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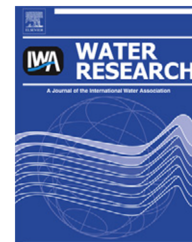

Enterococcus species composition determined by capillary electrophoresis of the *groESL* gene spacer region DNA

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ABSTRACT

Marine recreational beaches are monitored for fecal contamination by *Enterococcus* spp. (ENT) counts. Although different ENT species in the environment tend to thrive in and originate from distinct hosts, the current monitoring method does not differentiate among species. Time-consuming isolation-based species identification precludes routine analysis of environmental ENT communities. Therefore, an isolation-independent DNA fingerprinting method was developed to characterize environmental ENT communities using DNA length polymorphism of the spacer region between the *groES* and *groEL* genes common to most ENT species. Capillary electrophoresis resulted in distinct peak sizes of PCR products that carried polymorphic *groESL* spacers (300–335 bp in length) among 8 different ENT species (*Enterococcus avium*, *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Enterococcus mundtii*, *Enterococcus hirae*, *Enterococcus faecium*, *Enterococcus durans*, and *Enterococcus faecalis*). Distortions in true species ratios observed in electropherograms were caused by PCR biases arising in a mixed ENT community DNA template. *E. faecalis* was overestimated and *E. avium* and *E. faecium* were underestimated compared to the original species ratios in the mixed community. The PCR product bias was constant between species, so good approximation of the species ratio in ENT communities is possible. In environmental samples, a high percentage of *E. faecalis* (96%) together with high total ENT counts were observed in samples collected from a sewer line and from several sites in a storm drain system where sewage leaks were suspected. In contrast, samples with <400 CFU 100 ml^{-1} ENT were either dominated by *E. mundtii* or had 4 or more ENT species. The latter ENT community profiles are considered to be signatures of enterococci rarely associated with animals with low or of non-fecal origin.

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1. Introduction

Enterococcus spp. (ENT) are employed as the standard bacterial indicator to monitor fecal contamination for marine recreational beaches since ENT are abundant in the digestive tracts of warm-blooded animals including humans, and their abundance in the water column correlates to the cases of gastrointestinal illnesses (Cabelli et al., 1982). Beach managers

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assume that a change in the numbers of fecal indicator bacteria represents a change in human-related fecal inputs that threaten human health, and that indicators behave similarly to human pathogens in the environment.

Although the current ENT monitoring method counts bacteria that belong to the genus *Enterococcus*, not all species in the genus are residents of human intestinal tracts. Different species of ENT, although species overlap hosts, tend to be specific to certain ranges of host animals. For example, *Enterococcus faecalis* and *Enterococcus faecium* are common in human or dogs, while *Enterococcus avium*, *Enterococcus hirae*, *Enterococcus durans*, and *Enterococcus gallinarum* are rarely found or absent in human hosts (Devriese et al., 1987; Layton et al., 2010; Meier, 1998; Wheeler et al., 2002). ENT species such as *Enterococcus casseliflavus* and *Enterococcus mundtii* also thrive in non-intestinal environments such as meadows grasses (Müller et al., 2001) or in livestock manure (Graves et al., 2009).

Further, recent studies suggest long-term survival of ENT in the environment (Anderson et al., 2005; Lleo et al., 2005; Lleo et al., 2001; Kline et al., 2007; Pettibone et al., 1987; Signoretto et al., 2005). The survival rates may vary depending on species- or strain-specific characteristics. In the environment, species composition of ENT varies (Bonilla et al., 2006), probably reflecting fecal inputs from different host animals, different environmental survival rates of individual ENT species and strains, and non-fecal inputs of environmental species. Therefore, not only the number, but also the species structure of ENT communities provides valuable information for determining the sources of ENT inputs, and thus, sources of fecal contamination to recreational waters.

To determine species composition of environmental ENT communities, differentiating ENT species by culture-dependent methods is time-consuming and labor-intensive, requiring a large number of isolates to be typed to obtain statistically significant data. Furthermore, ENT species identification by biochemical tests is not fully reliable at the species level (Freney et al., 1992; Moore et al., 2006; Winston et al., 2004). Therefore, current methods for studying or monitoring ENT communities by typing individual environmental isolates are not practical or accurate.

