MST methods, terminal restriction fragment length polymorphism (TRFLP) is attractive because it is relatively accessible and inexpensive (Cao *et al.* 2011b) compared to either pyrosequencing (Unno *et al.* 2010) or phylogenetic microarrays (Wu *et al.* 2010, Dubinsky *et al.* 2012). In the TRFLP method, community DNA is first PCR-amplified using fluorescently labeled primers to produce terminally labeled PCR products. Terminal restriction fragments (TRFs) are then generated by digesting the PCR products using restriction enzymes (Liu *et al.* 1997). TRFs are separated and their lengths and abundances determined via capillary electrophoresis, producing a semi-quantitative profile of microbial community composition that is then analyzed by appropriate multivariate statistical approaches (Cao *et al.* 2011a).

Widely employed for characterization of bacterial communities in microbial ecology (Schütte *et al.* 2008), TRFLP also has a long history in MST. An early application of TRFLP for MST was to compare microbial communities in deer feces and sand (Clement *et al.* 1998). Later, individual peaks were used for source identification, to select primer sites for source specific PCR assays (Bernhard and Field 2000, Field *et al.* 2003), and more recently in an integrated community analysis approach for source tracking in the field (Cao *et al.* 2011a).

Here, TRFLP were paired with a primer set that primarily targets the order of Bacteroidales to develop a new method (Bac-TRFLP) for potential MST applications. The order Bacteroidales was of interest because these bacteria belong to the class of Bacteroidetes that are prevalent in fecal material (Wu *et al.* 2010 and references therein) and many source-specific qPCR markers target organisms within this class (Wuertz *et al.* 2011). The performance of Bac-TRFLP was then evaluated for detection of fecal and human waste pollution, along with a previously described TRFLP method that targets most Bacteria (Univ-TRFLP; Cao *et al.* 2011a) in two laboratories to assess cross-laboratory stability. The two TRFLP methods were utilized for source identification of 64 unknown challenge samples as two separate methods, as well as one combined method (Univ&Bac-TRFLP) where the two answers from individual TRFLP methods were considered jointly to provide one collective answer for each unknown sample. Note that this manuscript aims to report method development and inter-laboratory evaluation of the performance of Bac-TRFLP 1) alone, 2) in comparison to Univ-TRFLP, and 3) in combination with Univ-TRFLP. TRFLP is a commonly used and relatively inexpensive way of profiling complex microbial communities. Other community analysis methods (e.g., microarray or next generation sequencing) are used in microbial ecology for both profiling and identifying taxa, to varying degrees of resolution. Readers are referred to Cao *et al*. (2013) for a cross-comparison, focusing on common source identification performance characteristics, of community analysis-based MST methods (including TRFLP, PhyloChip, and Illumina next generation sequencing) that vary as such in their data density.

Methods

Bac-TRFLP Method Development

A literature review was conducted to select primers for developing the Bac-TRFLP method. Potential primer pairs were checked for phylogenetic coverage using Probe Match (http://rdp.cme.msu.edu/probematch/search.jsp), and a primer set from Wood *et al.* (1998) was selected: rBacPre (5'-TCACCGTTGCCGGCGTACTC) paired with a universal bacterial primer fD1 (5'-AGAGTTTGATCCTGGCTCAG) to produce an amplicon of approximately 900 bp. According to Probe Match (last accessed September 7, 2012) the rBacPre primarily amplified three families of bacteria within the order Bacteroidales (91% within the order Bacteroidales; 98% within the phylum Bacteroidetes). The fD1 forward primer was modified for use in TRFLP by attaching a fluorophore (Hex) to the 5' end.

After primer selection, experiments were conducted to develop an overall Bac-TRFLP standard operating procedure (SOP) using representative fecal DNA from sewage, dog, and cow. The experimental parameters tested during optimization included annealing temperature, number of PCR cycles, and laboratory replication. Each 50 µl PCR reaction included 0.025 U Takara EX Taq, 1X PCR buffer (Takara Ex Taq kit, Takara, Mountain View, CA), 0.2 µM dNTP mix (Takara Ex Taq kit), 1 mg/ml BSA, 0.5 µM rBacPre, and 0.525 µM fD1-Hex (i.e., fD1 labeled with Hex at the 5′ end). Template concentrations of 16, 40, and 80 ng per 50 µl reaction were tested with gel electrophoresis following PCR, and no visible difference was observed. To conserve

DNA, the concentration of 16 ng per 50 µl reaction was selected for use.

Except for the parameters being optimized, previously described PCR conditions (Wood *et al.* 1998) were used: an initial cycle of 94°C for 5 minutes, 60°C for 2 minutes, and 72°C for 2 minutes, followed by 29 cycles (24 to 32 cycles for optimization) of 94°C for 2 minutes, 60°C (52 to 62°C for optimization) for 30 seconds, and 72°C for 2 minutes, with a final cycle step at 72°C for 10 minutes. Annealing temperatures were optimized using a temperature gradient followed by gel electrophoresis using the Flashgel DNA system (DNA cassette 1.2%, loading dye, DNA marker 100 bp-3 kb; Lonza, Allendale, NJ). The number of PCR cycles was tested at the optimal annealing temperature in duplicate, by comparing the final TRFLP results after capillary electrophoresis.

Because Bac- and Univ-TRFLP both target 16S rRNA genes and the same fluorophore (Hex) was used for labeling the forward primers, it was assumed that the rest of the TRFLP protocol following PCR would transfer well from Univ- to Bac-TRFLP. This assumption was considered appropriate if confirmation of successful and repeatable Bac-TRFLP runs was obtained. The development of the Bac-TRFLP method was conducted in one laboratory at Southern California Coastal Water Research Project (SCCWRP), after which the optimized protocol was shared with a second laboratory at the University of California Santa Barbara (UCSB). The two laboratories participated in the method performance evaluation described herein.

