Development of quantitative PCR assays targeting 16S rRNA gene of *Enterococcus* spp. and their application to the identification of *Enterococcus* species in environmental samples

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Abstract

The detection of environmental enterococci has primarily been determined using culture-based techniques that might exclude some enterococci species as well as those that are nonculturable. To address this, the relative abundance of enterococci was examined by challenging fecal and water samples against a currently available genus-specific assay (Entero1). To determine the diversity of enterococci species, 16S rRNA gene group-specific qPCR assays were developed and evaluated against eight of the most common environmental enterococci species. Partial 16S rRNA gene sequences of 439 presumptive environmental enterococci strains were analyzed to further study enterococci diversity and to confirm the specificity of group-specific assays. The group-specific qPCR assays showed relatively high amplification rates with targeted-species (>98%), although some assays cross-amplified with non-targeted species (1.3 - 6.5%). The results with the group-specific assays also showed that different enterococci species co-occurred in most fecal samples. The most abundant enterococci in water and fecal samples were *E. faecalis* and *E. faecium*, although we identified more water isolates as *E. casseliflavus* than any of the other species. The prevalence of the Entero1 marker was in agreement with the combined number of positive signals determined by the group-specific assays in most fecal samples, except in gull feces. On the other hand, the number of group-specific assays signals was lower in all water samples tested, suggesting that other enterococci species are present in these samples. While the results highlight the value of genus- and group-specific assays at detecting the major enterococci groups in environmental water samples, additional studies are needed to further determine the diversity, distribution, and relative abundance of all enterococci species in water.

Introduction

For more than a century the microbiological quality of environmental waters has been assessed using fecal indicator bacteria (FIB). While fecal

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coliforms and *Escherichia coli* are still widely used in environmental monitoring, enterococci are becoming a frequent target as they can be used to estimate health risks in both recreational marine waters and freshwaters. The *Enterococcus* genus includes more than 20 species, many of which are commonly associated with different mammals and birds while some species have been isolated from non-fecal sources (Muller *et al.* 2001). Studies looking at the enterococci diversity in environmental waters have identified most strains as *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus, Enterococcus hirae, Enterococcus durans, and Enterococcus mundtii* (Ferguson *et al.* 2005, Moore *et al.* 2008, Badgley *et al.* 2010). These findings have relied on isolating enterococci strains on selective culturing media (APHA 2005) followed by their classification which may involve biochemical (Facklam and Elliott 1995, Manero *et al.* 2002) and molecular techniques (Patel *et al.* 1998). Culture-based techniques are also used in regulatory activities to estimate the densities of enterococci in environmental waters. Since none of the enterococci media available can be used to discriminate between the different species, densities are recorded as general enterococci counts. Information on the environmental prevalence of enterococci species is not only relevant to confirm the presence of fecal enterococci but also it has been suggested that it can help identify primary fecal pollution sources (Wheeler *et al.* 2002). Different fecal sources can contribute to pollution of environmental waters, and each of them carries different health risks (Schoen and Ashbolt 2010). The general consensus is that human fecal sources are associated with higher risks, particularly due to host-specific pathogens such as enteric protozoa and viruses. However, non-human pollution sources are increasingly receiving attention by the health risk community in light of recent outbreaks in which they are implicated as the most likely source (Gardner *et al.* 2011) and due to their relevance to beach closures where the economic impact can be significant.

A quantitative PCR assay (qPCR) Entero1 has recently been used to estimate the levels of enterococci in recreational waters (Haugland *et al.* 2005). Originally developed by Ludwig and Schleifer (Ludwig and Scleifer 2000), the Entero1 assay targets the 23S rRNA gene. In most bacterial species, rRNA genes are present in multiple copies per genome, and therefore targeting such genes in environmental samples can improve assay sensitivity due to lower detection limits. However, less sequence information is available for the 23S rRNA gene than the 16S rRNA gene, precluding robust in silico validation. As a result, validation of the Entero1 assay has relied on testing the assay against a relatively small number of environmental strains isolated from of a limited number of different geographic locations (Haugland *et al.* 2005, Maheux *et al.* 2011). Moreover, similar to selective enterococci media, the Entero1 assay cannot be used to determine which of the major enterococci groups are present in a given sample.

To address some of these issues, we compared the relative occurrence and abundance of environmental and fecal enterococci using the Entero1 assay and several 16S rRNA gene-based group-specific PCR assays, most of which were developed as part of this study. Due to their reported prevalence in the environment, the group-specific assays targeted three of the major fecal enterococci groups (*E. faecalis*, *E. faecium*, and *E. casseliflavus*). The study was conducted by challenging the assays to fecal samples from diverse hosts and environmental waters with a history of fecal pollution. We also identified 439 strains isolated from surface water samples using 16S rRNA gene sequence analysis.

Methods

Bacterial Strains

The following strains were used as positive and negative controls: *E. casseliflavus* (ATCC 25788), *E. dispar* (ATCC 51266), *E. durans* (ATCC 19432), *E. faecalis* (ATCC 19433), *E. faecium* (ATCC 19434), *E. gallinarum* (ATCC 49573), *E. hirae* (ATCC 8043), *E. pseudoavium* (ATCC 49372), *Aeromonas eurenophila* (ATCC 23309), *Escherichia coli* (ATCC 25922), *Legionella sainthelensi* (ATCC 35248), *Proteus vulgaris* (ATCC 13315), *Salmonella typhimurium* (ATCC 14028), *Shigella flexneri* (ATCC 29903), *Staphylococcus aureus* (ATCC 29213), *Catellicoccus marimammalium*, *Citrobacter freundii*, *Escherichia coli O157:H7*, *Escherichia hermanii*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. Additionally, 439 presumptive *Enterococcus* sp. strains isolated on mEI agar (Messer and Dufour 1998) were used for evaluating enterococci assays. The latter strains were isolated from environmental waters collected from 15 US states (AZ, CO, FL, GA, KS, MD, MN, MT, NJ, NV, NY, OK, WA, WB, and WY). The identity of the environmental

enterococci strains was confirmed using 16S rRNA gene sequencing analysis.

