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Mitochondrial Multiplex Real-Time PCR as a Source Tracking Method in Fecal-Contaminated Effluents

JANE M. CALDWELL,*
MORGAN E. RALEY, AND JAY F. LEVINE
Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, North Carolina 27606

Multiplex real-time PCR amplifying fecal mitochondrial DNA (mtDNA) combined with rapid, crude DNA preparations are promising additions to surface water source tracking methods. Amplification of eukaryotic mitochondrial DNA identifies the fecal source directly and can be used in conjunction with other intestinal microbial methods to characterize effluents. Species-specific primers and dual-labeled probes for human, swine, and bovine NADH dehydrogenase subunit 5 (ND5) genes were created for multiplex real-time PCR in feces and effluent slurries. The linear range of the multiplex assay was 10^2−10^7 mtDNA copies for human, bovine, and swine effluent in combination (equal volumes). PCR amplification efficiencies for bovine, human, and swine mtDNA when assayed in combination were 93, 107, and 92% respectively. Linear regression correlation coefficients (r^2) were 0.99 for all standard curves except for human mtDNA in combination ($r^2 = 0.95$). Multiplex amplification of bovine, human, and swine mtDNA (ND5) exhibited no cross-reactions between the effluents from three species of interest. Also, no cross-reactions were observed with effluents of other vertebrates: sheep, goat, horse, dog, cat, Canada goose, broiler, layer, turkey, and tilapia. Performed as a blind test, the PCR operator was able to correctly identify all but two effluent challenge samples (10/12 or 83% correct) with no false positives (22/22 or 100% correct). The multiplex assay had a tendency to detect the species of highest mtDNA concentration only. Better detection of all three species in a combination of human, bovine, and swine effluents was accomplished by running each real-time PCR primer/probe set singly. Real-time PCR detection limit was calculated as 2.0×10^6 mitochondrial copies or 0.2 g of human feces per 100 mL effluent. Some carry-over mtDNA PCR signal from consumed beef, but not pork, was found in feces of human volunteers.

1. Introduction

Contamination with enteric pathogens from animal and human feces limits the use of surface waters for recreation, commercial shellfish harvesting, and the irrigation of agricultural crops for human consumption. The potential sources of fecal contamination are numerous and reflect the breadth of mammalian activity on the planet. Spills from municipal and residential waste handling, runoff from fields used for spreading livestock waste, livestock waste lagoon leaks, feedlot runoff, and wildlife contamination add to the total fecal load in surface waters.

Fecal pollution of surface waters is considered a nonpoint source problem. However, mitigating the effects of fecal waste contamination in surface waters requires identification of its source. Source tracking methods have traditionally used indicator organisms (IO) such as thermotolerant coliforms, *Escherichia coli* and *Enterococcus* spp., to detect fecal contaminants. However, fecal coliforms such as *E. coli* are not diagnostic of particular animal species and are not good indicators of viruses and other pathogens that may be present in surface waters (*1*−*3*). Finding host-specific microorganisms has been the holy grail of microbial source tracking (MST). *Bifidobacterium adolescentis* (*4*, *5*), *Bifidobacterium dentium* (*6*), human adenovirus and human enterovirus (*7*), human polyomavirus (*8*), *Enterococcus* spp (*9*−*13*), and enterococcal surface protein from *Enterococcus faecium* (*14*) have been used as indicators of human fecal pollution. Teschovirus has been used as an indicator of porcine fecal contamination (*15*) and bovine enteric virus as indicator of bovine fecal contamination (*3*). Antibiotic resistance of various indicator organisms for source tracking has also been examined (*16*−*18*) but requires the generation of an extensive library of isolates. Many expensive and time-consuming nucleic acid-based molecular methods for source tracking have been tested, including ribotyping, length heterogeneity PCR, terminal-restriction fragment length polymorphism, repetitive PCR, pulsed-field gel electrophoresis, and amplified fragment length polymorphism (*19*). However, no single indicator organism or molecular test works for all eukaryotic species.

Biochemical methods evaluated for source tracking include fecal sterols (*20*), stable isotopic ratios (*21*), and whitening agents (*22*). Blanch and co-workers (*23*) suggested that no single parameter is able to discriminate between human and non-human sources of fecal pollution and recommended a "basket" of four or five parameters including microbiological, phage, and chemical assays. A combination of microbial and chemical indicators has been suggested (*24*) and other researchers have recommended identifying new genomic targets and quantification methods for microbial source tracking (*25*).

