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Potential Use of a Host Associated Molecular Marker in *Enterococcus faecium* as an Index of Human Fecal Pollution

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Several genotypic and phenotypic microbial source tracking (MST) methods have been proposed and utilized to differentiate groups of microorganisms, usually indicator organisms, for the purpose of tracking sources of fecal pollution. Targeting of host-specific microorganisms is one of the approaches currently being tested. These methods are useful as they circumvent the need to isolate individual microorganisms and do not require the establishment of reference databases. Several studies have demonstrated that the presence and distribution of *Enterococcus spp.* in feces seems to be influenced by the host species. Here, we present a method for detection of genetic sequences in culturable enterococci capable of identifying human sources of fecal pollution in the environment. The human fecal pollution marker designed in this study targets a putative virulence factor, the enterococcal surface protein (*esp*), in *Enterococcus faecium*. This gene was detected in 97% of sewage and septic samples but was not detected in any livestock waste lagoons or in bird or animal fecal samples. Epidemiological studies in recreational and groundwaters have shown enterococci to be useful indicators of public health risk for gastroenteritis. By identifying the presence of human fecal pollution, and therefore the possible presence of human enteric pathogens, this marker allows for further resolution of the source of this risk.

Introduction

The enterococci have been used as indicators of fecal pollution for many years and have been especially valuable in the marine environment and recreational waters as indicators of potential health risks and swimming-related gastroenteritis (*1*, *2*). Although some associations have been made between high levels of microbial indicators and health risks, the enterococci and traditional indicators of fecal pollution fail to predict specific sources of pollution and are often unreliable indicators of specific human pathogens and associated health risks (*3*).

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Several genotypic and phenotypic methods have been proposed and utilized to differentiate groups of microorganisms, usually indicator organisms, on the basis of the host animal or environment from which they were derived. This methodology has collectively been termed "Microbial Source Tracking" (MST), and the current state of this technology has been recently reviewed (*4*−*6*). By pinpointing specific sources of fecal pollution, MST methods are useful tools for remediation of impaired water systems and in risk assessment.

One drawback of many MST methods is the requirement for the construction of a host-origin database, which contains genotypic or phenotypic "fingerprints" from microorganisms of known sources to which fingerprints from environmental isolates of unknown origin can be compared. This is a tedious task, as recent research has indicated that libraries must be extremely large and contain isolates from a broad geographic region to be considered representative (*7*, *8*). Targeting of host-specific microorganisms circumvents the need to establish a database, and methods have been developed and have been used successfully for tracking specific sources of fecal pollution (*9*−*16*).

Several studies have demonstrated that the presence and distribution of *Enterococcus spp*. in feces seems to be influenced by the host species (*17*, *18*), but the diversity of this genus makes it a challenging target for research directed at identifying bacterial species that may be associated with a particular host animal. Similar to the approach used by Khatib et al. (*13*, *14*) we present a method for using PCR detection of a putative virulence factor, the enterococcal surface protein (*esp*) in *Enterococcus faecium*, as an index of human fecal pollution.

Materials and Methods

Sample Collection. Composite fecal samples were collected from animal wastewater lagoons at poultry (Bushnell, FL), dairy (Hague, FL; Ingham County, MI), and swine (Gainesville, FL; Ionia County, MI) farms. Human fecal samples were collected from septic tanks in Gainesville, FL and the Florida Keys. Domestic raw sewage, secondary sewage effluent, and filtered wastewater were collected from wastewater treatment facilities in Florida, Arizona, and Michigan. Individual scat samples were collected from swine, poultry, dairy cattle, beef cattle, and Canada geese at Michigan State University. Seagull fecal samples were collected in Grand Haven, Michigan and the Florida Keys. Pelican fecal samples were collected in the Florida Keys.

