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Real-time PCR method to detect *Enterococcus faecalis* in water

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Abstract

A 16S rDNA real-time PCR method was developed to detect *Enterococcus faecalis* in water samples. The dynamic range for cell detection spanned five logs and the detection limit was determined to be 6 cfu/reaction. The assay was capable of detecting *E. faecalis* cells added to biofilms from a simulator of a water distribution system and in freshwater samples. Nucleic acid extraction was not required, permitting the detection of *E. faecalis* cells in less than 3 h.

Introduction

Fecal enterococci are normal inhabitants of the gastrointestinal tract of animals (Devriese *et al.* 1992, Pourcher *et al.* 1991). Fecal enterococci densities correlate better with the incidence of gastrointestinal illnesses in recreational bathers than fecal coliform densities, and, consequently, they have been suggested to be a superior bacterial indicator of fecal contamination in recreational waters (Dufour & Ballentine 1986). *Enterococcus faecalis* is frequently isolated from environmental waters and has recently been used as a human fecal indicator for microbial source tracking (Wheeler *et al.* 2002). Moreover, in conjunction with *E. faecium*, *E. faecalis* has been linked to the recent increases in vancomycin-resistant enterococci (VRE) strains isolated from clinical samples (Nelson *et al.* 2000). The membrane filtration technique, which is conventionally used to enumerate fecal enterococci in environmental waters, can also be used to detect *E. faecalis.* However, the additional biochemical characterization needed to confirm the identity of environmental isolates, means that detection of *E. faecalis* strains can take a minimum of 72 h.

One problem associated with growing bacteria in artificial media relates to the poor culturability of injured and stressed organisms. This problem is exacerbated when selective media are used as selective agents can have an inhibitory or toxic effect on injured targeted bacteria. PCR methods based on 16S rDNA provide an alternate means of detecting bacteria without requiring isolation by pure culture techniques (Wang *et al.* 1996). However, conventional PCR methods also require additional steps to confirm the presence of the target gene in an environmental sample (e.g., cloning and sequencing). Recently, a real-time PCR method was developed to rapidly determine the presence of microorganisms in complex samples, including environmental and clinical samples (Cox *et al.* 1998, Oberst *et al.* 1998, Sen 2000). This method relies on the cumulative detection of the fluorescent signal that results from the degradation of a fluorescently labeled oligonucleotide probe capable of hybridizing to one of the DNA strands within the two PCR primer regions. The specificity introduced by this oligonucleotide probe introduces a high level of confidence concerning the detection of target microorganisms in environmental samples. The objective of this study was to develop and evaluate the use of real time PCR to detect *E. faecalis.* We demonstrate the specificity of the primers against other fecal enterococci species and determine the sensitivity of the method in different water samples.

Materials and methods

Bacterial growth conditions and strain information

The following species were used in this study: *Enterococcus faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *E. dispar*, *E. mundtii*, *E. seriolicida*, *E. sulfureus*, *E. raffinosus*, *E. saccharolyticus*, *E. malodoratus*, *E. dispar*, *E. casseliflavus* and *E. gallinarum.* These strains were obtained from R. Patel (Mayo Clinic, Rochester, MN) and were previously identified using 16S rDNA sequencing analysis (Patel *et al.* 1998). Bacteria were grown in Tryticase Soy Broth (Difco, Detroit, MI) overnight, washed twice in autoclaved distilled water via centrifugation, and resuspended to a final cell density of 1–2 $\times$ 10^8 cfu ml^{-1}.

Development and evaluation E. faecalis *specific assay*

The approach described by Haugland *et al.* (1999) was used to develop primers and a TaqMan probe specific to *E. faecalis* 16S rDNA. Regions with considerable sequence differences between *E. faecalis* and other enterococci species were determined using Lasergene99 (Dnastar, Inc. Madison, WI). Optimal primers within the selected region and specific to *E. faecalis* were determined using the Oligo 6 Primer Analysis Software (Molecular Biology Insights, Inc., Cascade, CO). The TaqMan probe was developed using the ABI Primer Express program (Applied Biosystems, Foster City, CA) and labeled using 6-carboxyfluorescein as the reporter at the 5′ end and 6-carboxytetramethylrhodamine as the quencher at the 3′ end. The primers and probe sequences were challenged against gene sequences found in public databases (GenBank) using the Blast software (http://www.ncbi.nlm.nih.gov:80/BLAST) and the Sequence Match Program (Ribosomal Databases Project) (Maidak *et al.* 2000). The sequences used in this study were CGCTTCTTTCCTCCCGAGT, GCCATGCGGCATAAACTG, and CAATTGGAAAGAGGAGTGGCGGACG, for the forward primer, reverse primer, and internal oligonucleotide probe, respectively.