Molecular methods to differentiate species-specific DNA sequences among ENT bacteria can potentially be applied to the analysis of species structure of environmental ENT communities without prior isolation and subsequent analysis of individual isolates. Tsai et al. (2005) demonstrated that an intergenic spacer region between heat shock genes, *groES* and *groEL*, of ENT was polymorphic in length among different species. The length of *groESL* spacer is species-specific and varies from 17 bp to 57 bp among species tested. The objective of the current paper was to develop and evaluate an isolation-free ENT community analysis method based on high resolution DNA size differentiation, *i.e.*, capillary electrophoresis, which targets the *groESL* spacer of ENT species. First, genus specificity of the PCR primer set was confirmed *in silico* and by control PCR with ENT and non-ENT bacterial strains. Second, to optimize the PCR fragment size and to tag the DNA fragment with a fluorescent dye for high resolution capillary electrophoresis, new primers for the nested PCR were designed. Third, sensitivity and specificity of ENT species differentiation by capillary electrophoresis together with PCR bias in mixed communities were evaluated using type strains of eight ENT species. Finally, the method proposed in this study was applied to a storm drain pipe system, which received various incoming flows including suspected sewage leaks, to characterize their ENT communities.

2. Materials and methods

2.1. Control bacterial strains and DNA extraction

Eight ENT species were used as positive controls (*E. avium*: ATCC14025, *E. gallinarum*: ATCC49573, *E. casseliflavus*: ATCC25788, *E. mundtii*: ATCC43186, *E. hirae*: ATCC8043, *E. faecium*: ATCC19434, *E. durans*: ATCC19432, and *E. faecalis*: Carolina item no. 155600). Each species has a unique length in the *groESL* spacer region (Table 1). The three negative control species (*Streptococcus bovis*: ATCC33317, *Streptococcus equinus*: ATCC9812, *Streptococcus pyogenes*: Carolina item no. 155630A, and *E. coli* K12 MG1655: ATCC700926) were purchased from the American Type Culture Collection (Manassas, VA) and Carolina Biological Supply Company (Burlington, NC). Each ENT or *Streptococcus* strain was incubated in 1 ml of brain heart infusion broth at 35 °C overnight and centrifuged to harvest the cells. For *E. coli*, Luria Bertani broth was used for incubation. The bacterial cells were suspended in sucrose lysis buffer (Massana et al., 1997) and incubated with 1 mg ml^{-1} of lysozyme for 30 min at 37 °C followed by 3-h incubation with 500 μg ml^{-1} proteinase K, and 1% SDS at 55 °C. DNA was extracted using three cycles of phenol-chloroform-isoamyl alcohol (25:24:1) extraction followed by chloroform-isoamyl alcohol (24:1) extraction, and was precipitated with 0.15 M sodium acetate (pH 5.2) and 1:1 volume of 100% isopropanol. The DNA was washed with cold 70% ethanol, and eluted in

Table 1 – Expected and observed DNA fragment lengths of *groESL* spacer of eight different ENT species that were PCR-amplified by nested PCR primers, Ent-ESL-SPFW4 and 6FAM-RV1EntGroSP, and analyzed by capillary electrophoresis. Expected sizes were calculated from the *groESL* spacer length reported by Tsai et al. (2005) and by adding flanking regions to the site by nested PCR (276 bp). Observed spacer lengths from electropherograms were determined with 6-FAM-tagged PCR products of both single species and those of mixtures of known ENT species.

ENT species	*groESL* spacer (bp)	PCR amplicon size (bp)	
		Expected size	Observed size
E. avium	24	300	299[a], 300
E. gallinarum	28	304	303[a], 304
E. casseliflavus	32	308	308
E. mundtii	36	312	312
E. hirae	39	315	315
E. faecium	50	326	327
E. durans	52	328	328, 329[a]
E. faecalis	57	333	334, 335[a]

a Observed in multi-species mixtures.

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