Bacterial Strains. Several species of enterococci were kindly provided by Dr. Valerie J. Harwood at the University of South Florida. All were evaluated for the presence of the *esp* gene and to assess potential cross reactivity of the PCR primers developed in this study. Members of Enterobacteriaceae were also evaluated for cross-reactivity. The species tested were as follows: *Enterococcus faecium* (ATCC# 19434), *Enterococcus faecalis* (ATCC# 19443), *Enterococcus casseliflavus* (ATCC# 700327), *Enterococcus avium* (ATCC# 14025), *Enterococcus gallinarum* (ATCC# 49573), *Enterococcus durans* (ATCC# 6056), *Streptococcus bovis* (ATCC# 15351), *Escherichia coli* (ATCC# 15597 and 13706), and *Klebsiella pneumoniae* (ATCC# 43816). *E. faecium* strain C68, which contains the *esp* gene and was used as a positive control in all PCR reactions, was kindly provided by Dr. Louis B. Rice of the Louis Stokes Cleveland Veterans Affairs Medical Center in Cleveland, Ohio.

Concentration of Enterococci from Water and Fecal Samples. Enterococci were concentrated from water or fecal

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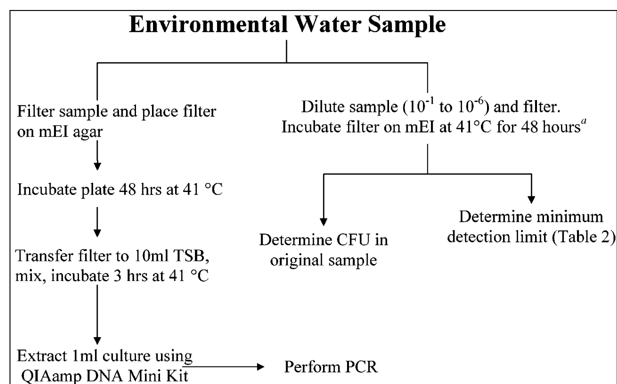

FIGURE 1. Schematic diagram showing processing of environmental samples. [a] Modification of EPA Method 1600.

slurries by membrane filtration. Filters were incubated for 48 h on mEnterococcus (mE) agar (Difco) supplemented with indoxyl substrate (Sigma, Inc.), according to the methodology outlined in USEPA Method 1600 (*19*). For analysis of fecal samples, 1 g was suspended in 10 mL of phosphate buffered saline (PBS), and the resulting slurry was serially diluted and concentrated by membrane filtration as described above. Membrane filtration was used to concentrate organisms and not as a means of isolating individual colonies. PCR reactions were performed on DNA extracts from washed membrane filters. Serial dilutions were used to estimate bacterial densities for purposes of enumeration.

Preparation of Template DNA for PCR Reactions. PCR reactions were performed on composite DNA samples extracted from membrane filters. Filters containing enterococci colonies (from above step) were lifted, suspended in tryptic soy broth (Difco), vortexed vigorously, and incubated for 3 h at 41 °C to wash bacteria from the filters and partially enrich the culture. The resulting bacterial culture contained both target and nontarget organisms, presumably including fungi and various bacterial species. Because of this, the validation of the method included an internal screening of nonspecific organisms for purposes of assessing cross reactivity of the PCR primers. DNA extraction was performed on the resulting culture of bacteria using a QIAamp DNA extraction kit according to manufacturer's instructions (Qiagen, Inc.). Sample preparation, DNA extractions, and PCR were performed according to the flowchart depicted in Figure 1.

PCR Primers and Reaction Conditions. Primers specific for the *esp* gene in *E. faecium* were developed after DNA sequence alignment of the two available variants of the gene (GenBank Accession Numbers AF444000 and AF443999) with the *esp* gene from *E. faecalis* (GenBank Accession Number AF034779). Unique differences were identified between the *E. faecium* and *E. faecalis esp* genes, and PCR primers were developed to specifically amplify only the *E. faecium* variant (Jellyfish Biowire Software). The forward primer designed in this study, which is specific for the *E. faecium esp* gene, is (5′-TAT GAA AGC AAC AGC ACA AGT T-3′). A conserved reverse primer (5′-ACG TCG AAA GTT CGA TTT CC-3′), developed previously by Hammerum and Jensen (*20*), was used for all reactions.

