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Evaluating the Utility of Enterococcus Specific Primers

KEY WORDS

fecal indicator bacteria

microbial source tracking

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ABSTRACT

Enterococci are the preferred fecal indicator bacteria (FIB) for monitoring the safety of recreational beaches. A reliable and cost-effective method to identify the species of origin for enterococci-contaminated rivers is essential for decreasing the risk to human health. In this study human and canine fecal samples were analyzed in polymerase chain reaction (PCR) studies with primers reported to amplify targets specific to enterococcal species with the goal of identifying the fecal source. While the primers successfully amplified the target sequences in many samples, amplification in non-target species made identifying one, or a small set of primers, that reliably discriminate between fecal source species more challenging. Alignment and comparison of PCR product sequences were conducted with the goal of designing novel primers with increased specificity. Analysis of multi-locus sequence typing (MLST) data suggested that specific nucleotide variations within loci found in species-specific enterococcal strains might be exploited to determine the source of contamination in local waterways. To this end, primers for two target loci were designed specifically for nucleotide sequences more frequently isolated from canine enterococcal samples and initial screening assays were conducted to optimize conditions and discriminate between source DNA without success. Collection of additional species-specific bacterial samples and additional control type strains are needed to better distinguish between the species of interest in this study.

INTRODUCTION

Contamination of recreational waters with bacteria from fecal contamination poses a significant health risk to humans (Cabelli et al. 1979). Increasing water temperatures driven by climate change, increased incidence and severity of rain events bringing more runoff, larger impervious surfaces resulting in less absorption of runoff before entering waterways, and the aging sewer infrastructure, all contribute to more frequent occurrences of beach closures due to high bacterial concentrations (Rose et al. 2001). To develop programs that decrease contamination and to better understand the risk to humans, it is essential to not only quantify the bacterial load in water, but to identify the relative contribution from different contributing species.

Enterococcus sp. are prevalent in bird, mammal and to some extent, insect and reptile fecal material, and comprise approximately 1% of the bacteria in the human large intestine (Dubin and Pamer, 2014). Other species contain a similarly complex and varied array of bacteria (Layton et al. 2010; Harwood et al. 2014).

The correlation between levels of fecal bacteria and illness in humans has long been recognized, and the EPA has identified enterococci as fecal indicator bacteria (FIB), the measurement of which are used to determine the safety of recreational swimming beaches and seafood harvesting waters (Cabelli et al. 1979; US EPA, 2012). High levels in recreational waters can result in beach closures and halt fish and oyster harvests. The standard method for tracking FIB levels utilizes selective media and direct colony counting (US EPA, 2009). Monitoring for all possible pathogens that may be in contaminated water is an impossibility, so the use of FIB has made it possible to track a common set of organisms, compare many locations and set thresholds for safety (Leclerc et al. 2001).

Microbial source tracking (MST) has previously been used to identify the source of enterococci and other bacteria associated

with fecal contamination found in environmental waters (Leclerc et al. 2004) and has been used to identify the source in bacterial infection outbreaks from sources including food and water (McRobb et al 2015). Identification of the contamination source is necessary for developing plans to eliminate the source, such as repairing leaks, upgrading septic systems and educating the public on pet waste clean-up. MST methods, such as restriction analysis, quantitative polymerase chain reaction (qPCR), and DNA sequencing of one or several loci, have been used with varying success (Foley et al. 2009; Homan et al. 2002; Ruiz-Garbajosa, 2006). Polymerase chain reaction (PCR) potentially provides an inexpensive way to identify the source of fecal contamination. Many target organisms have been proposed for PCR-based MST (Harwood et al. 2014). However, methods that target species other than *Enterococcus* require processing of the sample without initially quantifying the level of contamination, adding cost and wasted effort. A method that first screens for enterococcal contamination followed by MST, would be more efficient. To this end a project was initiated to identify or design primer sets that discriminate between fecal source species responsible for *Enterococcus* contamination.