Study Design for Performance Evaluation

The evaluation study design was as described elsewhere (Cao *et al.* 2013). Sixty-four blind challenge samples and 12 reference samples were created from freshly collected fecal material from 12 sources: human, sewage, septage, dog, horse, cow, deer, pig, chicken, goose, pigeon, and gull. The 64 challenge samples, i.e., a blind duplicate set of 32 blind samples, contained either a single fecal type (38 singletons) or two fecal types (26 doubletons). Each source was a composite of at least 12 individuals (or nine sewage sources, or six septage sources) with equal contribution from 4 representative California geographies. Each source was suspended and blended in a selected volume of bacteria-free artificial freshwater to create a singleton slurry intended to contain 1000 *Enterococcus* per 100 ml of slurry. A best attempt was made, based on *Enterococcus* abundances reported in the literature and on initial testing of collected source material, to compose the slurry to achieve this *Enterococcus* concentration. However, the resulting singleton slurries contained 2.65 to 6.24 Log_{10} colony forming units of *Enterococcus* per 100 ml (measured by EPA method 1600) and 2.41 to 8.18 Log_{10} copies of Bacteroidales 16S gene per 100ml (measured by the GenBac3 qPCR assay (Siefring *et al.* 2008)). The concentrations of fecal material in singleton slurries as measured by other methods (wet-mass, total DNA, *E. coli* and Enterococcus via culture or qPCR) are reported elsewhere (Ervin *et al.* 2013).

The 38 singleton challenge samples included 24 full strength and fourteen 1:10 strength singletons, which were created by filtering 200 ml and 20 ml of the corresponding singleton slurry, respectively. Each doubleton was created by filtering 200 ml of a doubleton slurry created by mixing 90% and 10% (by volume) of the corresponding singleton slurries. An additional set of 12 full strength singleton samples, one for each of the 12 sources, was created from the same singleton slurries and provided as known reference samples. More details on field fecal material collection and laboratory sample preparation are described elsewhere (Boehm *et al.* 2013).

Bac-TRFLP (developed herein) and Univ-TRFLP (Cao *et al.* 2011a) were run by two laboratories (SCCWRP, UCSB). Each laboratory received a set of known reference samples and a set of unknown challenge samples. All samples were processed and analyzed by both TRFLP methods in both laboratories following the same corresponding standard operating procedures (SOPs). Source(s) in the 64 blind challenge samples were determined through comparing microbial communities in the challenge samples to those in the reference samples. Source identification answers were provided by each TRFLP method. Additionally, the two answers from individual TRFLP methods were considered jointly to provide one collective answer by the combined method (Univ&Bac-TRFLP).

DNA Extraction

DNA was extracted from all samples using the DNA-EZ ST kit (GeneRite, North Brunswick, NJ) following the manufacturer's protocol. Extracted DNA was quantified by fluorometry (Quant-iT™ dsDNA High Sensitivity Assay Kit; Invitrogen, Carlsbad, CA) and checked for quality by the

A260/A280 ratio (Nanodrop 1000, Thermo Fisher Scientific, Wilmington, DE). A few DNA extracts had A260/A280 ratios outside the 1.6 to 2.2 range, but PCR amplification was successful with gel electrophoresis indicating distinct bright bands, i.e., showing no sign of inhibition. Therefore, no further purification was implemented. DNA extracts were stored at -20°C until analysis.

TRFLP Laboratory Analysis

Bac-TRFLP was performed following the optimized PCR protocol described above. Univ-TRFLP was performed as described previously (Cao *et al.* 2006). Briefly, genes encoding 16S rRNA were PCR amplified using universal bacterial primers 8F Hex (fluorescently labeled forward primer; 5'-AGAGTTTGATCCTGGCTCAG) and 1389R (5'-ACGGGCGGTGTGTACAAG). Duplicate 50 µl PCR reactions were performed on a CFX96 cycler (BioRad, Hercules, CA) using the Taq PCR Core kit (Qiagen, Valencia, CA) following the manufacturer's protocol with 25 ng extracted sample DNA (or a maximum of 5 µl) and a final concentration of 0.525 µM for each of the primers. The PCR thermal program included an initial denaturation (95°C, 240 seconds), annealing (58°C, 60 seconds) and extension (72°C, 90 seconds) cycle, followed by a hold at 72°C for adding the TaqQ polymerase mix (Taq PCR Core kit). The program then continued with 28 cycles of denaturation (94°C, 45 seconds), annealing (58°C, 45 seconds) and extension (72°C, 90 seconds), followed by a final extension at 72°C for 10 minutes. Successful amplification was confirmed by gel electrophoresis with a Flashgel® DNA system (DNA cassette 1.2%; Lonza).

PCR products from duplicate reactions were pooled, then purified using a QIAquick PCR purification kit (Qiagen) following the manufacturer's protocol, with the exception that the final elution was with 35 µl 0.1x (instead of 50 µl 1x) elution buffer. Purified DNA was quantified by fluorometry (Quant-iT™ dsDNA Broad Range Assay Kit, Inivtrogen) before restriction enzyme digestion. Two separate digestions were conducted, using either *Hha*I or *Msp*I (New England BioLabs, Ipswich, MA) as described previously (Cao *et al.* 2011a). These two enzymes were selected because they were shown to be effective in resolving 16S gene sequence differences in Bacteria (Engebreston and Moyer 2003). Approximately 600 ng purified PCR products were used in each 40 µl digestion reaction with 6 Units of *Hha*I or *Msp*I, and appropriate buffer and/or BSA following the manufacturer's instructions. The digestions were conducted at 37°C (*Hha*I: overnight for16 hours, *Msp*I: 4 hours) on a thermal cycler (CFX96, BioRad). *Hha*I digests were deactivated (65°C, 20 minutes) and desalted (QIAquick Nucleotide Removal kit; Qiagen), following the manufacturer's protocol, and stored at -20°C. *Msp*I digests were stored at -20°C or desalted immediately following digestion to stop the enzymatic reaction.