Environmental Sample Collection and DNA Extraction

The environmental monitoring value of the group-specific assays was tested against water samples (n = 311) collected from different locations in California, North Carolina, and Puerto Rico. Water samples from California and North Carolina were collected from estuarine sites primarily impacted by gulls, whereas water samples from Puerto Rico were collected from sites within the Rio Grande de Arecibo watershed presumably impacted by cattle, human, and wildlife. Additionally, the assays were challenged against fecal samples (n = 497) from 4 domesticated animals (goat, horse, monkey, and pig), 13 wildlife species (chipmunk, coyote, fox, marmot, yellow-bellied marmot, mule, mule deer, rabbit, jack rabbit, raccoon, snowshoe hare, squirrel, and ground squirrel), and 7 avian species (chicken, duck, guineafowl, gull, pelican, swan, and turkey). Water samples (100 ml) were collected and filtered onto polycarbonate membranes (0.4 μm pore size, 47 mm diameter; GE Water and Process Technologies, Trevose, PA). Fecal samples were collected aseptically, transferred to sterile tubes, and transported to the laboratory in ice coolers. Frozen filters and fecal samples were shipped overnight on dry ice to the USEPA, Cincinnati, Ohio (OH) and stored at –80°C until further processing. DNA extraction from filters and fecal samples was performed using Mo Bio PowerSoil kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's protocol. Avian fecal samples (i.e., gull and turkey) from France were extracted using the Fast DNA spin kit for soil (MP Biomedical, Illkirsh, France), according to the supplier's instructions, except that an additional wash using the SEWS-M reagent was performed. DNA concentration was measured using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). DNA extracts were stored at -20°C until further processing.

Sequencing Analyses

Sequences from 16S rRNA gene PCR products generated using universal bacterial primers (8F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 787R: 5'-CGACTACCAGGGTATCTAAT-3') were used to determine the identity of the 439 environmental isolates from mEI culture and reference bacteria. Briefly, PCR assays were performed in 25 μl using the polymerase TaKaRa Ex TaqTM (Takara Bio Inc.) in a Tetrad2 Thermal Cycler (Bio-Rad, Hercules, CA) under the following cycling conditions: one initial denaturation step at 95°C for 5 minutes and 25 cycles of 1 minute at 95°C, 1 minute at 56°C, and 1 minute at 72°C. PCR products were sequenced in both directions in the Children's Hospital DNA Core Facility (Cincinnati, OH) using an Applied Biosystems Prism 3730XL DNA analyzer. Raw sequences were processed using Sequencher software (Gene Codes, Ann Arbor, MI). For 16S rRNA gene sequences, homology searches of DNA sequences in the GenBank (NR) database were undertaken with the National Center for Biotechnology Information (NCBI) BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al.* 1997). Representative sequences were deposited in GenBank under the following accession numbers: JQ804941-JQ804949.

Assay Development and Performance Evaluation

Fifteen different assays were tested in this study: five genus-specific assays, three *E. faecalis*-specific assays, three *E. casseliflavus*-specific assays, and four *E. faecium*-specific assays (Table 1). Eleven assays were tested as conventional PCR assays and four were tested as qPCR (TaqMan-based) assays (one genus-specific, Entero1; and one for each of the enterococci groups, Faecalis1, Casseli1, and Faecium1). Entero1 and Faecalis1 qPCR assays were developed and evaluated in previous studies (Santo Domino *et al*. 2003, Haugland *et al.* 2005). To develop new enterococci assays, a phylogenetic tree that included 16S rRNA gene sequences from reference enterococci strains (Patel *et al.* 1998) and environmental strains was generated using a neighbor-joining algorithm in ARB (Ludwig *et al.* 2004). Unique phylogenetic clades were identified (Figure 1) and candidate primers were then chosen to target three major environmental clades (i.e., *E. faecalis*, *E. faecium*, and *E. casseliflavus*) using the primer design algorithm in ARB (Table 1). Additionally, 16S rRNA gene enterococci sequences were used to design two group-specific qPCR assays using Primer Express Software (Applied Biosystems, Foster City, CA, USA; Table 1). The assays were optimized through temperature gradients and tested for their specificity and sensitivity against reference bacterial strains and environmental enterococci

Table 1. Summary of oligonucleotide primers and probes for PCR and TaqMan qPCR.

Target Organisms	Assay*	Primer and Probe Sequences (5' to 3')	T_a (°C)**	Size (bp)***	Reference
Enterococcus spp.	Entero1	ECST748F: AGAAATTCCAAACGAACTTG ENC854R: CAGTGCTCTACCTCCATCATT GPL813TQ: 6FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA	60	92	Ludwig and Schleifer (2000)
	Ent1	Ent151F: ACACTTGGAAACAGGTGC Ent376R: TCGGTCAGACTTKCGTCC	65	243	This study
	Ent2	Ent151F: ACACTTGGAAACAGGTGC Ent578R: TTAAGAAACCGCCTGCGC	65	445	This study
	Ent3	Ent240F: TGCATTAGCTAGTTGGTG Ent578R: TTAAGAAACCGCCTGCGC	63	356	This study
	Ent4	Ent376F: GGACGMAAGTCTGACCGA Ent578R: TTAAGAAACCGCCTGCGC	65	220	This study
Enterococcus faecalis	Faecalis1	FaecalF: CGCTTCTTTCCTCCCGAGT FaecalR: GCCATGCGGCATAAACTG FaecalP: 6FAM-CAATTGGAAA GAGGAGTGGCGGACG-TAMRA	60	143	Santo Domingo *et al.* (2003)
	Faecalis2	Ent151F: ACACTTGGAAACAGGTGC Faecal449R: AGTTACTAACGTCCTTGTTC	64	318	This study
	Faecalis3	Ent240F: TGCATTAGCTAGTTGGTG Faecal449R: AGTTACTAACGTCCTTGTTC	63	229	This study
Enterococcus casseliflavus	Casseli1	CasselF: GGAGCTTGCTCCACCGAA CasselR: TTTCTTCCATGCGGAAAATAGT CasselP: 6FAM-CGAACGGGTGAGTAACACGTGGGTAA-TAMRA	60	132	This study
	Casseli2	Cassel190F: GGAAGAAAGTTGAAAGGC Ent376R: TCGGTCAGACTTKCGTCC	60	204	This study
	Casseli3	Cassel190F: GGAAGAAAGTTGAAAGGC Ent578R: TTAAGAAACCGCCTGCGC	60	406	This study
Enterococcus faecium	Faecium1	CiumF: TTCTTTTTCCACCGGAGCTT CiumR: AACCATGCGGTTTYGATTG CiumP: 6FAM-AGTAACACGTGGGTAACCTGCCCATCAGA-TAMRA	60	141	This study
	Faecium2	Cium84F: TGCTCCACCGGAAAAAGA Ent240R: CACCAACTAGCTAATGCA	63	174	This study
	Faecium3	Cium84F: TGCTCCACCGGAAAAAGA Ent376R: TCGGTCAGACTTKCGTCC	64	310	This study
	Faecium4	Cium84F: TGCTCCACCGGAAAAAGA Ent578R: TTAAGAAACCGCCTGCGC	65	512	This study