METHODS

Fecal sample collection

Canine fecal samples were obtained from local veterinarians (D samples) and dog owners (S samples). Each D sample contained fecal matter combined from 4 to 10 dogs (n=17). Individual human samples were obtained from anonymous volunteers (n=3; P002, P003, P004), and sewage samples were provided by several Anne Arundel County Water Reclamation Facilities (WRF) (n=8). *Enterococcus faecalis* NCTC 775, a positive control for *Enterococcus faecalis*-specific primers, was obtained from Biomerieux. *Enterococcus* faecium 700221, a positive control for *E. faecium*-specific primers, was obtained from American Type Culture Collection. Environmental

samples were collected from local waterways that contained high concentrations of enterococci (over 1000 bacteria/100 mL, approximately 10 times above the acceptable threshold).

Enterococcus isolation and genomic DNA isolation

Approximately 1 mL of liquid WRF influent or 0.1 mg of fecal matter suspended in sterile water and passed through a sterile 0.45-micron filter. Filters were placed on mE agar (Difco) selecting for *Enterococcus sp*. After incubation at 41 degrees Celsius for 24 hours, colonies with a blue halo were scraped, combined and suspended in sterile water. The Amresco Cyclo-Prep Genomic DNA Isolation kit was used for all DNA extractions (Avantor).

Primer selection and Polymerase Chain Reaction conditions

The primers chosen, their reported specificity and references are shown (Table 1). Primers were purchased from Integrated DNA Technologies (Coralville, USA).

TABLE 1

Primer target and specificity.

Primer Set	target	Reported Specificity	reference
Ent 376	16s rRNA	Enterococcus species	Ryu et al.2012
Ent	16s rRNA	*Enterococcus faecalis*	Ryu et al.2012
Cium	16s rRNA	*Enterococcus faecium*	Ryu et al. 2012
IS16	Insertion Sequence 16	nosocomial human *Enterococcus faecium*	Werner at al. 2011
esp	Enterococci Surface Protein gene	human *Enterococcus faecium*	Ahmed at al. 2008
psts11	*psts* gene fragment	canine *Enterococcus faecium*	This study
atpa15	*atpa* gene fragment	canine *Enterococcus faecium*	This study

Amplification reactions included 1 unit of Taq polymerase (New England Biolabs), 1X buffer, 300 nM dNTPs, 1.5 mM MgCl2, 1mM forward primer, 1mM reverse primer, 2 µl of the

template in a final volume of 50 µl. Samples were placed in a thermocycler and run for 30 cycles. Each cycle incubated samples for 60 sec at 94°C, 60 sec at an annealing temperature specific for a given primer set (Table 2), and 60 sec at 74°C.

TABLE 2

Primer sequences, conditions and predicted product size.

Primer set name	Annealing temp (°C)	Product Length (bp)	Forward Primer	Reverse Primer
esp	51	680	TAT GAA AGC AAC AGC ACA AGT T	ACG TCG AAA GTT CGA TTT CC
IS16	56.3	547	CAT GTT CCA CGA ACC AGA G	TCA AAA AGT GGG CTT GGC
ENT	56.3	229	TGC ATT AGC TAG TTG GTG	AGT TAC TAA CGT CCT TGT TC
ENT376	61	220	GGA CGM AAG TCT GAC CGA	TTA AGA AAC CGC CTG CGC
CIUM	57	512	TGC TCC ACC GGA AAA AGA	TTA AGA AAC CGC CTG CGC
psts11	56	181	CAA AGA TAC AGG TGT CAA AGA TAT CAC A	TAT AGG TGT GGC ACC ATC TAA
atpa15	56	248	GCC AAT CGG ACG CGG A	ATG GTG CGA TAT AAA GTA ATG GT

Analysis of PCR products

Aliquots of reactions were analyzed on 1.5% agarose alongside a 100 base pair standard (Amresco EZ-vision) and stained with ethidium bromide to estimate amplification product size. Samples that resulted in amplification of a product of the expected size were classified as positives. A sample agarose gel in Figure 1 highlights the expected product sizes. Samples that did not result in amplification, therefore no band on the agarose gel, were classified as negatives, and those with multiple bands were placed into a separate group. Once samples were verified they were sent out to Genewiz for sequencing to further verify that target sequences were amplified and to compare sequences.