Mitochondrial DNA (mtDNA) in conjunction with PCR or real-time PCR is used extensively in the fields of phylogenetics (*26*, *27*), forensics (*28*, *29*), and medicine (*30*). Martellini and co-workers (*31*) first used mtDNA with conventional and nested PCR to differentiate between human, bovine, porcine, and ovine sources in surface water source tracking. The advantages of targeting mtDNA as a source tracking tool are substantial. Polymerase chain reaction of mitochondrial DNA can be used to identify the animal species directly rather than microbial species it may host. Feces contain large amounts of exfoliated epithelial cells (*32*) and mtDNA has many copies per cell (*33*, *34*). Healthy human peripheral blood mononuclear cells were reported to have 1000 (*35*) and normal human endometrial cells have 158−2625 mtDNA copies/cell (*36*). Therefore, mtDNA genes give robust PCR signals similar to 16S rRNA genes. Detectable DNA persists even after cellular death. Martellini et al. (*31*) were able to detect human DNA for up to 15 days after addition of mtDNA to a water source. Since we are detecting host DNA, debates about indicator organism host relevance, cultivability, and viability are moot.

* Corresponding author phone: 919-513-6368; fax: 919-513-6464; e-mail: jane_caldwell@ncsu.edu.

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TABLE 1. Mitochondrial PCR Primers and Probes

primer or probe[a]	nucleotide sequence (5′ to 3′)	Tm (°C)	location within target[b]	amplicon size (bp)
human forward	CAGCAGCCATTCAAGCAATGC	57.9	497−517	195
human reverse	GGTGGAGACCTAATTGGGCTGATTAG	58.8	666−691	
human probe	TATCGGCGATATCGGTTTCATCCTCG	59.9	528−553	
bovine forward	CAGCAGCCCTACAAGCAATGT	58.1	497−517	191
bovine reverse	GAGGCCAAATTGGGCGGATTAT	58.0	666−687	
bovine probe	CATCGGCGACATTGGTTTCATTTTAG	56.9	528−552	
swine forward	ACAGCTGCACTACAAGCAATGC	58.6	497−517	196
swine reverse	GGATGTAGTCCGAATTGAGCTGATTAT	56.5	666−692	
swine probe	CATCGGAGACATTGGATTTGTCCTAT	56.7	528−553	

[a] Species-specific primers and probes were designed using IDT Primer Quest software (http://scitools.idtdna.com/Primerquest/) and adjusted for mismatch amplification mutation assay (*37*) for multiplex PCR in primers and species-specificity in probes. The dual-labeled probes were conjugated with Quasar 570, Cal Red, and FAM at the 5′ ends for human, bovine, and swine probe, respectively. The probe 3′ ends utilized Black Hole quenchers (BioSearch Technologies). [b] Positions of the oligonucleotides are listed relative to the numbering of the human mitochondrial gene NADH dehydrogenase subunit 5 (ND5) in VectorNTI (Version 10.1, Invitrogen Corp., Carlsbad, CA). ND5 nucleotide sequences were retrieved from GenBank (http://www3.ncbi.nlm.nih.gov) under accession numbers AY972053 (human), NC_006853 (bovine), and AF034253 (swine).

The purpose of this study was to validate the real-time PCR primers and probes on known positive fecal effluents. We have developed a triplex real-time PCR assay to distinguish among three fecal contaminators: human, cow, and pig. This process simply and quickly indicated the major source of fecal contamination and could potentially add to the database of cultural, molecular, and biochemical methods. This assay was much faster than conventional or nested PCR and could quantify up to three eukaryotic species per reaction. Furthermore, we quantified real-time PCR signal in human feces after consumption of beef and pork products.

2. Materials and Methods

2.1. Samples: Feces, Influents, and Effluents. Feces from farm animals were obtained from the NCSU College of Veterinary Medicine Teaching Animal Unit (TAU). Sixteen human volunteers provided fecal samples and completed a questionnaire concerning meals eaten for the previous 48 h for carry-over studies to determine if consumed meat products would provide mtDNA signal in human feces. The research with human subjects was in compliance with all relevant policies (NCSU Institutional Review Board: 172−05-9). All fecal samples were frozen at −20 °C.

Human, bovine, swine, and piscine influent and effluent grab samples (250 or 500 mL) were collected from the NCSU experimental farms and the Holly Springs Department of Water Quality wastewater treatment plant (WWTP). When species-specific effluents were unavailable (applies to sheep, goat, horse, dog, cat, Canada goose, broiler, layer, and turkey), feces or poultry litter (25−55 g) were mixed with 500 mL distilled water and incubated at room temperature overnight to hydrate the feces and create an effluent solution.