*Entero1 targets 23S rRNA gene, whereas the other assays target 16S rRNA gene.
**Optimum annealing temperatures determined using temperature gradient PCR
***Approximate product size determined *in silico* data

isolates described above. Applicability of the PCR and qPCR assays in environmental monitoring was also evaluated against the aforementioned set of water and fecal samples.

For the conventional PCR assays, all water and fecal samples were tested as previously described (Ryu *et al.* 2011) with the following modifications: 0.5 to 1 ng/µl of DNA extracts were used as templates and 10-fold dilutions of each DNA extract were used to test for PCR inhibition. PCR assays were performed in 25 µl using TaKaRa Ex TaqTM (Takara Bio Inc.) in a Bio-Rad Tetrad2 Peltier Thermal Cycler (Bio-Rad, Hercules, CA) under the following cycling conditions: one initial denaturation step at 95°C for 5 minutes and 25 cycles of 1 minute at 95°C, 1 minute at optimum annealing temperature (Table 1), and 1 minute at 72°C. PCR products were visualized in 1.5% agarose gels using GelStar Nucleic Acid gel stain (Lonza, Rockland, ME, USA).

The Taqman qPCR assays were performed in 25-µl reaction mixtures containing 1X TaqMan universal PCR master mix with AmpErase uracil-N-glycosylase (Applied Biosystems, Foster City, CA), 0.2 µg/µl bovine serum albumin, 0.2 µM (final concentration) of each primer and FAM (6-carboxyfluorescein)-labeled hydrolysis probe. The amplification protocol involved an initial incubation at 50°C for 2 minutes to activate uracil-N-glycosylase, followed by 10 minutes of incubation at 95°C to activate AmpliTaq Gold enzyme, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The qPCR assays were performed using a 7900 HT Fast Real-Time Sequence Detector (Applied Biosystems, Foster City, CA, USA). All assays were