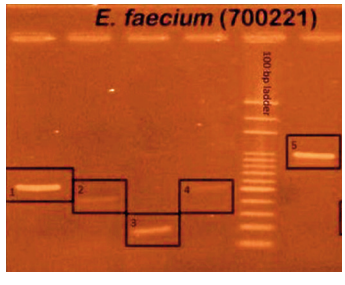

FIGURE 1

Agarose gel illustrating expected amplification product sizes. Each reaction contained Enterococcus faecium strain 700221 genomic template with a different primer set: 1-Esp (680 bp); 2-CIUM (512 bp); 3-ENT376 (220 bp); 4-IS16 (547 bp); 5-VanA (1029bp).

Analysis of MLST

MLST is a technique introduced in the early 1990s utilizing a limited number of short sequences from several loci within bacterial genomes capable of assigning a sample to a specific strain (Maiden et al. 1998). Sequence data from the Public Database for Molecular Typing and Microbial Genomic Diversity (pubMLST) suggested that specific nucleotide variations within loci found in all enterococcal strains might be exploited to determine the source of enterococci in contaminated local waterways. The MLST strain typing method typically employs sequence comparisons at seven loci to assign a sample to a specific strain. In database searches of sequences from many sources, it was found that the sequences of two loci, *psts* and atpa, were aligned and compared, and primers were designed specifically for nucleotide sequences more frequently isolated from canine enterococcal samples. Sequences in the database were aligned to look for individual nucleotide differences prevalent between loci amplified from bacteria from different host species. The analysis was performed in Ugene (Okonechnikov et al. 2012).

RESULTS

PCR results

Amplification results from assays performed to evaluate species selectivity of primer sets were promising. Type strain controls, *E. faecalis* NCTC775 and *E. faecium* 700221, performed as expected with each primer set (Table 3). NCTC775 is a non-virulent strain that does not contain the *esp* gene, while *E. faecium* 700221 is known to contain both the *esp* and *IS16* locus. The number of bacterial DNA samples from individuals was very small in this study (n=3), and none of the samples obtained were from clinical settings. Two of the three samples showed amplification with ENT, ENT376 and CIUM primers, as would be expected.

Sewage samples collected from waste reclamation facilities

around Anne Arundel County contain fecal matter from large populations, so reflect the complexity of bacterial populations in humans. One sewage sample, PAT, was negative for amplification by the ENT376 primer set, although those primers are the most inclusive, reported to amplify sequences from a variety of *Enterococcus* species. The sequence targets associated with potentially more virulent enterococcal species, *esp* and *IS16*, were found in 57% and 86% of sewage samples, respectively (Table 3).

TABLE 3

Summary of PCR Results.

Source Species	Sample Name	Primer set Name				
		esp	*IS16*	ENT	ENT 376	CIUM
E. faecalis	Type strain NCTC775					
E. faecium	Type strain 700221					
Sewage	SP1, SP2, MAYO					
Sewage	BROAD, COX					
Sewage	PAT					
Sewage	COX2					
Human	P002, P003					
Human	P004					
Dog	D1, D2					
Dog	D3					
Dog	D4					
Dog	S1					
Dog	S3					
Dog	S4					
Dog	S5, S8, S15, S16					
Dog	S6, S14					
Dog	S9, S13, S17					
Dog	S12					
Environmental	CG					
Environmental	SS, EGO					
Environmental	BH					
Environmental	HW					
Environmental	HC					
Environmental	CG 2					
KEY	AMPLIFICATION OF TARGET		NO AMPLIFICATION OF TARGET		AMPLIFICATION & extra bands	

The amplification results with bacterial DNA from dog fecal samples, representing over 35 individual dogs, were encouraging

for several reasons. All of the samples from dog fecal material successfully amplified the ENT376 target, confirming the reports that ENT376 is the least selective of the primer sets used. Over 88% of the samples from dogs were positive for the *E. faecium* target (CIUM primer set), while only 69% were positive for *E. faecalis* (ENT primer set). Of note, only 13% of the samples obtained from dog feces were positive for the presence of the *esp* gene, and 29% were positive for *IS16*.