2.2. Centrifugation. Clear effluents with low amounts of floating solids were concentrated by centrifugation using a Sorvall RC-5B plus superspeed centrifuge (Thermo Electron Corp., Asheville, NC). Samples were collected in 250 or 500 mL sterile centrifuge bottles and spun at 9000*g* for 15 min. Pellets were resuspended in remaining liquid (∼5−25 mL) after supernatant aspiration. Concentrations obtained were ∼2.5-fold as determined by DNA concentrations before and after centrifugation.

2.3. DNA Extraction. DNA was extracted from frozen human feces using the QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA) with two alterations: supernatant was incubated at 56 °C for 1 h with 25 μL proteinase K and spin/collection columns were washed twice with buffer AW1.

Effluent samples were frozen at −20 °C overnight, and then thawed at room temperature to form a crude DNA preparation. Samples high in organic matter or solids were diluted 1:100 in RT-PCR water (Ambion, Austin, TX) to eliminate PCR inhibitors such as humic acids, bilirubin, or bile salts. A NanoDrop (NanoDrop Technologies, Wilmington, DE) spectrophotometer was used to quantify and evaluate the quality of commercially extracted and crude DNA. Samples with high DNA concentrations (>35 ng/ul) were diluted 1:10 or 1:100 so as not to overwhelm the PCR reaction. Prior to real-time PCR, effluent samples were assigned random numbers by a non-interested third party to create a blind test for the PCR operator (Table 2).

TABLE 2. Multiplex Real-time PCR Assay of Collected Effluents[a]

sample	DNA conc. ng/μL	copies mtDNA/PCR reaction: human	bovine	swine
bovine sources:				
dairy farm I	2686		3.5×10^3	
dairy farm II	475		6.5×10^3	
dairy farm III	155		2.5×10^3	
beef barn feces	239		7.0×10^3	
human sources:				
WWTP influent I	761	1.4×10^3		
WWTP influent II	25	7.6×10^1		
WWTP influent III	23			
WWTP lift station I	43			
WWTP lift station II	34	1.0×10^4		
swine sources:				
swine feces	3177			8.0×10^2
swine settling basin	117			2.2×10^2
swine litter	748			7.9×10^3
mixtures:[b]				
Mix I	N/A		1.1×10^3	
Mix II	N/A		2.5×10^3	

[a] Detection of mtDNA in crude preparations of eukaryotic effluents. DNA was extracted using the freeze/thaw method and assayed by multiplex real-time PCR using three species-specific primer and probe sets (bovine, human, and swine). DNA concentrations determined by Nanodrop spectrophotometer. The following mammalian, avian and fish effluents gave no PCR cross-signals: sheep, goat, horse, dog, cat, Canada goose, broiler, layer, turkey, and tilapia. Wastewater treatment plant (WWTP) microbial- and UV-treated wastewater discharges gave no human mtDNA signal. [b] Human, bovine, and swine effluents combined in equal volumes in Mix I and II. All numbers corrected by dilution and concentration factors.

2.4. Primer and Probe Design. Three species-specific primer and dual-labeled probe sets (human, bovine, and swine) for the multiplex real-time PCR assay were designed with Primer Quest software (http://scitools.idtdna.com/Primerquest/) for amplification of mitochondrial gene NADH dehydrogenase subunit 5 (ND5) (Table 1). Multiplex primers were adjusted for mismatch amplification mutation assay (*37*) utilizing penultimate primer mismatch kinetics to increase their specificity (Table 1). Primers were purchased

from IDT (http://www.idtdna.com). Dual-labeled probes were purchased from BioSearch (www.biosearchtech.com) with 3′ black hole quenchers, (BHQ), and 5′ fluorophores Quasar 570 and Cal Red corresponding to Cy3, and Texas Red, respectively (Table 1). All oligonucleotides were reconstituted in TE buffer (pH 7.5) and stored at −20 °C prior to use.

2.5. Standard Curves and Assay Specificity. Standard curves were generated using 10-fold serial dilutions (10^7-10^1) of mtDNA copies generated from double PCR amplifications of human, swine, and bovine mitochondrial ND5 clones. Amplicons were cloned using the TOPO TA kit (Invitrogen, Carlsbad, CA), sequenced (see Section 2.8) and NCBI BLAST searches were performed to verify sequence identities.