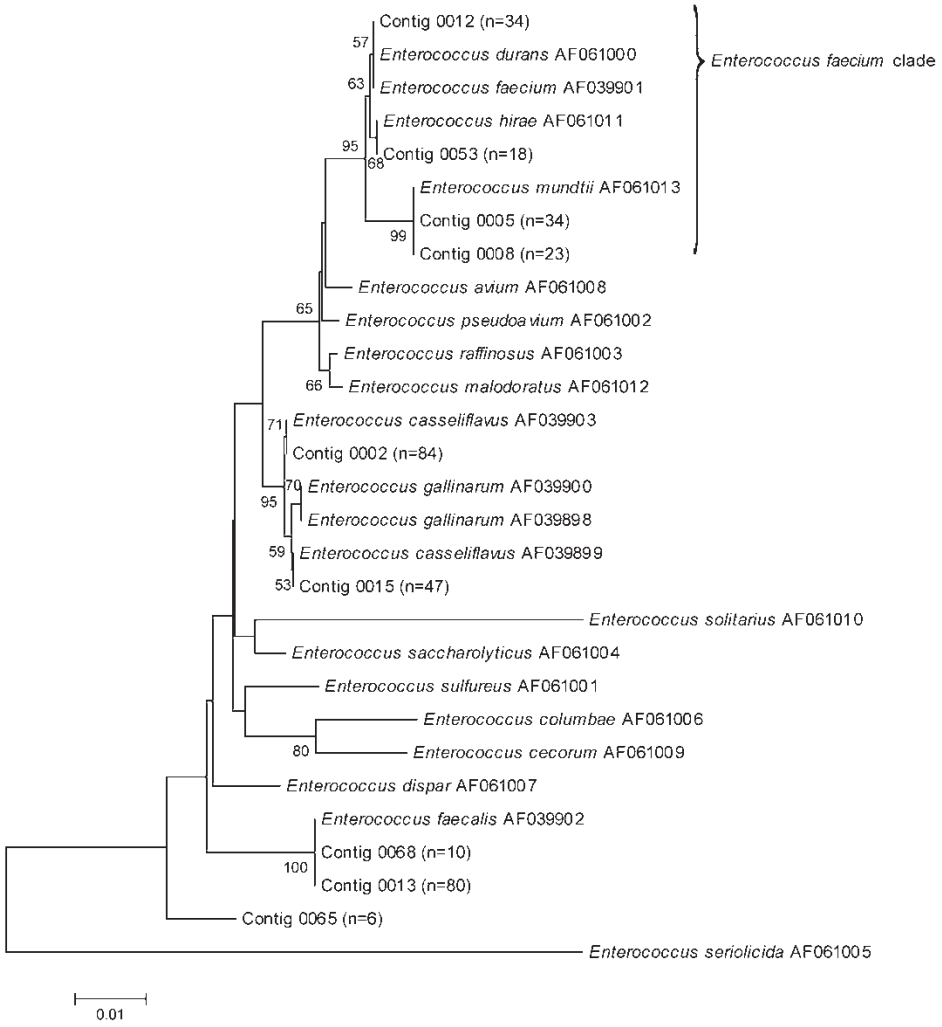

Figure 1. Unrooted neighbor-joining tree of 16S rRNA gene sequences obtained from *Enterococcus* environmental isolates. The number of sequences for each contig is included within parentheses (n), and the contigs of less than five sequences were not presented in the phylogenetic tree. Reference bacteria with their GenBank accession numbers and 1000-replicate bootstrap values are shown in the tree. The bootstrap values are reported as percentage greater than 50%. The scale bar corresponds to 0.01 changes per nucleotide.

performed in triplicate in MicroAmp Optical 96-well reaction plates with MicroAmp Optical Caps (Applied Biosystems, Foster City, CA, USA). PCR data were analyzed using ABI's Sequence Detector software (version 2.2.2). Four independent standard curves for each qPCR assay were generated by plotting threshold cycle (CT) values against the number of target copies corresponding to serially diluted plasmid standards purchased from IDT integrated DNA technologies (Coralville, Iowa, USA). The target copy numbers (T) were estimated by the following equation:

$$T = [D/(PL \times 660)] \times 6.022 \times 10^{23}$$

where D (g/µl) is plasmid DNA concentration, and PL (bp) is plasmid length in base pairs.

Each standard curve was generated from at least five 10-fold plasmid dilutions in triplicates. Percent amplification efficiencies were calculated by the instrument manufacturer's instructions (Applied Biosystems). Two no-template controls per PCR plate were used to check for cross-contamination.

Venn Diagram

The relation among the genus- and species-specific qPCR assays against fecal and water samples was determined using Venn diagrams as previously described (Ryu *et al.* 2012). Briefly, two Venn diagrams were constructed sequentially. The first diagram was used for calculating the prevalence of three species-specific markers. The second diagram was used to establish the relation between the

genus-specific assay and the three species-specific assays combined.

Results and Discussion

Rationale for Assay Development

Phylogenetic trees that included sequences from reference and environmental enterococci strains were generated to identify 16S rRNA gene sequences that could be used to develop multiple enterococci species-specific assays (Figure 1). This approach indicated that it was difficult to develop assays that discriminated *E. faecium*, from *E. mundtii*, *E. durans*, *E. hirae*, *E. dispar*, and *E. casseliflavus* from *E. gallinarum*. However, the analysis demonstrated that most of the fecally relevant species belong to three major enterococci clusters, i.e., *E. faecalis*, *E. faecium*, and *E. casseliflavus/gallinarum*. We proceeded to identify primers for both conventional and qPCR assays for each of these groups. A total of nine novel group-specific assays were developed in this study. We compared these groups-specific assays to determine which of the assays were better suited at detecting fecally relevant enterococci in environmental waters. We also identified sequences that were present in most enterococci species and tested them as potential general enterococci assays. We then compared these assays with the currently available Entero1 assay against a variety of animal fecal samples and environmental waters collected from different geographical locations. Based on the number of enterococci isolates, fecal samples, and environmental waters tested in this study, this represents one of the largest studies using molecular assays aiming at identifying and detecting enterococci from water and fecal samples.

We focused on 16S rRNA gene-based assays as these genes are part of multiple operons and therefore the detection limits (i.e., per genome copy) of these assays are expected to be higher than single copy genes. One of the assays (Entero1) targets the 23S rRNA gene which is part of the same operon as the 16S rRNA gene. Although less sensitive, non-ribosomal genes can be used to discriminate different enterococci species (Jackson *et al.* 2004, Vermette *et al.* 2010). However, only a handful of non-ribosomal genes have been used in environmental studies to detect or identify enterococci (Scott *et al.* 2005, Maheux *et al.* 2011, Ahmed *et al.* 2012). More importantly, the sequence database for function-specific genes of environmental enterococci and other phylogenetically related genera is much more limiting than 16S and 23S rRNA genes. Moreover, sequence conservancy in functional genes is considerably lower than in rRNA genes, which explains why it is difficult to develop genus- and group-specific assays unless comprehensive sequence databases are developed.

Identification of Environmental Strains

Based on 16S rRNA gene sequence analyses of the 439 environmental isolates used in this study, approximately 91% were identified as *Enterococcus* sp., whereas others were classified as non-enterococci (7%) or unclassified bacteria (2%). These results are in agreement with other studies using mEI agar as the isolation media for environmental enterococci (Messer and Dufour 1998, Mote *et al.* 2012), although Nayak *et al.* (2011) reported relatively lower false-positive rates (i.e., 1.6%) in subtropical waters. The latter study was based on 61strains isolated from two lakes on two different dates, which may explain the lower false positive rate.

Based on sequence identity of the environmental isolates tested in our study, the most dominant enterococci species were *E. casseliflavus* (34%) *E. faecalis* (25%) and *E. mundtii* (15%), while *E. faecium* and *E. hirae* were identified to a lesser extent (i.e., 5%; Table 2). Several enterococci species have been detected in environmental waters but their overall prevalence varies considerably. For example, Mote *et al.* (2012) found that the most dominant enterococci species were *E. faecalis* (31%), *E. mundtii* (31%), and *E. casseliflavus* (16%), while *E. faecium*, and *E. gallinarum* were identified less frequently (i.e., 10 and 4%, respectively). Moore *et al.* (2008) and Grammenou *et al.* (2006) also found different environmental enterococci species but *E. faecalis* and *E. faecium* were most dominant species in many water samples. In spite of these differences, these results clearly indicate that multiple enterococci species can be present in the same water sample. The differences in enterococci species occurrence may be associated with different *in situ* growth and environmental survival rates (Lleo *et al.* 2001) and preferential host distribution of different enterococci species in different animals. Other studies have suggested that that the environmentally relevant enterococci species detected in this study are found in a wide variety of hosts (Layton *et al.* 2010). Altogether, these data suggest that enterococci

Table 2. Classification of environmental isolates from mEI culture using 16S rRNA gene sequencing.