Environmental samples collected from area waterways on days associated with high concentrations of *Enterococcus sp.* were analyzed and compared to look for patterns that might suggest the species responsible for the contamination. Six of the seven samples were positive for amplification with ENT376, suggesting the presence of at least one species of *Enterococcus*. The two CG samples, CG and CG2, were collected on different days. Both were positive for ENT376, while CG was positive for ENT, suggesting the presence of *Enterococcus* faecalis, and CG2 was positive for CIUM, suggesting the presence of E. faecium. None of the environmental samples were positive for amplification of *esp*, and only two, SS and EGO were positive for *IS16*.

Sequence Analysis

To further analyze and compare DNA targets that were amplified, PCR products from a sampling of reactions were sequenced, and compared to sequences with the National Library of Medicine's National Center of Bioinformatics (NLM NCBI) database to confirm that the correct targets were amplified (Table 4). In each case the expected product was amplified with near 100% identity to predicted sequences (Table 2), with one caveat. The *IS16* primer set was designed to recognize human pathogenic, clinical E. faecium strains, but bacterial DNA template from both dog and human fecal samples resulted in amplification of identical products with the highest similarity to a human isolate, with a very

close second match to a dog isolate.

Sample	Primer Set	Product length (bp)	Top BLAST hit/s	% identity	Correct target gene?	Correct target species?
CG	ENT	186	*E. faecalis* strain AaR12 16S ribosomal RNA gene (soil)	100	yes	yes
p004	ENT376	178	*E. faecium* strain g4 16S ribosomal RNA (fish)	100	yes	yes
Dog1 SP2 (identical)	*IS16*	489	1-*E. faecium* strain NMVRE-001 plasmid p1 (human clinical isolate) 2- *E. faecium* strain V13-21-E11-012-001 plasmid pK21EFM001(canine)	99	yes	yes
SP1	*esp*	616	*E. faecium* strain VVEswe-R (human clinical sample)	100	yes	yes

TABLE 4

Sample PCR products sequenced.

Sequence data from a subset of amplification products were aligned and compared to each other. Of the 11 amplification products analyzed, only S1 and S15 products with ENT primers contained nucleotide variations. More variation was observed when comparing the sequences from amplification with the ENT376 primer set. Of 15 samples that were sequenced 5 of them contained at least one nucleotide difference. Only two samples from amplifications with *ESP* primers were sequenced and the sequences were identical. Both of these samples were from sewage effluent, MAYO and SP1. From the *IS16* primer set there were 4 samples sequenced. These had variations in at least two of the four samples, but because of low quality sequence data confidence in the variations was also low.

MLST database alignments and primer design

Alignment of a portion of the E. faecium *psts* locus revealed that of the 105 *psts* alleles in the pubMLST, alleles 11 and 7 were most frequently associated with bacterial DNA from canine sources, while allele 1 was more often associated with bacterial DNA

from human sources (Jolley et al. 2018). Nucleotide differences were used to design primers able to specifically amplify DNA from *psts* allele 11 (Figure 2 and Table 2). One such primer is indicated with yellow highlighting. Initial assays involved varying PCR conditions, specifically using different annealing temperatures that would affect the stability of primer binding. Higher annealing temperatures require a perfect match between primer and target and lower temperatures, potentially allow binding and amplification even if there are mismatches between the primer and the target. In amplification reactions comparing templates from sewage samples, SP1 and Mayo, and dog samples, D3 and S5, an annealing temperature that was able to differentiate between sources, therefore allowing amplification from templates of one species but not the other, was not found (data not shown).