Multiplex primers and probes were tested for real-time PCR cross-reactions with other avian and mammalian effluents or feces in addition to plant, fungal, and bacterial DNA extracts. Annealing temperatures, thermal cycler background, and cycle threshold levels were optimized for each primer/probe set to eliminate possible noise caused by high levels (stationary phase of microorganisms) of gram negative and positive bacteria, yeast, fungus, plant, freshwater algae, and aquatic plant DNA.

2.6. Statistical Analyses. PCR amplification efficiency (E) was determined using the slope of the standard curve:

$$E = (10^{-1/\text{slope}}) - 1$$

Data analysis of the real-time PCR standard curves was performed using Origin software version 7.5 (OriginLab Corp., Northampton, MA). Goodness-of-fit of linear regression correlation coefficient (r^2) and slope were used to assess the quality of each real-time primer and probe set.

2.7. Multiplex Real-Time PCR. Multiplex real-time PCR was run in 25 μL volume reaction tubes (Cepheid; Sunnyvale, CA) with OmniMix Bead (TaKaRa Bio Inc.; Madison, WI), (1.5U TaKaRa hot start Taq polymerase, 200 μM dNTP, 4 mM $MgCl_2$, 25 mM HEPES pH 8.0), all six oligonucleotide primers (300 nM each), human, swine, and bovine probe (320 nM each) (Table 1), additional 1.5 mM $MgCl_2$ (5.5 mM final $MgCl_2$) 5 μL of template DNA (crude or extracted), and RT-PCR water (Ambion, Austin, TX) to final volume.

Amplifications were performed in a Cepheid Smart Cycler II thermal cycler (Cepheid, Sunnyvale, CA) with the following conditions: 95 °C for 120 s; 40 cycles of 94 °C for 10 s, 60 °C for 12 s, and 72 °C for 10 s. Three fluorophore channel optics were on during annealing: FAM, CY3, and Texas Red. The rapid real-time PCR assay runs ca. 40 min.

No template (NTC) and positive controls (10^3-10^5 ND5 amplicon copies) were used for all assays. For a sample to be considered positive, its Ct value must be less than all negative control reactions and its corresponding amplification curve had to exhibit the three distinct phases of real-time PCR: lag, linear, and plateau. Internal amplification controls (IAC) were employed with effluent and feces to check for PCR inhibitors: 10^3 copies of human ND5 amplicon was added to a sample aliquot and compared to the human mitochondrial copy number standard curve or another IAC sample with only water and master mix added.

2.8. Amplicon Sequencing. Real-time PCR amplicons and mitochondrial clones were sequenced following the recommended protocol with the ABI BigDye v. 3.1 sequencing kit (Applied Biosystems; Foster City, CA). Sequencing reactions were purified using an ethanol/ammonium acetate precipitation protocol (*38*) and visualized using an ABI 3130XL Automated Sequencer (Applied Biosystems, Foster City, CA). Sequences were compiled in Sequencher version 4.5 (Gene Codes Corp., Ann Arbor, MI) and NCBI BLAST searches were performed to verify sequence identities.

3. Results

3.1. Linear Range and Amplification Efficiency of the Assay. Standard curves were generated using serial dilutions of known ND5 copy numbers to determine the linear range and amplification efficiencies of the real-time PCR assay (Section 2.5). Multiplex assays were run with dilutions from each species separately (human, bovine & swine mtDNA copies) and all three species combined in equal copy numbers. The assay had a linear range between 10^1 and 10^7 copies when run separately and 10^2 and 10^7 copies when run in combination (Figure 1), which is comparable to ranges in clinical real-time PCR literature. Polymerase chain reaction amplification efficiencies for bovine, human and swine mtDNA were 95, 92, and 100%, respectively. Amplification efficiencies for bovine, human, and swine mtDNA when assayed in combination were slightly less robust but still acceptable: 93, 107, and 92% respectively. Linear regression correlation coefficients (r^2) were 0.99 for all standard curves except for human mtDNA in combination ($r^2 = 0.95$).

3.2. Specificity of the PCR Assay. Mitochondrial clones created for standard curves of human, swine, and bovine mitochondrial ND5 (NCBI accession numbers DQ926980-DQ926982, respectively) exhibited 100% sequence identity to their species of origin when subjected to NCBI BLAST analysis (data not shown). Multiplex PCR amplicons (191−196 bp) were found to have 100% identity to their designated species when subjected to NCBI BLAST analysis (data not shown). Detection of mitochondrial DNA was species-specific in fecal samples and effluents above 10^2 copies (See section 3.3).