PCR reactions were performed in a 50 μL reaction mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each of the four deoxyribonucleotides, 0.3 μM of each primer, 2.5 U of HotStarTaq DNA polymerase (Qiagen), and 5 μL of template DNA. Amplification was performed with an initial step at 95 °C for 15 min (to activate Taq polymerase), followed by 35 cycles of 94°C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. PCR products were separated on a 1.5% agarose gel stained with GelStar nucleic acid stain (BioWhittaker) and viewed under UV light. The PCR product is 680 base pairs in length.

TABLE 1. Detection of the *esp* Gene Sequence in Composite DNA Samples Extracted from Enterococci Isolated from Domestic Sewage, Birds, and Livestock

host/source	sample type	no. of samples analyzed	PCR results positive	PCR results negative
human/sewage	primary influent	40	40	0
human/sewage	secondary effluent	10	10	0
human/sewage	filter effluent	5	5	0
human	septic tank	10	8	2[a]
poultry	feces/lagoon	6	0	6
swine	feces/lagoon	9	0	9
dairy cattle	feces/lagoon	28	0	28
beef cattle	feces	4	0	4
Canada goose	feces	12	0	12
seagull	feces	28	0	28
pelican	feces	7	0	7
wild birds	feces	8	0	8
total human		65	63[b]	2
total animal		102	0[b]	102

[a] Total *Enterococcus* levels were $<1 \times 10^2$ CFU and showed atypical colony morphology. [b] Statistically significantly different as shown by chi square analysis $p < 0.001$.

The PCR product from the *E. faecium* C68 was purified using a QIAquick PCR Purification Kit (Qiagen, Inc.). The purified PCR product was cloned using the TOPO TA Cloning Kit (Invitrogen). Sequencing of the product was performed at the Genomics Technology Support Facility at Michigan State University using an ABI Prism 3100 Genetic Analyzer to verify the sequence of positive control and specificity of the primers.

Minimum Detection Limit. Five sewage, septic, and marine environmental samples from the Florida Keys suspected of containing sewage contamination were collected. The samples were serially diluted, and 1 mL of each dilution was passed through membrane filters and placed on mEI media as described above (Figure 1). Each filter was enumerated for total enterococci and was prepared for analysis by PCR as described above. Total viable enterococci were then compared to PCR results in order to estimate the colony densities that must be present to ensure detection of the human associated marker.

Persistence of Marker in Environmental Samples. To associate the presence of the *esp* gene and its detection with direct colony counts, autoclaved freshwater and simulated marine (Instant Ocean) water samples were spiked with primary sewage influent containing approximately 10^4 enterococci. Each type of water sample (n=3) was then divided equally (25 mL) into 10 separate 50 mL polypropylene tubes and were incubated in a water bath at 30 °C. At intervals of 0, 3, 5, 7, and 10 days, individual sample tubes were processed and analyzed for total enterococci and the *esp* gene by plate counts and PCR, respectively, as described above.

Statistical Analysis. All statistical analyses (Chi-squared, t-tests) were performed using Pro-Stat statistical software (polysoftware international, Inc.).

Results

Presence of *esp* in Culturable Enterococci from and Human and Animal Fecal Samples. Primers specific for the *E. faecium esp* gene were developed in this study as a marker of human derived fecal pollution. The primers were used to screen composite fecal samples from humans, birds, and various livestock to test for cross-reactivity with sources other than humans. A total of 167 samples containing 10^2-10^5 enterococci colonies per membrane were screened. The gene was detected only in samples from primary sewage influent (n=40), secondary sewage effluent (n=10), filtered wastewater (n=5), and septic tanks (n=10) and not in any enterococci cultured from birds or livestock (n=102) (Table 1). Overall,

TABLE 2. PCR Results (±) for the Naturally Occurring *esp* Gene in Diluted Sewage, Septic, and Environmental Samples

sample type (no. of samples)	no.[a] of *Enterococci* screened[b] (no. of samples positive)				
	$>10^4-<10^5$	$>10^3-<10^4$	100−1000	10−100 ($\mu = 58 \pm 24$)	1−10 ($\mu = 5 \pm 3$)
primary influent (5)	+ (5)	+ (5)	+ (5)	+ (5)	-
septic tank (5)	+ (5)	+ (5)	+ (5)	+ (5)	-
water samples[c] (5)	NA[c]	+ (5)	+ (5)	+ (5)	-

[a] Higher values extrapolated from plates in countable range. [b] CFU/100 mL. [c] Water samples contained ~1.5 × 10^3 CFU/100 mL and were collected from a marine environment (Florida Keys) with a defined sewage input. NA indicates counts were not in this range for any of these samples.