The specificity of the TaqMan assay was determined by testing it against fourteen different enterococci species. Aliquots (5 μl) of *E. faecalis* resuspended cells were added to the final reaction mix and processed as described below. *E. faecalis* cells were also added to water samples from Burnet Woods pond (located approx. 0.5 km east of the EPA laboratory, Cincinnati, OH) to determine the specificity and efficiency of the assay in a natural water sample. Fecal enterococci counts were determined using mEI agar (Difco) and the membrane filtration method as suggested by Messer & Dufour (1998). Total heterotrophic bacterial counts for Burnet Woods samples were determined using R2A agar (Reasoner & Geldreich 1985).

Real-time PCR studies

All reactions were performed as described by Haugland *et al.* (1999) with the following modifications. Briefly, DNA template consisted of 5-μl from water samples containing bacterial cells. The final concentrations of the primers and probe were 1 μM and 80 nM, respectively. A total volume of 25 μl was used in the assays and a total of 40 cycles were performed using an ABI 7700 sequence detector. In order to minimize potential contamination, all TaqMan reagents were manipulated in a biological hood that had been UV treated. Serial dilutions were performed using autoclave-sterilized deionized water that was irradiated with UV light. Pure cultures resuspended in deionized water (5 μl of 1–2 $\times$ 10^7 cfu ml^{-1}) were initially added to the reaction mix to empirically test the specificity of the TaqMan procedure. Unammended deionized water was used as negative control.

The potential effect of PCR inhibitors present in environmental samples was tested by spiking different densities of *E. faecalis* into water samples from the Burnet Woods, to biofilm material obtained from a simulated water distribution system and to a human stool sample containing low densities of fecal enterococci. The biofilm and human stool material were diluted 10-fold with phosphate buffered water and double distilled water, respectively, prior to adding the *E. faecalis* cells. In some experiments, 100 μl were taken from seeded samples and a 10 fold dilution series was performed prior to the TaqMan reactions. Aliquots (5 μl) from these samples were then added to the TaqMan mixture.

The presence of culturable *E. faecalis* in the Burnet Woods pond was determined by first enriching for fecal enterococci using the membrane filtration method. Pond water (i.e., 1 ml, 10 ml, and 100 ml) was fil-

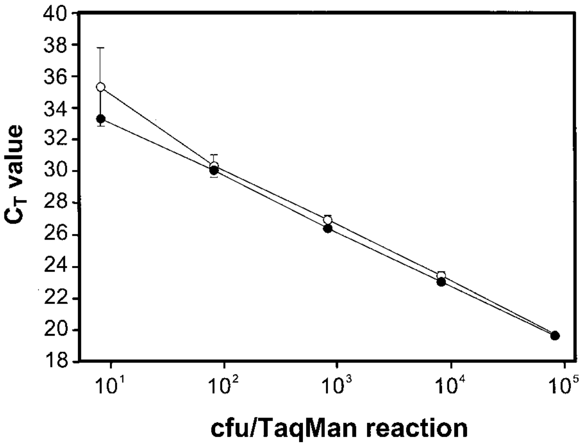

Fig. 1. Dynamic range for the detection of *E. faecalis* with the TaqMan assay. Cells were added to sterile deinonized water (○) or Burnett Woods pond water (●). In both cases the R^2 was greater than 0.98. Each data point represents the cycle threshold (C_T) average of three samples per reaction.

tered onto cellulose acetate membranes, which were then placed on mEI agar and incubated at 42 °C. After 24 h, membranes were transferred to sterile tubes containing 2 ml of sterile water, and vortex for 30 s to remove microbial biomass. Aliquots from these samples were used in the Taqman assays as described above. In another experiment, *E. faecalis* cells (approx. 10^9 cfu ml^{-1}) were serially diluted and spiked into sterile water samples that were filtered onto cellulose acetate membranes and processed as described above, with the exception that membranes were only incubated for 4 h.