Digests were stored at -20°C until shipment on dry ice to the Genomics Technology Support Facility (Michigan State University) where the terminal restriction fragment (TRF) length and abundance were determined with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

TRFLP Data Analysis for Source Identification

Data processing was conducted as previously described (Cao *et al.* 2011a). Briefly, individual TRF peak heights were normalized to percentages of total height for each sample, and peaks with a relative height of less than 1% were discarded. The TRFs were aligned using the crosstab macro written by Dr. C. Walsh (http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls). After alignment, two multivariate datasets (one for each of the two restriction enzymes *Hha*I and *Msp*I, with samples as rows and relative abundance of TRFs as columns) were generated for each of the TRFLP methods.

Two multivariate analysis techniques (a graphical approach and a numerical approach) were performed on each dataset to identify the reference fecal sample to which an unknown challenge sample was most similar. The fecal source represented by the most similar reference sample was deemed to be present in the unknown sample. The graphical approach was based on detrended correspondence analysis (DCA), where source identification was achieved by observing the proximity of an unknown sample to the reference samples on DCA plots (Supplemental Information (SI) Figure SI-1a, available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar13_475_490SI.pdf) as described previously (Cao *et al.* 2011a). Samples (unknown and reference) closer to each other on the plot are deemed to have more similar microbial communities than those more distant. The numerical approach was based on Bray Curtis (BC) similarity coefficients (Clarke and Warwick 2001),

where source identification was achieved by determining the reference sample with which an unknown challenge sample had the highest BC similarity coefficient (Figure SI-1b). When BC coefficients between an unknown and several reference sources (in most cases, just two) were similarly very high, both sources were deemed present in the unknown. A BC coefficient was not used for source identification unless it was at least higher than the minimum of all BC coefficients for the 12 inter-laboratory pairs of reference samples, for each restriction enzyme within each TRFLP method. DCA and BC analyses were performed in CANOCO (Microcomputer Power, Ithaca, NY, USA) and Primer 6 (Primer-E; Plymouth Marine Laboratory, Plymouth, UK), respectively. The 24 TRFLP profiles of the reference samples produced from both laboratories were pooled such that 24 reference samples were included in DCA or BC analyses. However, the inclusion of the other laboratory's reference samples was mainly for quality control purposes; source identification in each laboratory was based on its own reference samples. Guidelines for selecting multivariate statistical techniques are discussed elsewhere (Cao *et al.* 2011b).

For either Bac- or Univ-TRFLP, four preliminary source assignments from analyzing TRFLP data from each of the two enzymes using two multivariate techniques (i.e., DCA of either *Hha*I or *Msp*I data, and BC coefficient analyses for either *Hha*I or *Msp*I data) were combined using assigned qualitative scores to yield a final source identification answer for each of the 64 challenge samples. Namely, each preliminary source assignment was given a qualitative score of "high confidence" or "medium confidence" based on the extent to which the graphical (DCA) or numerical (BC) evidence supported the preliminary assignment. When preliminary assignments contradicted each other, the preliminary assignment with the higher qualitative score was reported as the final source identification answer. For example, a "high confidence" score was given to a preliminary DCA assignment if that assignment was supported by distinct clustering on the DCA plot according to either of two criteria: 1) the symbol representing an unknown overlaid the symbol representing a reference sample, or 2) the distance between an unknown and one reference was several times further than that between the unknown and other reference samples. Similarly, a high BC coefficient between an unknown and a reference sample (or a BC coefficient much higher with one reference than with other reference samples) yielded a "high confidence" BC-based preliminary assignment. For Univ-TRFLP, all four preliminary source assignments were conducted in both laboratories. For Bac-TRFLP, one laboratory conducted all four assignments while the other laboratory performed only the two assignments based on BC analyses.

Additionally, the pair of final answers from Univ- and Bac-TRFLP for each sample was considered together to report a final answer from combining both TRFLP methods (Univ&Bac-TRFLP) in each laboratory. A final Univ&Bac-TRFLP answer was identical to the final answer for each method if both TRFLP methods identified the same source(s). When only one TRFLP method provided an answer, the Univ&Bac-TRFLP reported the same final answer. When different sources were identified by Univ- and Bac-TRFLP, both sources were generally listed in the final answer by Univ&Bac-TRFLP. However, if the BC similarity coefficient between the inter-laboratory pair of reference samples was low (<40) for either Bac- or Univ-TRFLP, this indicated that there was high variation in sample processing in either laboratory. In that case, the source identified using the corresponding reference samples and TRFLP method was not included in the final answer by Univ&Bac-TRFLP.

TRFLP data analysis was primarily conducted using one data handling approach, i.e., *Hha*I and *Msp*I as separate datasets based on peak height as described above. Nevertheless, two alternative data handling approaches were applied to data from one laboratory to assess their potential effects on source identification results: 1) Source identification was repeated with a concatenated single dataset from *Hha*I and *Msp*I peak height data, via the BC analysis; and 2) Source identification via BC analysis was also repeated with peak area data using the same data processing procedure as that for peak height.

Performance Evaluation

The TRFLP methods performed in both laboratories were assessed for percentage of correct source identification among the 64 challenge samples, for sensitivity and specificity for each of the 12 fecal sources, and for reproducibility between the two laboratories. Assessments were conducted by comparing the reported source identification answers to truth (i.e., the key) and by comparing the source identification answers from the two participating

laboratories. The key included what source(s) were present (for both single- and dual-source samples) in the challenge samples and at what percentages (for dual-source samples only). The percentage was calculated as percent DNA contribution from the two sources to total DNA concentration of the doubleton. Briefly, concentrations of total DNA measured in the single-source samples were used to approximate the proportion of total DNA contributed by each source to the dual-source samples based on a 90% and 10% (by volume as the dual-source challenge samples were prepared) *in silico* mixing. A source was considered dominant if its percent contribution was greater than two times the contribution of the second source.