TABLE 3. Persistence of the Naturally Occurring *esp* Gene in Culturable Enterococci in Fresh Water and Simulated Seawater[a]

water type	total culturable Enterococci (CFU, $\mu \pm$ sd)				
	day 0	day 3	day 5	day 7	day 10
freshwater (*n*=3)	1.80 (±0.53) × 10^4 [c]	2.51 (±0.64) × 10^2 [c]	1.22 (±0.30) × 10^2 [c]	1.9 (±0.30) × 10^1 [c]	9.0 (±0.4) × 10^0 [c]
PCR results (±)	+	+	+	-	-
simulated seawater (*n*=3)	1.34 (±0.42) × 10^4 [c]	5.01 (±0.81) × 10^2 [b]	2.65 (±0.46) × 10^2 [b]	1.32 (±0.41) × 10^2 [b]	7.1 (±0.3) × 10^1 [b]
PCR results (±)	+	+	+	+	+

[a] Values in the same column followed by the same letter are not statistically different at $p < 0.05$.

this gene was detected in 97% of sewage and septic samples but was not detected in any livestock waste lagoons or in bird or animal fecal samples. In addition, six reference strains of enterococci along with three strains from other Enterobacteriaceae were screened to test for cross-reactivity. The primers did not amplify any PCR products in any of the reference strains.

Minimum Detection Limit. A membrane filtration/PCR method was developed, and the detection limits of the naturally occurring *esp* gene in five separate sewage, septic, and ambient water samples were determined. As shown in Table 2, on average 58 ± 24 enterococci CFU were necessary to ensure detection of the *esp* gene and to determine if human fecal pollution is present.

Persistence of the Marker in Environmental Samples. Survival studies using naturally occuring enterococci in raw sewage inoculated into freshwater and simulated seawater were conducted, and the results are shown in Table 3. High temperature (30 °C) incubation was used to enhance die-off rates. The number of culturable enterococci dropped rapidly in a short period of time (3 days), fell to 19 CFU per membrane (per mL) by day 7 in freshwater, and was no longer detectable by day 10. In simulated seawater the level of culturable enterococci declined to 132 CFU by day 7, to 71 CFU by day 10, and was no longer detectable by day 15 (data not shown). The *esp* marker was only detectable up to day 5, when levels of enterococci were between 19 and 122 CFU/membrane in freshwater and up to day 10 at levels above 70 CFU/membrane in marine waters.

Discussion

In order for Microbial Source Tracking methods to be the most useful, they should be specific and applicable over a broad geographic region. Recent research has indicated that methods requiring a reference database may have limited utility for identification of specific sources of fecal pollution when isolates are collected from multiple watersheds (*7*). Molecular methods currently exist that identify specific sources of fecal pollution without relying on a database. These methods can circumvent the inherent drawbacks of existing, library-dependent MST methodologies (*9*, *10*, *13*, *14*). These targeted methods also generally cost less and can produce results within 1−2 days, which is significantly better than methods such as ribotyping, which can take 7−14 days.