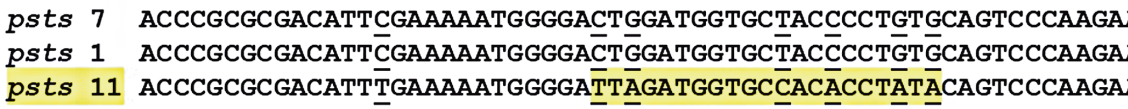

FIGURE 2

Comparison of psts *allele sequences from the pubMLST E. faecium database. Sequence differences are underlined and the sequence chosen for a potential species-specific primer is highlighted.*

In much the same way that the *psts* locus was analyzed, multiple *atpa* sequences from the pubmlst *E. faecalis* database were aligned to identify alleles frequently associated with canine sources. MLST allele 15 was more often associated with bacteria obtained from dogs than humans. Therefore, primers were designed that would target only allele 15. In PCR reactions containing the *atpa*-specific primers and DNA template from sewage samples, SP1 and Mayo, and canine samples, D3 and S5, varying annealing temperatures either resulted in successful amplification in all reactions or no amplification in all reactions. Similar to the results

observed in the *psts* assays, nucleotide differences were either not present in the template or not significant enough to cause temperature-dependent differential annealing of primers at the target sites (data not shown).

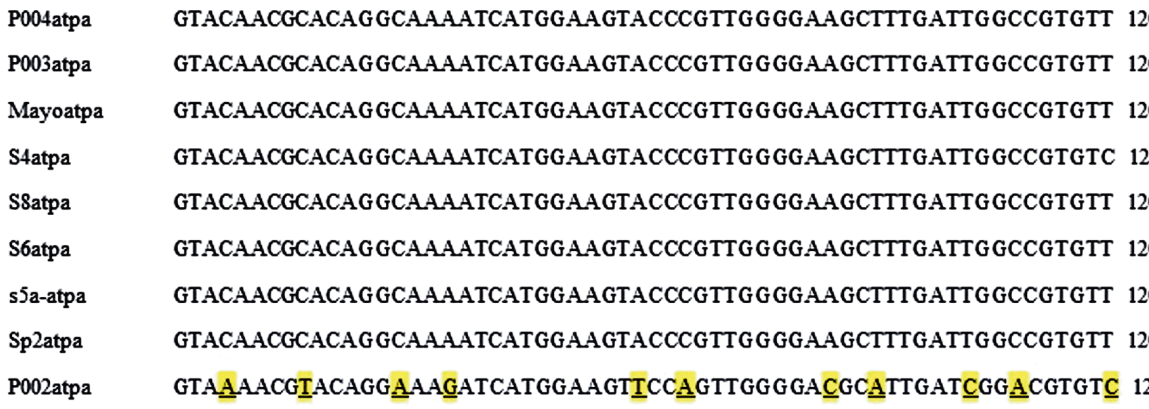

FIGURE 3

Sample of the atpa sequence alignment of E. faecium amplified using the atpal15 primer set and sequenced by Genwize. Nucleotide variations are underlined and highlighted.

Sequencing of a subset of amplification products revealed that only the product from bacterial sample P002 with primer set atpa15 contained a nucleotide difference (Figure 3). Interestingly P002 was also the only one of the 7 sequences amplified with the psts11 primer set that contained nucleotide differences (data not shown).

DISCUSSION

Determining bacterial concentration in a water sample, important to determining safety for recreational use, does not provide information on the source of contamination. Consequently, considerable effort has been made developing MST methods (Meays et al. 2004). *Enterococcus sp.* have emerged as the recommended FIB for both fresh and brackish waters, making them a convenient target for this study since samples identified as having high

FIB concentration can be targeted for MST without the need for collection of an additional sample, without the need for collection of a larger sample, and without the wasted effort of processing a sample that is later found to lack contamination.