Each source identification result was classified into one of five categories depending on how it compared to the key (Table 1). For singletons, the percentage correct identification was calculated as the number of samples where the source was correctly identified and no incorrect source was listed, divided by the number of samples where an answer was reported. For doubletons, the percentage of correct identification was calculated as the number of samples where the dominant source (or both sources) was correctly identified and no incorrect source was listed, divided by the number of samples where an answer was reported.

Sensitivity and specificity were calculated for each of the 12 sources as described elsewhere (Cao *et al.* 2013). Briefly, for each particular source A, sensitivity was calculated as the number of challenge samples correctly identified as containing source A divided by the total number of samples that contained source A; specificity was calculated as the number of challenge samples that were not falsely reported as containing source A divided by the total number of samples that did not contain source A. The three types of human source (human feces, sewage and septage) were considered both individually and together as a single source.

Direct comparison of source identification answers was performed to assess the level of agreement between the two laboratories on the three sets of final source identification answers: by Univ-TRFLP alone, by Bac-TRFLP alone, and by Univ&Bac-TRFLP. Overall agreement ("agreed") occurred when the source identification answers for an unknown sample from both laboratories were classified into the same category (listed in Table 1). More stringent agreement ("agreed and correct") occurred when the "agreed" criteria were met and the source listed was actually present in the unknown sample. These two assessments were conducted for all challenge samples together and for singletons and doubletons separately. In addition to assessing reproducibility at the source identification level, community TRFLP profiles of inter-laboratory replicates of each reference sample were compared to assess inter-laboratory reproducibility at the TRFLP data level.

Results

Bac-TRFLP Method Development

The 52 to 62°C range of annealing temperatures gave similarly bright bands at the expected size (approx. 900 bp), and PCR with 58, 60, or 61.1°C exhibited no visible difference in band size and band intensity on the gel. The annealing temperature of 60°C was therefore selected for optimizing PCR cycle numbers. Based on gel electrophoresis, >27 cycles provided the brightest bands of the expected size. A further experiment involving the whole process of TRFLP was conducted to compare community profiles generated by 27, 29, and 31 PCR cycles, each in duplicate. Within each of the

Table 1. Five categories of how reported results compared to the key for challenge samples.

Category	For Singletons	For Doubletons
Correct	Source correctly identified	Dominant source (or both sources) correctly identified
Minor Source	n/a	Minor source correctly identified but dominant source not identified
Partially Wrong	Two sources listed but only one correct	Correct dominant source listed with one or more incorrect source(s) OR correct minor source listed with one or more incorrect source(s)
Wrong	Source(s) listed not present in sample	
No Answer	No answer provided	No answer provided

three fecal DNA sources (sewage, cow, and dog) with the same cycle number, the duplicate TRFLP profiles were nearly identical in that the raw TRFLP electropherograms visually overlapped. For each of the three fecal DNA sources, different cycle numbers also yielded very similar TRFLP profiles in terms of number and size (in base pairs) of TRFs. The TRFLP profiles from sewage, cow, and dog were clearly different. The default PCR thermal conditions from Wood *et al*. (1998) were therefore deemed sufficient for Bac-TRFLP and used in the SOP.

The full Bac-TRFLP runs were successful and repeatable between PCR replicates, indicating TRFLP protocols following PCR amplification transferred well from Univ-TRFLP to Bac-TRFLP. Bac-TRFLP therefore adopted the same standard operating procedure as that for Univ-TRFLP with the exception of the initial PCR steps, the utilization of 300 ng of purified PCR products in 20 µl digestions, and the elution of the de-salted digests in 30 µl. The choice to scale down the procedure during two steps in the Bac-TRFLP SOP, compared to Univ-TRFLP, was due to logistics (to conserve sample DNA and reagents): 16 ng community DNA (vs. 25 ng for Univ-TRFLP) per 50 µL reaction was used for PCR and 300 ng of purified PCR product per 20 µl digestions (vs. 600 ng in 40 µl digestions for Univ-TRFLP).

Overall Source Identification

Source identification results for all 64 challenge samples are presented in Table 2 (for singletons) and Table 3 (for doubletons). The two alternative data handling approaches produced results similar to those produced using the default data handling approach. Concatenating the *Hha*I and *Msp*I data sets together as one dataset yielded the same primary identification results to those obtained via analyzing *Hha*I and *Msp*I data sets separately. Similarly, analysis on data sets based on peak area vs. peak height also did not change dominant source identification. Therefore, only results from the default data handling approach (*Hha*I and *Msp*I as separate datasets based on peak height) are presented. Bac-TRFLP showed high percentages of correct identification (>88%) in both laboratories (Table 4). General performance was better for singletons than for doubletons in both laboratories. Overall, there were 15 and 5 samples for which no answer was provided, for Laboratory 1 and Laboratory 2, respectively.

Compared to Bac-TRFLP, Univ-TRFLP showed similarly high percentages of correct identification (>89%), and increased performance for singletons rather than for doubletons, in both laboratories (Table 4). Overall, there were 8 and 13 samples for which no answer was provided, for Laboratory 1 and Laboratory 2, respectively.

Combining the Univ- and Bac-TRFLP methods increased the overall percentage of correct identification (>92%) as compared to using Bac- and Univ-TRFLP separately, reduced the number of samples for which no answer was provided and showed little performance difference between singletons and doubletons (Table 4). Univ&Bac-TRFLP had a similar percentage of correct identification of singletons to that of Bac- or Univ-TRFLP alone, but a marked increase in correct identification was observed for doubletons and the number of "wrong" and "no answer" results were greatly reduced (Table 4; Figure 1). When reporting Univ&Bac-TRFLP answers, i.e., by considering the source answers from both Bac- and Univ-TRFLP jointly, Univ- and Bac-TRFLP reinforced each other 74% of the time; different sources were listed and reported as multiple sources 9% of the time, and only one TRFLP method provided an answer 17% of the time.