3.3. Detection of mtDNA in Effluents. The freeze/thaw method was used to extract DNA from eukaryotic effluents. Multiplex real-time PCR using three species-specific primer and probe sets (bovine, human, and swine mtDNA) was then used to detect mtDNA in the effluent samples. Total DNA concentrations were determined by NanoDrop spectrophotometer and ranged from 23 to 34 ng/μL in clear human effluents to 2686 and 3177 ng/μL in dairy and swine effluents containing large amounts of suspended solids (Table 2). Effluents with low DNA concentrations were concentrated ~2.5-fold by centrifugation if PCR signal was not obtained for non-concentrated samples. Effluents with high DNA concentrations were diluted 1:10 or 1:100 to eliminate possible PCR inhibitors including nontarget DNA. The quality of DNA for all crude effluent preparations was low as indicated by 260/280 ratios of 0.9−1.5 (data not shown). Low ratios could affect the precision of the DNA quantification. However, the purpose of quantifying DNA in crude preparations was to estimate concentrations for sample loading in real-time PCR. Concentrations of crude DNA between 5 and 100 ng/25 μL reaction worked well in real-time PCR. Similar concentrations were used with extracted fecal DNA of higher quality.

The primer and probe sets developed for multiplex amplification of bovine, human, and swine mtDNA (ND5) exhibited no cross-reactions among the effluents from three species of interest (Table 2). Also, no cross-reactions were observed with effluents of other vertebrates: sheep, goat, horse, dog, cat, Canada goose, broiler, layer, turkey, and tilapia. Microbial- and UV-treated WWTP discharges gave no human mtDNA signal as expected (Table 2).

Performed as a blind test, the PCR operator was able to correctly identify all but two of the effluent challenge samples (10/12 or 83% correct) with no false positives (22/22 or 100% correct) in the effluent challenge samples including 10 other vertebrate samples (Table 2). Two human effluent sources, WWTP influent III and WWTP lift station I, gave no human mtDNA signal even after concentration by centrifugation.