Starting with primers previously reported to have specificity for one, or a subset of FIB species (Table 1), studies were undertaken to assess the feasibility of similar studies with samples from local sources including *Enterococcus* bacterial DNA from human, canine, sewage treatment facilities, and local rivers. The ENT and ENT376 primer sets target the 16s rRNA gene in *Enterococcus* faecalis and multiple *Enterococcus* species, respectively. The CIUM primer set is specific for the 16s rRNA gene in *Enterococcus* faecium, while the *esp* and *IS16* primer sets target sequences originally associated with virulence genes in virulent strains of E. faecium, but also present in some *E. faecalis* strains. In addition, reports utilizing *esp* and *IS16* primers relied on the association of their targets with bacterial samples from human clinical settings, both of which have been linked to vancomycin resistance (Werner et al. 2011; Willems et al. 2001).

Looking more closely at the amplification results in Table 3, the control type strain *E. faecalis* NCTC 775 illustrated the expected pattern of primer specificity, positive for amplification by primers specific for *Enterococcus* faecalis and multiple *Enterococcus* species, ENT and ENT376, respectively, and lacking amplification of the *esp* and *IS16* targets, associated with bacteria from clinical human samples (Mohamed et al. 2018; Scott et al. 2005; Werner et al. 2011). E. faecium 700221 genomic template resulted in amplification of the CIUM target, specific for E. faecium, and the virulence specific *esp* and *IS16* targets as expected (Table 3 and Figure 1).

The low number of individual human fecal samples (n=3) in this study complicates statistical analysis of the results. While the sewage effluent (n=8) provided a larger human population, the

material entering treatment plants does not only contain human fecal matter. Sewage influent potentially contains animal feces and chemicals that may remove some bacterial species of study. To be confident in correlations between primer specificity and human fecal sources, future studies will require additional human samples from both community and clinical settings.

Although the *ESP* and *IS16* primer sets were not able to distinguish between human and canine fecal sources with 100% selectivity, this finding is not entirely surprising. First, work by Ahmed (2008) evaluating sensitivity of the *ESP* primer set, demonstrated that about 91% of sewage and septic samples were *esp* positive with sensitivity between 67% and 100% depending on the type of sample. The findings in this study showed *esp*-positive results in 60% of human and sewage samples tested, a value not significantly lower than earlier results. Second, a recent study reported that 29% of *Enterococcus* from canine fecal samples were *esp*-positive (Stępień-Pyśniak et al. 2021). In this study 13% of canine samples were *esp*-positive. These findings suggest that *esp*-carrying *Enterococcus* strains are moving from human clinical settings to human and animal populations outside of clinical settings, which will adversely affect the success of using *esp* as a species-selective target. An increase in genetic similarities in the bacteria found in humans and pet hosts will continue to rise as we live in close proximity to each other (Song et al. 2013). Results with *IS16* were similar. In this study 60% of bacterial samples from human and sewage samples were positive for *IS16*, and 29% of samples from dogs were positive for *IS16*. While a study by Werner reported 100% sensitivity in over 100 samples obtained from humans in a clinical setting, less than 5% of samples collected outside of hospitals were positive for *IS16* (Werner et al. 2011). In another study evaluating a transposon related to the *IS16* sequence in samples from dogs, researchers proposed exchange between humans and dogs to explain canine samples positive for the transposon (Simjee

et al. 2002).

As reported in several other studies, some samples collected from canine and human fecal samples for this study demonstrated similar amplification patterns when using primers that were designed to discriminate between species (Song et al. 2013, Stępień-Pyśniak et al. 2021). These findings may make some molecular MST methods invalid in the coming years. Consequently, additional nucleotide differences need to be identified, using alignments such as those performed in this study with *atpa* and *psts* alleles (Figures 2 and 3). Future studies will include larger sample sizes to better analyze the specificity of the atpa15 and psts11 primer sets and a wider range of annealing temperatures to identify allele-specific amplification conditions. Other methods such as exploiting known single nucleotide polymorphisms (SNPs), which are changes in a single nucleotide, will be explored. Building from a study using known SNPs (Rathnayake et al. 2011), a series of primers could be designed to recognize SNPs specific to *Enterococcus* from a single species.

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