Sensitivity and Specificity to Each Source

Regardless of source(s), Bac-TRFLP exhibited excellent specificity in both laboratories (Table 5). Sensitivity was more variable within each laboratory and between laboratories, depending on the source. Both laboratories had near perfect sensitivity and specificity for deer, horse, and pig (Tables 2, 3, and 5). Neither laboratory identified goose (sensitivity = 0) or most of the gull (sensitivity = 0.17) challenge samples. The majority of the challenge samples where no answer was provided contained these avian fecal sources (Tables 2 and 3). Among the 12 challenge sources, Bac-TRFLP profiles from the known reference samples for three bird sources (gull, goose, pigeon) were also most distinct from the rest of the sources (Figure SI-2A and B). PCR for these avian samples had to be repeated to obtain enough product for the TRFLP steps; moreover, sources containing gull and pigeon could not be distinguished and were reported as a single category (i.e., gull and/or pigeon). Chicken, although also an avian source, did not experience difficulty in PCR and could be distinguished from other avian sources by Bac-TRFLP. Additionally, the 1:10 dilution of singletons resulted in one false negative for sewage and dog sources, and two false negatives for gull (one

Table 2. Reported source identification results[§] for singletons. An underline indicates that the identified source was not present in the unknown sample.

Singletons [a]	Bac-TRFLP		Univ-TRFLP		Univ&Bac-TRFLP	
	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2
chicken	chicken	chicken	chicken	chicken	chicken	chicken
chicken[b]	chicken & sewage	chicken	chicken & sewage	-	chicken & sewage	chicken
cow	cow	cow	cow	cow	cow	cow
cow	cow	cow	cow	cow	cow	cow
cow 1:10	cow	cow	cow	cow	cow	cow
cow 1:10	cow (minor?)	cow	goose	goose	cow/goose	cow
deer	deer	deer	deer	deer	deer	deer
deer	deer	deer	deer	deer	deer	deer
dog	dog	dog	dog	dog	dog	dog
dog	dog	dog	dog	dog	dog	dog
dog 1:10	dog	dog	dog	dog	dog	dog
dog 1:10	-	dog	dog	dog	dog	dog
goose	gull/pigeon	-	pig	-	pig	-
goose	-	-	-	gull/pigeon	-	gull/pigeon
gull	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon
gull	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon
gull 1:10	-	-	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon
gull 1:10	-	-	gull	gull/pigeon	gull	gull/pigeon
horse	horse	horse	horse	horse	horse	horse
horse	horse	horse	horse	horse	horse	horse
human	-	human	human	human	human	human
human	human	human	human	human	human	human
human 1:10	-	human	human	human	human	human
human 1:10	human	human	human	human	human	human
pig	pig	pig	-	-	pig	pig
pig	pig	pig	pig	-	pig	pig
pig 1:10	pig	pig	-	-	pig	pig
pig 1:10	pig	pig	-	-	pig	pig
pigeon	-	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon
pigeon	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon	gull/pigeon
septage	septage	septage	septage	septage	septage	septage
septage	septage	septage	septage	septage	septage	septage
septage 1:10	septage	septage	septage	septage	septage	septage
septage 1:10	septage	septage	septage	septage	septage	septage
sewage	sewage	human/sewage	sewage	sewage	sewage/human	sewage
sewage	chicken/sewage	human/sewage	sewage	sewage	sewage	sewage
sewage 1:10	sewage	sewage	sewage	sewage	sewage	sewage
sewage 1:10	-	sewage	sewage	sewage	sewage	sewage

[§] "/" in the source identification answer denotes "and/or". "&" in the source identification answer denotes "and". "-" denotes no answer was reported. TRFLP mostly only reported a dominant source unless overwhelming evidence suggested presence of two sources.

[a] Samples included blind duplicates of 19 singletons (full strength or 1:10 strength).

[b] This challenge sample for Lab 1 was suspected to be contaminated with sewage during sample preparation; however, performance evaluation was conducted as-is.

Table 3. Reported source identification results[§] for doubletons. An underline indicates that the identified source was not present in the unknown sample. Italic font in the doubletons column indicates that neither source met dominance criteria (i.e., DNA contribution from source 1> two times that from source 2), and an answer was considered correct if it reported either source.

Doubletons [a]	Bac-TRFLP		Univ-TRFLP		Univ&Bac-TRFLP	
	Lab 1	Lab 2	Lab 1	Lab 2	Lab 1	Lab 2
chicken:sewage 92:8	chicken & sewage	chicken	sewage & chicken	chicken	chicken & sewage	chicken
chicken:sewage 92:8	chicken & sewage	-	sewage & chicken	-	sewage & chicken	-
cow:human 92:8	cow	cow	cow	-	cow	cow
cow:human 92:8	cow	cow	-	-	cow	cow
dog:human 62:38	dog	human & dog	human	human	human/dog	human
dog:human 62:38	human	human & dog	-	-	human	human & dog
goose:human 91:9	human	human	goose	gull/pigeon	human/sewage	human
goose:human 91:9	human	human & pig	goose	goose	human/goose	goose/human/pig
gull:human 56:44	human	human	human	human	human	human
gull:human 56:44	-	human	human	human	human	human
gull:sewage 92:8	-	sewage	gull	gull/pigeon	sewage/gull	gull/pigeon
gull:sewage 92:8	-	sewage	gull & sewage	gull/pigeon	gull/sewage/pigeon	gull/pigeon
horse:septage 82:18	horse	horse	horse	-	horse	horse
horse:septage 82:18	horse	horse	horse	horse	horse	horse
human:cow 88:12	-	human	goose	-	sewage/human	human
human:cow 88:12	-	human	-	human	-	human
human:dog 98:2	human	human	human	human	human	human
human:dog 98:2	human	human	human	human	human	human
human:gull 98:2	cow	human/sewage	-	human	cow	human
human:gull 98:2	human	human	human	human	human	human
pig:sewage 99:1	pig	pig	pig	-	pig	pig
pig:sewage 99:1	pig	pig	pig	pig	pig	pig
sewage:gull 88:12	-	human/sewage	sewage	sewage	sewage/human	sewage
sewage:gull 88:12	-	sewage	sewage	sewage	sewage	sewage
sewage:pig 54:46	pig	pig	goose	goose	pig	pig
sewage:pig 54:46	pig	pig	goose	goose	pig	pig

[§] "/" in the source identification answer denotes "and/or". "&" in the source identification answer denotes "and". "-" denotes no answer was reported. TRFLP mostly only reported a dominant source unless overwhelming evidence suggested presence of two sources.