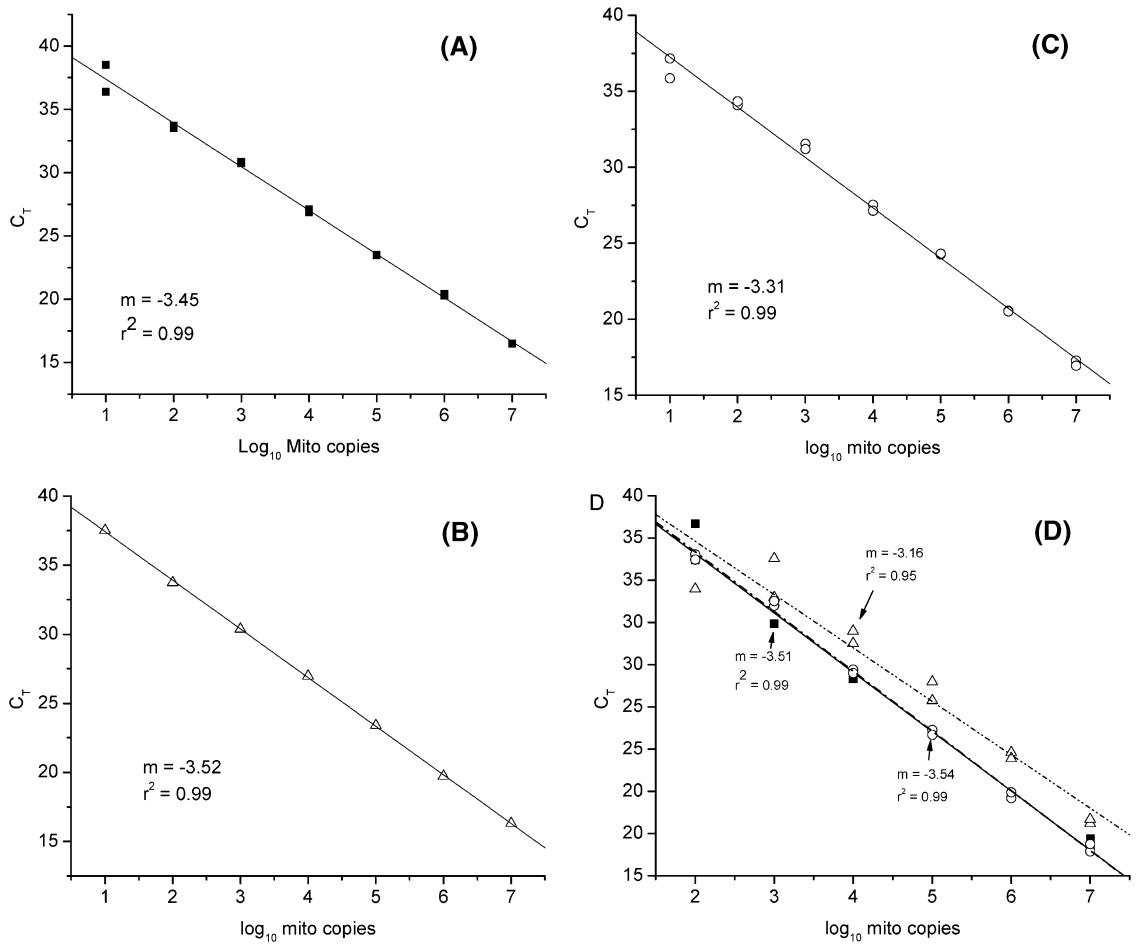

FIGURE 1. Standard curves for the real-time multiplexed PCR assay. The linear range of the assay was determined using bovine mtDNA (A, ■), human mtDNA (B,△), swine mtDNA (C, ○), and bovine, human and swine mtDNA together (D). In all cases, the PCR reaction contained primers and probes for all three. The threshold values (C_T) were plotted against the corresponding mitochondrial copy numbers, and the slope (m) and goodness-of-fit of the linear regression correlation coefficient (r^2) were determined. Mitochondrial copies of DNA from bovine, human, and swine were diluted 10-fold in RT-PCR water (Ambion; Austin, TX) to construct the standard curves. The PCR amplification efficiencies were 95, 92, and 100% for bovine (A), human (B), and swine (C), respectively. PCR amplification efficiencies for bovine, human, and swine assayed together (D) were 93, 107, and 92% respectively. PCR efficiencies were calculated by the formula: $E = (10^{(-1/\text{slope})}) - 1$.

Internal amplification controls (IAC) in similar aliquots of these false-negative human samples gave expected Ct values, so PCR inhibitors were not a factor. However, a recent addition to the crude DNA protocol using heat treatment of thawed effluent (99 °C with shaking at 300 rpm for 5 min) resulted in 6/6 true positives, or 100% correct identification of human influent (data not shown). Therefore, further modifications to DNA extraction and concentration methods are indicated.

Two mixtures (Mix I and II) using equal volumes of bovine, human, and swine effluents were created in the lab (Table 2). When assayed by multiplex real-time PCR, only bovine mtDNA signal was detected. The bovine primer/probe set might have a higher binding affinity than the human and swine sets. To test this, separate real-time PCR assays (Table 3) were conducted with two new mixtures using equal volumes of bovine, human, and swine effluents (Mix III and IV). In these effluent mixes, human influent was concentrated ~2.5-fold by centrifugation prior to combination with equal volumes of bovine and swine effluents. Both multiplex and "singleplex" real-time PCR were performed with these mixtures. When assayed singly, all three species were detected. However, the swine effluent was not detected in Mix III. When run in multiplex PCR, human concentrated effluent was detected, bovine effluent was detected only in Mix III, and swine effluent was not detected in either mixture.

TABLE 3. Single and Multiplex Real-time PCR of Combined Effluents[a]

	copies of mtDNA/PCR reaction					
	single qPCR			multiplex qPCR		
sample	human	bovine	swine	human	bovine	swine
Mix III	5.5×10^2	3.3×10^3		9.7×10^1	1.7×10^3	
Mix IV	3.2×10^2	5.7×10^3	3.9×10^3	1.7×10^2		

[a] Detection of mtDNA in crude preparations of mixed eukaryotic effluents. DNA was extracted using freeze/thaw method and assayed using either single primer/probe set or multiplex real-time PCR using three species-specific primer and probe sets (human, bovine, and swine). Effluents from human, bovine, and swine sources were combined in equal amounts. Prior to mixing, human effluent was concentrated ~2.5× by centrifugation (Section 2.2). All numbers corrected by dilution and concentration factors.