Bacteria	# of Isolates	GenBank Accession Number*
E. casseliflavus	152 (34%)	DQ333294.1
E. faecalis	111 (25%)	AB534553.1
E. mundtii	68 (15%)	NR_024906.1
E. faecium	23 (5%)	EU003447.1
E. hirae	20 (5%)	Y17302.1
Enterococcus spp.	21 (5%)	NR_036922.1, NR_037082.1, NR_042054.1
Aerococcus spp.	24 (5%)	HM582941.1
Lactococcus garvieae	3 (0.7%)	AY699289.1
Pediococcus pentosaceus	4 (0.9%)	CP000422.1
Streptococcus gallolyticus subs. pasteurianus	4 (0.9%)	AB457024.1
Unclassified bacteria	9 (2%)	Not Available
Total	439 (100%)	

*All sequences for enterococci isolates are >99% identical to GenBank reference

speciation might not be an adequate approach to fecal source identification.

In this study four non-enterococci species were identified (i.e., >99% identical to reference sequences) among the environmental isolates, namely *Aerococcus sp., Lactococcus garvieae, Pediococcus pentosaceus,* and *Streptococcus gallolyticus subs. pasteurianus*. Other studies have reported on the presence of some of these genera in mEI media. For example, Maraccini *et al.* (2012) showed that most non enterococci mEI isolates were identified as *Aerococcus viridans* (i.e., 17%) with fewer isolates identified as *S. mutans*, *S. gallolyticus, Leuconostoc* sp., and *Pediococcus acidilactici*. The samples from the latter study were collected within a three-day period from one marine site. While it is not known how predominant these non-enterococci species are during an entire beach season, these data suggest that some non-enterococci species may be highly abundant in recreational marine waters, potentially resulting in over-estimation of enterococci densities when using culture-based methods. In another study, Viau and Peccia (2009) showed that mEI also supported the growth of bacteria from biosolids that wereidentified as *Bacillus* sp., *Vagococcus* sp., and *Desemzia incerta*. As biosolids and animal fecal waste (i.e., treated manure) are used in agricultural activities, the results from these studies suggest that non-enterococci species might interfere with culture-based methods used to estimate fecal pollution levels. In our study, the bacterial water strains tested were isolated from waters presumed to be impacted by wastewater treatment plants and to a lesser extent by agricultural activities, although wildlife fecal pollution sources cannot be ruled out. Our results further suggest that culture based methods can support the growth of non-enterococci species present in freshwater samples and that further studies are needed to better determine the identity and prevalence of these non-targeted species in fecal and water samples.

Validation of Genus-Specific Enterococci PCR Assays

The specificity of the *Enterococcus* genus- and group-specific PCR assays was evaluated against a subset of the enterococci strains sequenced in this study. This subset (n = 153) included several strains from the most common *Enterococcus* species identified in this study, non-enterococci species obtained from culture collections (n = 13), and non-enterococci strains isolated from this study (n = 4; Table 3). All of the genus-specific assays successfully amplified the enterococci type strains (ATCC). Additionally, four of the genus-specific assays generated positive signals with greater than 97% of the environmental strains tested in this study and in most cases cross-amplified relatively few non-enterococci strains (i.e., 0 - 24%; Table 3). Two of the assays, Ent2 and Ent3, did not show cross-amplification with non-enterococci strains, and therefore may prove useful as confirmatory tests. However, Ent2 only detected 59% of the enterococci strains tested, suggesting that it cannot be used as a stand-alone enterococci assay.

Interestingly, the Entero1 assay showed a low level of cross amplification with *C. marimammalium.*

Table 3. The number of positives and percent positives in parentheses by the *Enterococcus* assays against environmental isolates and ATCC strains. n = number of samples.

Target Organisms	Assay	*Enterococcus casseliflavus* n = 50[a]	*Enterococcus faecalis* n = 39	*Enterococcus faecium* n = 11	*Enterococcus hirae* n = 5	*Enterococcus mundtii* n = 40	Other Enterococci Species n = 8[b]	Non-Enterococci Species n=17[c]
Enterococcus spp.	Entero1[c]	50 (100)	39 (100)	11 (100)	5 (100)	40 (100)	8 (100)	1 (6)
	Ent1	50 (100)	39 (100)	11 (100)	5 (100)	39 (98)	7 (88)	4 (24)
	Ent2	33	19 (49)	8 (73)	4 (80)	18 (45)	8 (100)	0
	Ent3	50 (100)	37 (95)	11 (100)	4 (80)	39 (98)	8 (100)	0
	Ent4	50 (100)	39 (100)	11 (100)	5 (100)	40 (100)	8 (100)	2 (12)
Enterococcus faecalis	Faecalis1[d]	5 (10)	39 (100)	2 (18)	0	6 (15)	1 (13)	0
	Faecalis2	0	16 (41)	0	0	0	0	0
	Faecalis3	0	25 (64)	0	0	0	0	0
Enterococcus casseliflavus	Casseli1[d]	49 (98)	0	1 (9)	1 (20)	0	0	0
	Casseli2	28 (58)	4 (10)	1 (9)	0	4 (10)	0	0
	Casseli3	48 (96)	0	1 (9)	0	0	0	0
Enterococcus faecium	Faecium1[d]	2 (4)	0	11 (100)	5 (100)	40 (100)	8 (100)	0
	Faecium2	27 (54)	2 (5)	10 (91)	3 (60)	35 (88)	8 (100)	0
	Faecium3	30 (60)	1 (3)	11 (100)	5 (100)	28 (70)	8 (100)	0
	Faecium4	0	0	11 (100)	4 (80)	19 (48)	7 (88)	0

[a]All enterococci species were identified by NCBI blast as such, with the exception of *E. casseliflavus* which sequences are nearly identical to *E. gallinarum*.