[a] Samples included blind duplicates of 13 dual-source challenge samples. Numeric ratios following the two sources indicate percentage DNA contribution from each source.

in each laboratory), but did not affect specificity for any source (Table 2).

Similar to Bac-TRFLP, Univ-TRFLP exhibited excellent specificity in both laboratories regardless of source(s), but variable sensitivity within each laboratory and between laboratories depending on the source (Table 5). Univ-TRFLP had perfect (i.e., 100%) sensitivity and specificity for deer in both laboratories (Tables 5). While performance with deer, human sources, dog, and goose was similar between Bac- and Univ-TRFLP, Univ-TRFLP was better at identifying gull and/or pigeon, and Bac-TRFLP was better at identifying horse, pig, and cow. The majority of the challenge samples where no answer was provided by Univ-TRFLP contained cow or pig feces (Tables 2 and 3). Among the 12 challenge sources, Univ-TRFLP profiles from the known reference samples for septage were most distinct from the rest of the sources (Figure SI-2C and D). Additionally, the 1:10 dilution of singletons led to two false positives for goose, but did not affect sensitivity for any source (Table 2).

Table 4. Overall performance.

TRFLP Method	Lab	Singleton (n=38)		Doubleton (n=26)		All (n=64)
		% correct [a]	no answer [b]	% correct [a]	no answer [b]	% correct [a]
Bac	1	90%	8	84%	7	88%
	2	100%	4	84%	1	93%
Univ	1	91%	4	86%	4	89%
	2	94%	6	84%	7	90%
Univ&Bac	1	92%	1	92%	1	92%
	2	97%	1	92%	1	95%

[a] The percentage of correct identification was calculated as the number of samples where all source(s) or the dominant source was correctly identified ("correct", Table 1) and no incorrect source was listed divided by the number of samples where an answer was reported.

[b] The no answer column lists the number of challenge samples where no answer was provided by the TRFLP method.

Univ&Bac-TRFLP demonstrated at least equivalent and mostly improved sensitivity and specificity for all sources compared to Bac- and Univ- alone (Table 5). An exception was that minor decreases (1 - 4%) in specificity were observed, mostly in one laboratory. Nevertheless, overall specificity remained excellent (rarely <96%). Sensitivity increased for 9 and 5 sources for Laboratory 1 and Laboratory 2, respectively, and decreased occasionally (but never in both laboratories) for goose, dog and sewage. As with Bac- or Univ-TRFLP, Univ&Bac-TRFLP also could not distinguish gull and pigeon and reported gull and/or pigeon when either source was identified. The 1:10 dilution of singletons led to one false positive for goose, but did not affect sensitivity for any source (Table 2).

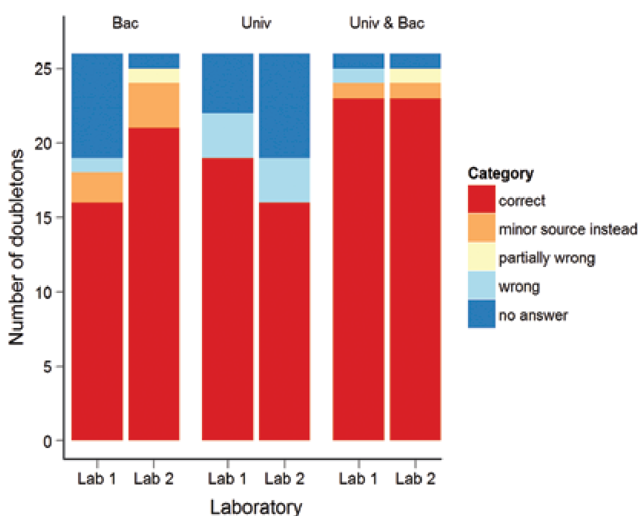

Figure 1. Performance evaluation of doubletons for all three TRFLP methods (Bac-, Univ-, and Univ&Bac-) in two laboratories (Lab 1 and Lab 2). Definitions of performance categories are as in Table 1.

Reproducibility across Laboratories

For Bac-TRFLP, the final source identification answers were fairly reproducible (72% agreed) between laboratories (Table 6). Laboratory 1 had substantially more challenge samples for which no answer was provided than did Laboratory 2 (Tables 2 and 3) and this limited reproducibility. Among the challenge samples where both laboratories provided source identification answers by Bac-TRFLP (31 singletons and 18 doubletons), 100% and 94% agreement were achieved for singletons and doubletons, respectively. However, reproducibility at the TRFLP data level (Table SI-1) was generally lower than that at the source identification level. BC coefficients (reported on a scale of 0 to 100 (Clarke and Warwick 2001)) for the inter-laboratory pairs of reference samples generally ranged from 50 to 74, but were low (<40) for goose, gull, pigeon with both restriction enzymes, and for deer, sewage and septage with either *Msp*I or *Hha*I.

The % agreed was higher for Univ-TRFLP than for Bac-TRFLP. However, among the challenge samples where both laboratories provided source identification answers by Univ-TRFLP (34 singletons and 17 doubletons), 100% and 94% agreement were achieved for singletons and doubletons, respectively, which is the same as the reproducibility observed for Bac-TRFLP (Tables 2 and 3). Similar to Bac-TRFLP, reproducibility at the Univ-TRFLP data level (Table SI-1) was generally lower than that at the source identification level. BC coefficients for the inter-laboratory pairs of reference samples generally ranged from 50 to 87, but were low (<40) for deer and human with *Msp*I.