Since human mtDNA was detected singly and in multiplex PCR after concentration by centrifugation, total concentration of target DNA seems to be the major factor in producing a real-time PCR signal. Based on PCR amplification efficiencies (Figure 1), binding affinities for all three primer/probe sets should not be dissimilar. But when tested on environmental samples, the swine primer/probe set appears to be the least robust of the three sets. Since the multiplex assay had a tendency to detect only the species of highest mtDNA

TABLE 4. Multiplex Real-Time PCR for Human Feces[a]

	copies mtDNA/g feces		
sample	human ($\times 10^6$)	bovine ($\times 10^4$)	swine
1	8.7		*b*
2	13.0		
3	3.7	*b*	*b*
4	2.1		
6	1.2	1.9, *b*	*b*
8	24.0		
9	20.0		*b*
10	13.0		
11	27.0		
14	0.28		*b*
15	7.0	27.0, *b*	*b*
16	10.0	*b*	
17	13.0		
18	15.0		*b*
22	4.4		*b*
24	11.0		

[a] Detection of mtDNA in feces of 16 human volunteers. DNA was extracted using the Qiagen DNA stool kit and assayed by multiplex real-time PCR using species-specific primers and probes. [b] Meat consumed by the volunteers in the 24 h preceding stool collection.

concentration, better detection of all three species could be accomplished by running each real-time PCR primer/probe set singly. Also, it should be noted that duplicate replicates are not always sufficient for real-time PCR detection of mtDNA, multiplex or singly. Replicate numbers should be determined empirically by each laboratory utilizing this protocol based on predetermined confidence intervals, risk assessments, and cost.

3.4. Human Feces Carry-Over. Sixteen human volunteers provided fresh feces samples and returned a questionnaire citing their meals taken for the 48 h prior to sampling. After DNA was extracted using the Qiagen DNA stool kit and assayed by mitochondrial multiplex real-time PCR, there were two instances of carry-over mtDNA signal from meat consumed (Table 4). The mtDNA signals collected corresponded to only the beef products consumed within the last 24 h and were 2×10^4 and 30×10^4 copies mtDNA per gram of feces. The human mtDNA signals for the same samples were $1 \times 10^6$and 7×10^6 per gram of feces, respectively. Thus the human mtDNA signal from human feces was 1−2 orders of magnitude greater than the consumed beef. Seven other volunteers consumed beef and pork within 24 h of sampling with no carry over effect. Human feces were calculated to contain $\sim 1.1 \times 10^7$ copies human mtDNA/g feces. Another report using Trypan blue and a hemocytometer to count both viable and nonviable cells cited 1.5×10^6 human colonic epithelial cells/g stool (*39*). Our larger figure may be due to multiple copies of mitochondria per cell. Carry-over mtDNA signal from consumed meat was 1−2 orders of magnitude lower than human mtDNA signal for beef only. There was no mtDNA carry-over signal for pork. It is obvious that carry-over signal from consumed meats by humans must be considered when analyzing triplex mitochondrial real-time PCR results.

4. Discussion

Current source tracking methods rely on indicator organisms, expensive chemical assays, or molecular methods requiring extensive data libraries to differentiate among sources of fecal contamination. Amplification of eukaryotic mitochondrial DNA identifies the fecal source directly and can be used in conjunction with other methods to characterize contaminants. Mitochondrial DNA has many copies per cell (*33*−*36*) and can be amplified in a robust manner, much like 16S rDNA. Real-time PCR is quantifiable and can detect mtDNA levels more rapidly than conventional PCR, as it does not require nested PCR runs or agarose gels. This report is the first to utilize real-time PCR to quantify and differentiate among three common fecal contamination sources: human, cow, and pig.

Our triplex mitochondrial real-time PCR assay combined with crude DNA preparations has proven to be a quick and potentially powerful source-tracking assay. However, the limits of this assay must be tested empirically. Very low levels of signal that are potential false positives must be examined with care. Threshold levels and annealing temperatures for each species were optimized to eliminate low-level noise caused by nonspecific fluorescent signal, nonspecific DNA binding due to high levels of nontarget DNA. Using validated standard curves; the multiplex real-time assay has a detection limit of 10^2 mitochondrial copies per reaction (5 μL sample/reaction) for all three species. Water monitoring parameters are usually stated per 100 mL sample. Therefore, our real-time PCR detection limit translates into 2.0×10^6 mitochondrial copies per 100 mL effluent or surface water. Using data from our human fecal carry-over studies ($\sim 1.1 \times 10^7$ mtDNA copies per g human feces), this sensitivity can be described as a detection limit of 0.2 g of human feces per 100 mL effluent or surface water. Because pigs are omnivorous like humans, they would be expected to have similar sensitivity per gram feces. However, cows may require a larger volume of feces for detection due to the high percentage of roughage in their feces. Further study is warranted to determine lower limits on environmental surface water samples as compared to effluents.