[b]Sequences of the isolates are affiliated to the *Enterococcus faecium* clade (see Figure 1).

[c]Seven ATCC strains: *Aeromonas eurenophila*, *Escherichia coli*, *Legionella sainthelensi*, *Proteus vulgaris*, *Salmonella typhimurium, Shigella flexneri*, *Staphylococcus aureus*. Six laboratory strains: *Catellicoccus marimammalium, Citrobacter freundii*, *E. coli O157:H7*, *E. hermanii*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*. Four environmental strains: *Aerococcus* spp., *Lactococcus garvieae*, *Pediococcus pentosaceus*, *Streptococcus pasteurianus*

[d]TaqMan qPCR assays.

Several gull-specific assays target *C. marimammalium* 16S rRNA gene. Indeed, signals with these gull assays have frequently been detected in environmental waters with history of gull fecal contamination (Lu *et al.* 2008, Ryu *et al.* 2012). Signals detected against *C. marimammalium* DNA were approximately four orders of magnitudes lower than the enterococci species tested (Figure 2), suggesting that *C. marimammalium* may not contribute significantly to false positive signals. Entero1 cross-amplification signals have been observed with other lactobacilli species (Frahm and Obst 2003). These results are relevant to environmental monitoring as the Entero1 assay has been proposed as an alternate method for the rapid detection of *Enterococcus* spp. in recreational waters (USEPA 2010). As the overestimation of the Entero1 assay due to non-targeted bacteria could result in unnecessary beach closures, additional studies are needed to more accurately determine the levels of false positive signals in recreational settings. Future studies also need to determine whether these non-targeted populations are present in environmental waters frequently enough to interfere with risk assessment models.

Validation of Group-Specific Enterococci PCR Assays

Overall, the group-specific assays indicated a relatively high amplification with targeted enterococci species and low cross-amplification with non-enterococci (Table 3). Noteworthy, the Faecalis2 and Faecalis3 assays showed 100% specificity and amplified *E. faecalis* type strain, but it did not amplify some of *E. faecalis* environmental strains, suggesting that they may be used under limited cases as a group-specific-assays. The Faecalis1 TaqMan qPCR assay successfully amplified all of the tested *E. faecalis* strains (n = 39) and did not cross-react with seven non-*E. faecalis* species ATCC strains, as previously observed (Santo Domingo *et al.* 2003). Although the Faecalis1 assay cross-reacted with a low number of non-*E. faecalis* environmental stains (Table 3), signal intensities of these non-target bacteria were more than 4 orders of magnitude lower than those of *E. faecalis* strains (Figure 2). Tracking signal intensity will be important to determine the value of these assays in environmental applications. As cross-amplification signals are relatively low for some of these assays, scenarios showing high environmental signals are likely to be result of true-positive signals rather than false positive signals, unless cross-amplification targets are present in high abundance in a given environmental sample. This assumption needs to be tested with these newly developed assays as well as most published FIB-targeting assays.

The *E. casseliflavus*-specific assays showed relatively high specificity (i.e., low cross-amplification rates against non-target species) when compared to the other group-specific assays. The Casseli1 TaqMan qPCR assay successfully amplified 98% (49/50) of *E. casseliflavus* environmental strains and showed 1.7% (2/120) cross-amplification with non-target species. Moreover, the Casseli1 and the

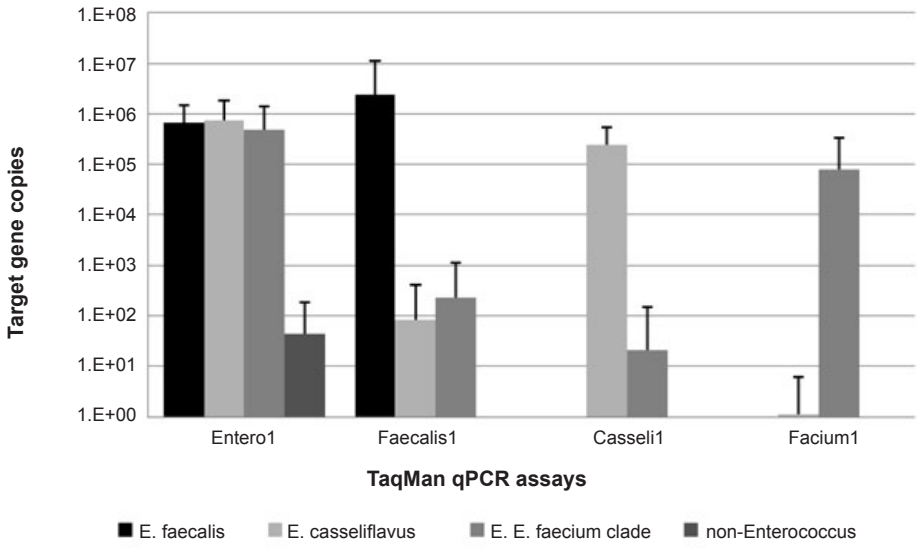

Figure 2. Mean copy number of target markers against environmental isolates of enterococci species and non-*Enterococcus* bacteria. To calculate mean concentrations, below detection limits were treated as zero. Error bars represent one standard deviation.

Casseli3 assays did not cross-amplify with any of non-*Enterococcus* bacteria tested in this study. The Casseli2 showed the least specificity and sensitivity (i.e., higher cross-amplification rate with non-target species and lower amplification rate with *E. casseliflavus*).