Table 5. Sensitivity and specificity[§] of the three TRFLP methods (Univ, Bac, Univ&Bac) as performed in two laboratories (Lab1, Lab2), for all sources calculated based on all 64 challenge samples.

Source	Bac-TRFLP				Univ-TRFLP				Univ&Bac-TRFLP			
	Sensitivity		Specificity		Sensitivity		Specificity		Sensitivity		Specificity	
	Lab1	Lab2	Lab1	Lab2	Lab1	Lab2	Lab1	Lab2	Lab1	Lab2	Lab1	Lab2
septage	0.67	0.67	1	1	0.67	0.67	1	1	0.67	0.67	1	1
sewage	0.36	0.57	0.98	0.98	0.64	0.43	0.98	1	0.71	0.43	0.94	1
human[a]	0.5	0.89	1	0.93	0.56	0.67	1	1	0.78	0.89	0.96	1
HUMAN[b]	0.47	0.74	0.96	1	0.61	0.58	0.96	1	0.74	0.68	0.96	1
chicken	0.75	0.75	0.98	1	1	0.5	1	1	1	0.75	1	1
cow	0.75	0.75	0.98	1	0.5	0.38	1	1	0.75	0.75	0.98	1
deer	1	1	1	1	1	1	1	1	1	1	1	1
dog	0.5	0.75	1	1	0.5	0.5	1	1	0.63	0.63	1	1
goose	0	0	1	1	0.5	0.25	0.93	0.95	0.25	0.25	0.98	1
gull[c]	0.17	0.17	0.96	0.96	0.5	0.5	0.96	0.92	0.5	0.5	0.96	0.94
horse	1	1	1	1	1	0.75	1	1	1	1	1	1
pig	1	1	1	0.98	0.38	0.13	0.98	1	1	1	0.98	0.98
pigeon[c]	0.5	1	0.95	0.97	1	1	0.95	0.87	1	1	0.94	0.89

[§] For each particular source A, sensitivity (%) was calculated as the number of challenge samples correctly identified as containing source A divided by the total number of samples that contained source A (i.e. sensitivity = true positive / (true positive + false positive); specificity (%) was calculated as the number of challenge samples that was not falsely reported as containing source A by the total number of samples that did not contain source A (i.e. specificity = true negative / (true negative + false negative).

[a] Lower case human refers to human feces.

[b] Capital HUMAN refers to all three human sources (human feces, sewage, septage) combined.

[c] Gull and pigeon could not be distinguished, and gull and/or pigeon were reported when either source was identified. A "gull and/or pigeon" answer was considered either a true positive or false positive, depending on the source being evaluated and whether or not that source was present in the unknown challenge sample. All other occasional "and/or" answers were evaluated similarly.

Table 6. Percentage of agreement between source identification answers from two laboratories.

	Bac	Univ	Univ&Bac
% Agreed [a]			
Singleton	79%	89%	89%
Doubleton	62%	69%	85%
All samples	72%	81%	88%
% Agreed and correct [b]			
Singleton	71%	79%	89%
Doubleton	58%	54%	81%
All samples	66%	69%	86%

[a] Overall agreement ("agreed") occurred when the source identification answers for an unknown sample from both laboratories were classified into the same category from Table 1.

[b] More stringent agreement ("agreed and correct") occurred when the "agreed" criteria was met and the source listed was actually present in the unknown sample.

Combining Univ- and Bac- TRFLP methods greatly improved reproducibility and correctness of source identification answers, leading to a high level of agreement between the two laboratories (Table 6). Such improvement was most evident for more complex challenge samples such as doubletons (Table 6). Additionally, the number of samples for which no answer was provided was reduced to two using Univ&Bac-TRFLP (Table 2; Figure 1).

Discussion

The new Bac-TRFLP method demonstrated good performance when challenged with 64 blind samples from 12 fecal sources. This is consistent with previous findings that the order Bacteroidales contains source-specific 16S rRNA genetic markers for many sources (Wuertz *et al.* 2011) and that *Bacteroides-Prevotella* populations are much more similar within host species than between host species (chicken, cow, deer, dog, horse, human, pig; Fogarty and Voytek 2005). However, previous group-specific TRFLP methods had less success in source identification evaluation studies (Field *et al.* 2003).

The improved performance of Bac-TRFLP may be attributed to two factors. First, our Bac-TRFLP likely provides a good balance between method resolution and information richness. Previous group-specific TRFLP methods mostly targeted *Bacteroides-Prevotella* genera, resulting in limited TRFs in the TRFLP community profiles (Field *et al.* 2003) that may have hindered source identification. A recent pyrosequencing study showed high abundance of Bacteroidales in fecal samples from humans, chickens, geese, cows, and pigs outside of these two genera (Unno *et al.* 2011). In fact, high relative abundance was observed for Bacteroidales from three families Bacteroidaceae, Prevotellaceae, and Porphyromonadaceae (Unno *et al.* 2011), which are the primary target of Bac-TRFLP. While TRFLP targeting all Bacteria contains more information, the higher intrinsic redundancy in Univ-TRFLP profiles (Engebreston and Moyer 2003) can lead to lower resolution for source identification (Cao *et al.* 2011a). Restricting TRFLP to a sufficiently large subgroup such as Bacteroidales may have provided a suitable balance between TRF redundancy and information richness.

A second factor contributing to improved MST performance may be more effective data analysis (Cao *et al.* 2011a). TRFs-based (i.e., individual peak-based) assessments, such as described in early studies (Bernhard and Field 2000, Field *et al.* 2003), can be more variable due to methodological choices, but conclusions regarding overall community patterns among different samples are often robust and consistent (Zhang *et al.* 2008) and have been shown to be more effective for source identification (Cao *et al.* 2011a).