Crude freeze/thaw DNA preparations were inexpensive, rapid, and produced no false positives or cross-reactions in real-time PCR. However, the real-time signal obtained from effluents was limited to the bottom third of the standard curve. Due to high nontarget DNA concentration from bacteria, many effluents were diluted to prevent inhibition of the PCR reaction. Extraction of DNA from effluents using QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA) was found to reduce the real-time PCR signal significantly and greatly impaired the specificity of the real-time PCR assay when compared to crude freeze/thaw extracts (data not shown). It is possible that degradation of DNA during kit extraction and clean up created false positives through chimerical amplification of short fragments.

Small sample size is a major disadvantage of all PCR methods, conventional and real-time. Our triplex real-time PCR assay used a 5 μL sample per reaction in duplicate with an IAC added to a third sample aliquot. Despite this small sample size, we reported 83% correct positive samples, including one sample exhibiting mtDNA signal below the assay detection level of 10^2. Empirical testing will determine the number of replicates needed to sufficiently quantitate eukaryotic mtDNA in effluents and reduce risks associated with fecal contamination at a reasonable cost.

We reported no false positives or cross-reactions with other similar or dissimilar species. High specificity is attributed to optimized annealing temperature and thermal cycler settings and the design of mismatch amplification mutation assay (MAMA) primers (*37*) that utilize primer penultimate base pair mismatches to decrease the likelihood of nontarget amplification. When run as a multiplex assay on effluent mixtures, we were able to identify and quantitate two out of three contaminants in one case. However, one contaminant usually dominated the multiplex assay and masked the other two. Therefore, we will consider the multiplex assay a screening procedure. Once fecal contamination is detected from one member of the triplex assay, the detection levels of other species can be improved by running each primer/probe set singly.

Other limitations to the assay include possible PCR inhibitors present in environmental samples. These limitations can be overcome through inclusion of internal amplification controls (IAC) to monitor PCR inhibitors (10^3 human mt copies). Most PCR inhibitors were eliminated by 1:100 dilution of the sample in RT-PCR water. Poultry manure, especially fresh litter with high ammonia concentrations, had the greatest inhibition of PCR. Even after dilution, certain poultry samples gave only 10% of expected IAC signal. High (nontarget) DNA concentration in effluents (>100 ng/ul) also inhibited PCR reactions. Again, dilution at 1:10 or 1:100 was successful. However, most DNA in effluent is microbial and efforts are now underway in our laboratory to concentrate the mitochondrial DNA targets.

Most humans are omnivorous. Could consumed animal products still contain nondegraded DNA in human feces, and could we pick up this carry-over mtDNA signal? Martellini and coworkers (*31*) could not detect ovine, porcine, or bovine PCR signal from the feces of one human volunteer who had eaten these meat products the previous day. However, we discovered carry-over signal for beef in two out of four of our human volunteers (Table 4) who consumed beef within 24 h of sampling. There was no carry-over signal for pork. The human signal was 1−2 orders of magnitude greater than the consumed beef signal. This carry-over effect should be considered when analyzing triplex mitochondrial real-time PCR data. When human signal is detected in combination with bovine or porcine signal it can reveal a mix of all three, human contamination alone, or some combination of human and livestock contamination. The order of magnitude of each of the three contaminates should be considered to determine the source. Presently, we are studying municipal raw sewage to determine its mtDNA signature. Furthermore, real-time PCR could conceivably detect mtDNA from non-fecal sources such as skin and sputum of swimmers; waste from kitchen garbage disposals and abattoir, or industrial manufacturing wastewaters (i.e., plastics which use stearic acids derived from cattle).

Fecal contamination of surface waters is a public health, environmental, and economic resource problem. Guidelines for water quality monitoring have traditionally focused on the use of bacterial indicators. However, efforts to effectively mitigate fecal contamination necessitate greater clarity in source recognition. When used in combination with other molecular, microbial, and chemical assays, mitochondrial real-time PCR points to the eukaryotic contaminator directly. Indeed, it represents a paradigm shift in source detection. Ongoing research to detect fecal waste sources includes development of new dual-labeled probes and primers for other species of livestock, domestic pets, Canada geese, and other wildlife. We are testing local surface waters for eukaryotic mtDNA to determine limits of these assays and meaningful baseline levels through comparison with other source tracking methods.

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