Results and discussion

All bacteria identified as *E. faecalis*, including all environmental strains, showed a cycle threshold (C_T) value for *E. faecalis* strains of 15–18 for 10^5 cfu per reaction. C_T is defined as the point when a PCR product has reached logarithmic amplification and is used to calculate the number of bacteria containing the gene target present in a given sample. The larger the population size of the targeted bacteria the fewer cycles it will take to reach logarithmic amplification and therefore the smaller the C_T value (Figure 1). No signals (i.e., $C_T = 40$) were detected when the assay was challenged against non-*E. faecalis* fecal enterococci bacteria at 10^7 cfu per ml. Although the *E. faecalis* TaqMan assay was tested against a limited number of bacterial species, both the forward and reverse primers and the internal oligonucleotide probe showed a minimum of two to three nucleotide mismatches with the closest non *E. faecalis* sequences available in GenBank. The poor sequence homology of the primers against other enterococci species, the stringency of the reaction, and the results obtained with closely related enterococci species strongly indicates that the TaqMan protocol developed in this study is highly specific for the detection of *E. faecalis.* An excellent correlation between *E. faecalis* identification via biochemical characterization using a commercially available kit (API Strep 20; BioMerioux, France) and TaqMan assay was observed for the 26 environmental strains tested in this study (data not shown).

C_T values showed a linear correlation with *E. faecalis* densities over a five log dynamic range (Figure 1). Culturable counts of *E. faecalis* were used to estimate of the number of cells added to the water samples. The results indicate that the sensitivity of this approach was on average 6 cfu of *E. faecalis* per assay. The C_T values obtained with freshwater samples and biofilm material from a water distribution system amended with *E. faecalis* cells were not different from the C_T values obtained in amended distilled water (Figures 1 and 2A), suggesting that no PCR inhibitors were present in these particular environmental samples. Since the total heterotrophic bacterial counts for the pond and water distribution system samples were approximately 10^7 and 10^5 cfu ml^{-1}, respectively, these results provide further evidence for the specificity of the *E. faecalis* procedure. In contrast, samples containing fecal material showed a considerable inhibitory effect, as the samples required a 1000-fold dilution before a signal was detectable (Figure 2B). In this particular case, a DNA extraction step that includes the removal of PCR inhibitors might increase the sensitivity of the reaction.

E. faecalis signals were not detected in any of the environmental water samples tested with the *E. faecalis* TaqMan assay. These results can be explained by the fact that the fecal enterococci density in each of the samples was below the detection limits of the TaqMan procedure. For example, the fecal enterococci densities in pond water samples fluctuated from 15 to 55 cfu/100 ml and only 36% of the enterococci strains isolated from the pond were identified as *E. faecalis* (data not shown). However, studies using biomass harvested from membrane enrichments showed the presence *E. faecalis* (Figure 3). Therefore, in some cases an enrichment step or biomass concentration via membrane filtration might be necessary in order