In comparison to Univ-TRFLP (Cao *et al.* 2011a), Bac-TRFLP demonstrated strength in identifying fecal contamination from cow, horse, and pig but had difficulty identifying feces from goose, gull, and pigeon sources. This may be explained by differences in the extent of microbial community divergence among the fecal sources. Fecal bacteria communities may be different at a much higher phylogenetic level (e.g., at the phylum level) between disparate categories of animals, but a lower phylogenetic level (e.g., at the order, family or even genus levels) within a category of animals. For example, the fecal microflora from certain avian sources (gulls, geese, pelican, pigeon, cormorant) has been found to contain high abundances of bacteria from the phylum Firmicutes (the classes of Bacilli and Clostridia), but low abundances of bacteria from the phylum Bacteroidetes (Lu *et al.* 2008, 2009; Dubinsky *et al.* 2012), while grazing animals (cow, horse, elk) have fecal communities dominated by both Firmicutes and Bacteroidetes (Shanks *et al.* 2011, Dubinsky *et al.* 2012). Communities have also been shown to differ between human sources of fecal contamination: human feces have a high abundance of Bacteroidetes (McLellan *et al.* 2010; Unno *et al.* 2010, 2011),

while sewage and septage communities are usually dominated by Firmicutes, Bacteroidetes, and Proteobacteria (McLellan *et al.* 2010, Wu *et al.* 2010, Dubinsky *et al.* 2012). Chicken fecal communities were found to be most different from those in human feces when fecal communities from chickens, geese, cows, pigs, and humans were compared (Lee *et al.* 2011). Therefore, a general TRFLP targeting the entire kingdom of Bacteria should be able to separate these drastically different fecal communities.

However, when attempting to separate TRFLP signatures from more closely related animals, it may be beneficial to filter out common species of bacteria and focus on a subgroup where host-associated species are most prevalent. Many Bacteroidales have been shown to be associated with certain mammals (Shanks *et al.* 2011, Unno *et al.* 2011, Dubinsky *et al.* 2012). One study found that 98% of pig-associated Bacteroidales sequences belonged to the genus *Prevotella* (Lamendella *et al.* 2009); another showed that pig fecal communities, compared to those from chickens, geese and cows, were most similar to fecal communities in human feces (Lee *et al.* 2011). Therefore, the better performance of Bac-TRFLP at identifying cow, horse, and pig fecal sources is consistent with current knowledge of fecal community composition across these mammalian fecal sources.

Filtering out non-Bacteroidales, although not tested in this study, may be advantageous when Bac-TRFLP is utilized in the field. For example, Proteobacteria has been shown to be a large component of non-fecal sources (McLellan *et al.* 2010, Unno *et al.* 2010, Wu *et al.* 2010). By excluding the Proteobacteria background, Bac-TRFLP may achieve higher sensitivity for fecal sources. Nevertheless, as exemplified by our results, Bac-TRFLP may not be suited for some avian sources (gull, pigeon, goose), likely due to the low abundance of Bacteroidales in these sources (Lu *et al.* 2008, 2009; Unno *et al.* 2010; Dubinsky *et al.* 2012; Ervin *et al.* 2013).

Combining Univ-TRFLP with the more targeted Bac-TRFLP can be very effective for fecal source identification. Individually, each TRFLP method, focused at a different phylogenetic scale, has inherent strengths and weaknesses. Combining the methods (i.e., Univ&Bac-TRFLP) made it possible to benefit from the strengths of both. This may explain the higher overall % correct identification, higher sensitivity and specificity metrics, and higher reproducibility in fecal source identification by Univ&Bac-TRFLP.

One concern with using community analysis methods for microbial source tracking is whether the performance and MST answers are consistent across laboratories, since these methods usually involve more steps than single host-associated marker assays. The variability inherent in individual DNA extraction procedures and PCR amplification are well known (Pan *et al.* 2010). Despite the additional complexity of the TRFLP methods, our results showed good source identification agreement between two laboratories. Standardization of the complete procedure from DNA extraction to data analysis for final source identification likely contributed to the consistent MST performance of the TRFLP methods across the laboratories, but a multi-laboratory intercalibration study will be necessary to more robustly address the question of across laboratory reproducibility. Nevertheless, given that inter-laboratory reproducibility at the TRFLP data level was generally lower than that at source identification level (this study) and that TRFLP data are highly repeatable (i.e., nearly identical or very similar TRFLP profiles from within-laboratory replicates (Fogarty and Voytek 2005, Zhang *et al.* 2008), it is preferable to perform source identification using TRFLP data within a single laboratory as done in the current study.

While this study demonstrated successful development of Bac-TRFLP and added two additional tools (Bac-TRFLP, Univ&Bac-TRFLP) to the MST toolbox, we recognize certain limitations in the applicability of our results to use of these methods in the field. First, challenge samples were prepared in artificial freshwater free of ambient bacteria that affect fecal source signals. Dilution in ambient water could lead to fecal signals being masked by abundant ambient microorganisms in the overall community profiles. However, combining a general Univ-TRFLP and a more targeted Bac-TRFLP may provide enough additional resolution to ameliorate this potential issue. Second, fresh fecal material was used to create both reference and challenge samples. Although a previous study has shown that TRFLP profiles of feces-amended pond water remained constant over a 12-day experiment under direct sunlight and aerobic conditions at 18°C, and were identical to the original TRFLP profiles for the original cow fecal sample (Fogarty and Voytek 2005), further study on how aging affects the stability of community profiles should be performed since changes during fecal aging may

impinge on the utility of community analysis in MST. Third, the pool of 64 challenge samples was centered around human fecal sources and the California geographic region (Boehm *et al.* 2013). The number of challenge samples per animal fecal source was relatively low (2 - 12 challenge samples per source), which can lead to high uncertainty in estimated sensitivity values. This is because having drastically more negative than positive challenge samples for a given source inevitably creates a stringent study design for assessing specificity, but an inadequate setting for assessing sensitivity for this source. More detailed evaluation with different animal sources and from other geographic regions will provide more information on method performance.

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Supplemental Information

Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar13_475_490SI.pdf.