Most MST methods attempt to characterize indicator microorganisms (either genotypically or phenotypically) on the basis of the host from which they were isolated. However, the ubiquitous nature of most of these indicators makes this task particularly challenging. The *Enterococcus* genus contains multiple species, and several researchers have reported on the variable distribution of species of enterococci in different animal hosts (*17*, *18*). Accurate speciation is difficult, however, as many biochemical tests currently used for identification of enterococci often produce ambiguous results (*21*). More specific molecular tests for speciation of enterococci and/or species and source-specific molecular markers would be extremely useful for targeted sampling of host associated molecular markers (HAMMs) within the enterococci group of bacteria. The *esp* gene target used in this study is believed to be located on the bacterial chromosome (personal communication, Dr. Louis B. Rice); therefore, the target is assumed to be stable and would be less likely to be transferred (via horizontal transfer) to nontarget microorganisms in the environment. However, recent laboratory-based research indicated that conjugative transfer of this gene between species is a possibility (*22*). Nevertheless, the current field study describes a method that may be useful for purposes of tracking sources of human fecal pollution. Much like the approach of Khatib et al. in their identification of bovine and porcine-specific biomarkers in *E. coli* (*13*, *14*), the intent of our investigation was to target potential enterococcal virulence factors that have been associated only with human clinical disease (*23*−*25*). Multiple targets were evaluated, and fecal samples from domestic sewage, septic tanks, birds, and various livestock animals were screened. By examining the entire population of enterococci in a water or fecal sample and specifically targeting a single HAMM, this test circumvented the need to isolate and characterize individual colonies of *Enterococcus* and allowed for the screening of millions of bacteria from multiple hosts. In agreement with previous literature (*20*, *26*−*28*), the primers specific for the *E. faecium* *esp* gene produced a product only in populations of microorganisms cultured from fecal samples of human origin. The marker was consistently detected when levels of human derived enterococci in a water or diluted fecal sample totaled at least 100 colony-forming units (CFU). This is useful, as the current recommendation for permissible levels of enterococci as a fecal indicator in marine waters is less than 104 CFU/100 mL for a single sampling event (*3*). Current water quality guidelines are based on total numbers of enterococci, regardless of composition. While these guidelines have been shown to be reliable as general indicators of water quality

and health risks (*1*, *2*), microbial source tracking methods should seek to better characterize the constituent species within this group in order to develop more specific tests for specific sources of fecal pollution.

Survival studies showed that the marker was detectable in both freshwater and simulated seawater for as long as total numbers of culturable enterococci were above 70 CFU, thus indicating that differential survival of enterococci populations in sewage did not significantly affect the utility of this test. The study was conducted at a relatively high temperature (30 °C), and enterococci were inoculated as primary sewage influent. Therefore, the experiment was a worst-case scenario of temperature and matrix (i.e. predation by sewage microorganisms) and was designed only to indicate the stability of the marker relative to culturable enterococci. In addition, the marker was no longer detected in filter concentrated cells once enterococci were no longer able to be cultured from the sample. The persistence of the genetic target determined by direct PCR of the sample is currently under investigation.

A literature review by Wade et al. supports culturable enterococci as indicators of human health risk in recreational waters (*29*), and culturable enterococci were also shown to be superior markers for risk of disease due to contamination of groundwater used for drinking when septic tanks were the source of contamination (*30*). Finally, culturable enterococci have been shown to be extremely useful indicators of the efficacy of wastewater treatment for purposes of reclamation (*31*).

Following along from the above-mentioned studies, we propose that the presence of culturable enterococci and the detection of the *E. faecium esp* gene in a water sample indicates the presence of human fecal contamination and potential human health risk, while the absence of the marker in association with high counts of enterococci could then also indicate an alternative source of contamination. Both results therefore will aid in the further investigation of the contamination and use of the water body. The method developed herein allows for identification of viable organisms as well as quantification and is compatible with current methods utilizing EPA approved membrane filtration or liquid-based methods (e.g. enterolert, data not shown).

The ideal source-tracking tool is one that employs multiple targets for each individual source group/animal. Overall, the results of the current study suggest that better genetic characterization of the communities of enterococci present in different animal hosts warrants further investigation. Future studies may reveal valuable information, which could subsequently be used to design more specific tests for the identification of multiple sources of fecal pollution. The use of PCR primers specific for the *esp* gene in *Enterococcus faecium* as an index of human fecal pollution may be a useful addition to the ever expanding microbial source tracking toolbox. As with all source tracking methods, as they are utilized in new regions of the country and with new potential sources of fecal pollution it is recommended that a set of field collected QA/QC samples (known sources of fecal contamination) be evaluated. The enterococci are becoming more useful indicators of water pollution and of public health risk, and the source of this risk should continue to be investigated by research directed at further characterization of these organisms.

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