Most *E. faecium* assays primarily amplified *E. faecium*, *E. durans*, *E. hirae*, and *E. mundtii* strains. This is compatible with the fact that these species formed a cohesive clade and that it is difficult to differentiate these species using 16S rRNA gene sequences (Figure 1). Specifically, the Faecium1 TaqMan qPCR assay amplified *E. faecium*, *E. hirae*, and *E. mundtii* strains. The Faecium2 and Faecium3 assays cross-reacted with *E. casseliflavus*, whereas the Faecium4 assay showed the best specificity (i.e., lower cross-amplification rate with *E. casseliflavus*; Table 3). Thus, the *E. faecium* assays developed in this study might be used as a multi-species specific assay. Future studies should focus on assessing the value of the conventional PCR assays developed in this study as qPCR assays.

Detection of Enterococci in Fecal and Environmental Water Samples

The Entero1, Faecalis1, Casseli1, and Faecium1 assays were used in further studies based on the overall specificity and sensitivity results and the fact that they can provide quantification data. Specifically, the assays were used to investigate the presence and abundance of enterococci in 497 fecal samples collected from four different geographic locations and from 24 different animals, and in 311 environmental water samples collected from California, North Carolina, and Puerto Rico (Table 4). To our knowledge, this represents the largest study in which different enterococci species have been detected from fecal samples via PCR assays without the need of an enrichment step.

The range of quantification (ROQ) for the Entero1 and Faecalis1 qPCR assays was 10^1 to 10^6 DNA copies per reaction. For the Faecium1 and the Casseli1 assays, 10 copies per reaction were below detection limit of the assay, and therefore, the ROQ of these assays was determined to range from 10^2 to 10^6 DNA copies. In order to evaluate assay sensitivity, four independent standard curves were used to calculate the percent amplification efficiency average. The Entero1 assay showed the greatest amplification efficiency, followed by the Faecalis1, the Faecium1, and the Casseli1 assays (i.e., average ±1 standard deviation: 94.8 ±0.8, 90.9 ±1.1, 88.5 ±2.1, and 85.2 ±1.3, respectively). All of the no-template controls were negative indicating the absence of cross-contamination in the qPCR experiments.

Approximately 74, 41, 25, and 49% of fecal samples were positive for the Entero1, the Faecalis1, the Casseli1, and the Faecium1 markers, respectively (Table 4). However, excluding gull samples, the number of positive samples for enterococci increased to 44% for the Casseli1 marker and 74 to 94% for the other markers, clearly suggesting that enterococci are normal inhabitants of most of the hosts tested. The results of group-specific assays showed that different enterococci species co-inhabit in most hosts, although the high prevalence of multiple enterococci species was evident in some hosts more than others. For example, each of the three group-specific markers was detected in more than 87% of pig feces, while a specific group predominated in gulls, horses, and wildlife. The prevalence of the Entero1 marker (i.e., n (G)) was in agreement with the combined number of positive signals (i.e., n (S) = n (A U B U C)) determined by the three species-specific markers in fecal samples (Table 4). In other words, combining the results from the genus- and group-specific assays (i.e., n (G U S) did not increase the number of enterococci positive samples in most feces types, with the exception of gull fecal samples in which an increased prevalence was observed (i.e., n (G U S) > n (G) > n (S)). There are two possible scenarios to explain the lower prevalence of the species-specific markers in gull feces. First, it is possible that there are environmental enterococci species that are detected by the Entero1 assay, but not detected with the group-specific assays tested in this study. This suggests that additional group- or species-specific assays are needed to further study the abundance and dynamics of these species in fecal samples and perhaps in environmental waters impacted by gulls. This may be important if these non-targeted enterococci species are noted to be important in recreational waters. A second scenario relates to the Entero1 assay cross-reacting with some of the indigenous non-*Enterococcus* bacteria such it may be the case for *C. marimammalium* which resides in the gull feces and for which signals have been detected in gull impacted waters. If the latter is of any significance, the Entero1 assay may be overestimating enterococci levels, although positively correlating with the presence of gull-fecal contamination. Thus further validation of the specificity of Entero1 against

Table 4. Detection of enterococci species in different fecal and water samples using TaqMan qPCR assays. n = number of samples; NA = not available.

Sample Type	Sampling Location(s)	n	Number of Positive Samples (% Positive)				Relationship between Assays[a]		
			Entero1 (G)	Faecalis1 (A)	Casseli1 (B)	Faecium1 (C)	n (S)	n (G ∩ S)	n (G U S)
Goat	Puerto Rico	32	32 (100)	18 (56)	7 (22)	30 (94)	32 (100)	31 (97)	32 (100)
Horse	Puerto Rico	28	28 (100)	7 (25)	3 (11)	22 (79)	24 (86)	24 (86)	28 (100)
Monkey	Puerto Rico	9	9 (100)	7 (78)	9 (100)	6 (67)	9 (100)	9 (100)	9 (100)
Pig	Puerto Rico	30	30 (100)	26 (87)	26 (87)	29 (97)	30 (100)	30 (100)	30 (100)
Wildlife[b]	California	77	61 (79)	13 (17)	9 (12)	39 (51)	49 (64)	49 (64)	61 (79)
Chicken	Puerto Rico	35	35 (100)	24 (69)	19 (54)	31 (89)	34 (97)	34 (97)	35 (100)
Duck	Puerto Rico	16	16 (100)	9 (56)	13 (81)	15 (94)	16 (100)	16 (100)	16 (100)
Guineafowl	Puerto Rico	11	11 (100)	1 (9)	2 (18)	6 (55)	6 (55)	6 (55)	11 (100)
Gull[c]	California, Delaware, and France	220	108 (49)	82 (37)	10 (4)	33 (15)	87 (40)	83 (38)	112 (51)
Pelican	California	10	10 (100)	10 (100)	7 (70)	9 (90)	10 (100)	10 (100)	10 (100)
Swan	Puerto Rico	22	22 (100)	6 (27)	11 (50)	18 (82)	19 (86)	19 (86)	22 (100)
Turkey	France and Puerto Rico	7	7 (100)	2 (29)	6 (86)	7 (100)	7 (100)	7 (100)	7 (100)
	Total	**497**	**369 (74)**	**205 (41)**	**122 (25)**	**245 (49)**	**NA**	**NA**	**NA**
Estuarine Water	California	65	55 (85)	24 (37)	3 (5)	24 (37)	31 (48)	31 (48)	55 (85)
Estuarine Water	North Carolina	109	107 (98)	68 (62)	9 (8)	34 (31)	76 (70)	76 (70)	107 (98)
Surface Water[d]	Puerto Rico	137	69 (50)	32 (23)	4 (3)	24 (18)	37 (27)	33 (24)	73 (53)
	Total	**311**	**231 (74)**	**124 (40)**	**16 (5)**	**82 (26)**	**NA**	**NA**	**NA**

[a]Results were calculated using a Venn diagram approach where n(U) = total number of samples, n(A) = number of positive samples with Faecalis1, n(B) = number of positive samples with Casseli1, n(C) = number of positive samples with Faecim1, and n(G) = number of positive samples with Entero1; n(S) = n(A U B U C).