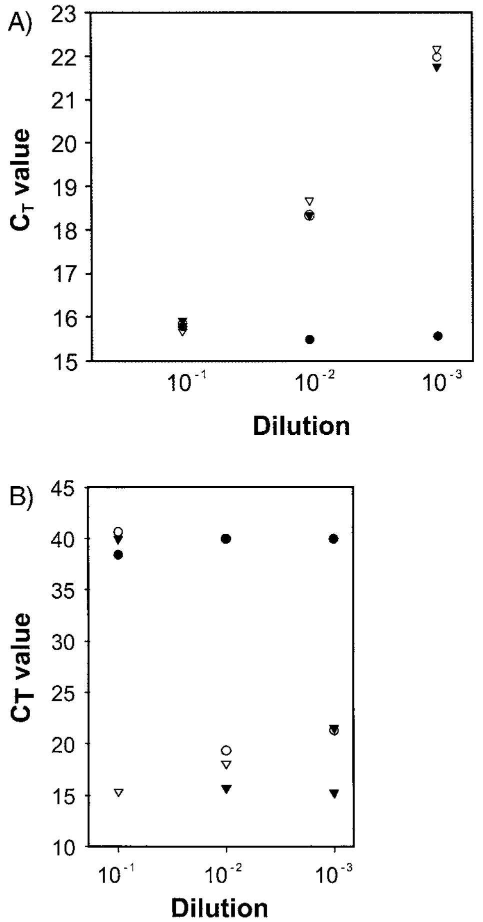

Fig. 2. Detection of *E. faecalis* in water distribution system biofilms (A). *E. faecalis* cells were added to previously 10 to 1000 fold diluted biofilm (●) or to undiluted biofilm (▼) which were then serially diluted. Alternatively, cells were first diluted 10 to 1000 fold in sterile water and aliquots were added to undiluted biofilm (○). The controls consisted in cells serially diluted in sterile water (▽). The assay was also performed in water samples containing fecal material (B). In this case the assays were performed against water samples contain diluted fecal material with no *E. faecalis* cells added (●), against water containing fecal material with *E. faecalis* cells added prior to the sample being serially diluted (○), and against water containing diluted fecal material that contained the same number of added *E. faecalis* cells (▼). The control consisted of *E. faecalis* cells serially diluted in sterile water (▽).

to detect *E. faecalis* in waters containing low densities of fecal enterococci. We found that a minimum of a 4 h incubation was needed to detect the presence of 60–100 cfu of *E. faecalis*/100 ml of water (Figure 4). Since it is possible to reduce the volume of water used to detach the cells from the filtration membrane, these results also suggest that considerable shorter incubation times might be required for the detection of enterococci at cell densities higher than

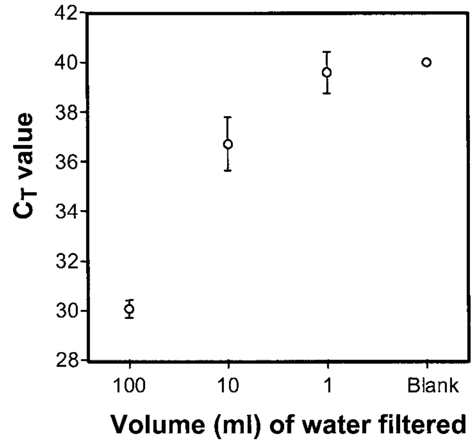

Fig. 3. Detection of culturable *E. faecalis* in freshwater samples with low levels of fecal enterococci. Water samples from Burnett Woods pond (100 ml, 10 ml, and 1 ml) were filtered onto cellulose acetate membranes and transferred to mEI media. After 24 h incubation, membranes were processed as described in the Materials and methods section. Control consisted in a sterile water sample (with no added cells) that was filtered onto a membrane and processed as indicated above (referred as the blank).

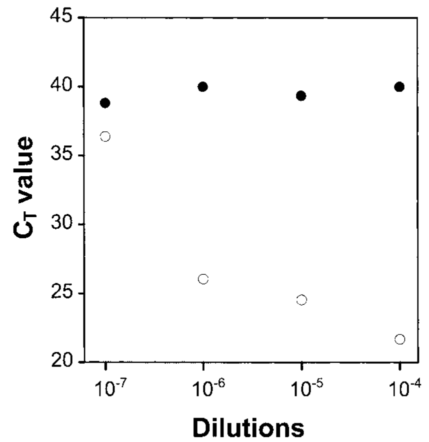

Fig. 4. Detection of *E. faecalis* using an enrichment step. *E. faecalis* cells were serially diluted and added to 100 ml of sterile water. Samples were then filtered onto cellulose nitrate filters, incubated for 4 h at 42 °C (○). Controls consisted in samples filtered and immediately stored at −20 °C (●). Numbers in the X-axis represent ten fold dilutions of the original culture. The 10^{-7} sample had an average of 60 cfu per dilution bottle. Experiments were performed in triplicates. Standard errors were less than 5%. Membranes were processed as in Figure 3.

600 cfu/100 ml.

In conclusion, the results from this study suggest that a TaqMan-based approach can be used to detect the presence of specific enterococci in environmental waters. The detection and phylogenetic identification of the targeted bacterial species using TaqMan chemistry could be performed in a matter of a few hours, either while the organisms are part of a microbial community or once they have been isolated in pure culture. Therefore, detection is attainable in substantially less time than required by most biochemical tests and phylogenetic methods, especially if a DNA extraction step is not required. Future improvements in TaqMan technology (e.g., time needed for each amplification cycle) may result in a further reduction in detection time (Belgrader *et al.* 1999). Because conventional phenotypic identification methods are cumbersome, require up to 72 h for results, and are sometimes inaccurate, the method described here has the potential for providing rapid detection of *E. faecalis* in water. The assay may prove useful for rapid assessment of recreational water quality increasing our knowledge regarding the ecology of *E. faecalis.*

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