[b]Thirteen different animals (chipmunk, coyote, fox, marmot, yellow-bellied marmot, mule, mule deer, rabbit, jack rabbit, raccoon, snowshoe hare, squirrel, and ground squirrel).

[c]Three species of gull from California (*Larus californicus*), Delaware (*L. atricilla* and *L. smithsonianus*), and France (*L. argentus*).

[d]Water samples were collected from twelve sampling locations in Arecibo watershed, Puerto Rico between September 2010 and January 2011 (thirteen sampling events).

broad range of non-*Enterococcus* bacteria is needed, particularly members of the Lactobacillales family as overestimation due to false positive signals is relevant in scenarios in which molecular assays are used as alternative to culture-based assays used to monitor recreational water quality.

Most water samples tested in this study (i.e., 74%) contained detectable enterococci signals. In general terms, among the group-specific assays used, *E. faecalis* was detected more frequently (40%) in the water samples than *E. faecium* (26%) and *E. casseliflavus* (5.1%), regardless of the sample origin. The prevalence of the genus and group-specific enterococci assays in estuarine water samples from California and North Carolina was higher than in tropical surface water samples (Table 4). Estuarine water samples tested in this study have historically been impacted by gulls while surface waters in Puerto Rico are primarily impacted with wastewater and cattle fecal sources, and to a lesser extent to domesticated animals such as chicken, pigs, horses, and goats. Interestingly, the Casseli1 marker was seldom detected in gull fecal samples which could explain the relatively low prevalence of the Casseli1 marker in the temperate water samples. However, the fact that Casseli1 marker was also seldom detected in tropical waters not impacted by gulls suggests that low detection of *E. casseliflavus* may not be indicative of low levels of waterfowl in environmental waters. This data suggests that some of the major enterococci species are cosmopolitan (i.e., present in various hosts), and therefore, the use of microbial source tracking (MST) methods targeting enterococci species might be difficult to justify in source allocation applications.

Unlike fecal samples, the prevalence of the Entero1 marker (i.e., n (G)) was higher than the combined number of positive signals determined by three species-specific markers (i.e., n (S) = n (A U B U C)) in all tested water samples from three different geographical locations (Table 4). Several factors could have contributed to these results. For example, some of the numerically dominant species were not detected with the group-specific assays used in this study. This means that species such as *E. raffinosus*. *E. saccharolyticus*, *E. avium*, *E. pseudoavium*, and *E. cecorum* might be present in some of these samples and contributed significantly to the genus-specific signals. Using Slanetz-Bartley agar one study showed that a relatively high number of *E. raffinosus*, *E. avium*, and *E. saccharolyticus* strains were isolated from environmental waters (Arvanitidou *et al.* 2001), while in another study 25% of the isolates were non typical enterococci species and only classified as *Enterococcus* sp. (Svec and Sedlacek 1999). Grammenou *et al.* (2006) also isolated *E. avium* from water samples but the strains represented approximately 2% of all enterococci isolates. Altogether, these results suggest that mEI (i.e., the media used in our study) favors the growth of some enterococci, which explains why *E. feacalis*, *E. casseliflavus*, and *E. faecium* are often isolated from mEI plates. On the other hand, Suzuki *et al.* (2012) recently showed that *E. faecalis* and *E. faecium* combined did not represent more than 32% of the mEI isolates from five Japanese rivers, potentially implicating the prevalence of other enterococci species.

An alternate explanation for the differences in prevalence between the Entero1 and the collective group-specific markers is that novel enterococci might have also been responsible for a fraction of the signals in the water samples. Indeed, novel enterococci species have been identified in recent years from water (Svec *et al.* 2001, 2005a,b) and fecal samples (Naser *et al.* 2005, Caravalho *et al.* 2006). While the relative abundance of novel enterococci species in water samples is unknown, these results indicate that there is a need of further investigating enterococci diversity in both fecal and environmental samples. The results also suggest that some enterococci species might be more adapted to persist outside of the gut environment than others (Badgley *et al.* 2010), which might lead to adaptation of fecal bacteria to secondary habitats (Gordon *et al.* 2002). The latter has important implications in conventional microbial water quality monitoring as well as in microbial source tracking applications using enterococci as targeted populations.

Overall, the results from this study are in agreement with previously published data demonstrating that animals frequently implicated in the fecal contamination of environmental waters shed different enterococci species. This study also suggests that while three major enterococci groups (i.e., *E. faecalis*, *E. faecium*, and *E. casseliflavus*) tend to dominate in fecally contaminated waters, additional enterococci species may be present and not detected with the currently available genus- and group-specific qPCR assays. Better understanding of the molecular diversity and occurrence of enterococci species in fecal samples and environmental waters will be critical in future evaluation studies of conventional as well as

molecular detection methods used in the application of ambient microbial water quality recommendations. The approach herein used is also suitable when studying the fate and transport of targeted microbial groups in environmental waters, and therefore in the improvement of current quantitative microbial risk assessment models. Future studies are needed to determine if enterococci species (group)-specific assays correlate better with risks than genus-specific assays and can then be of value in public health and environmental monitoring studies.

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