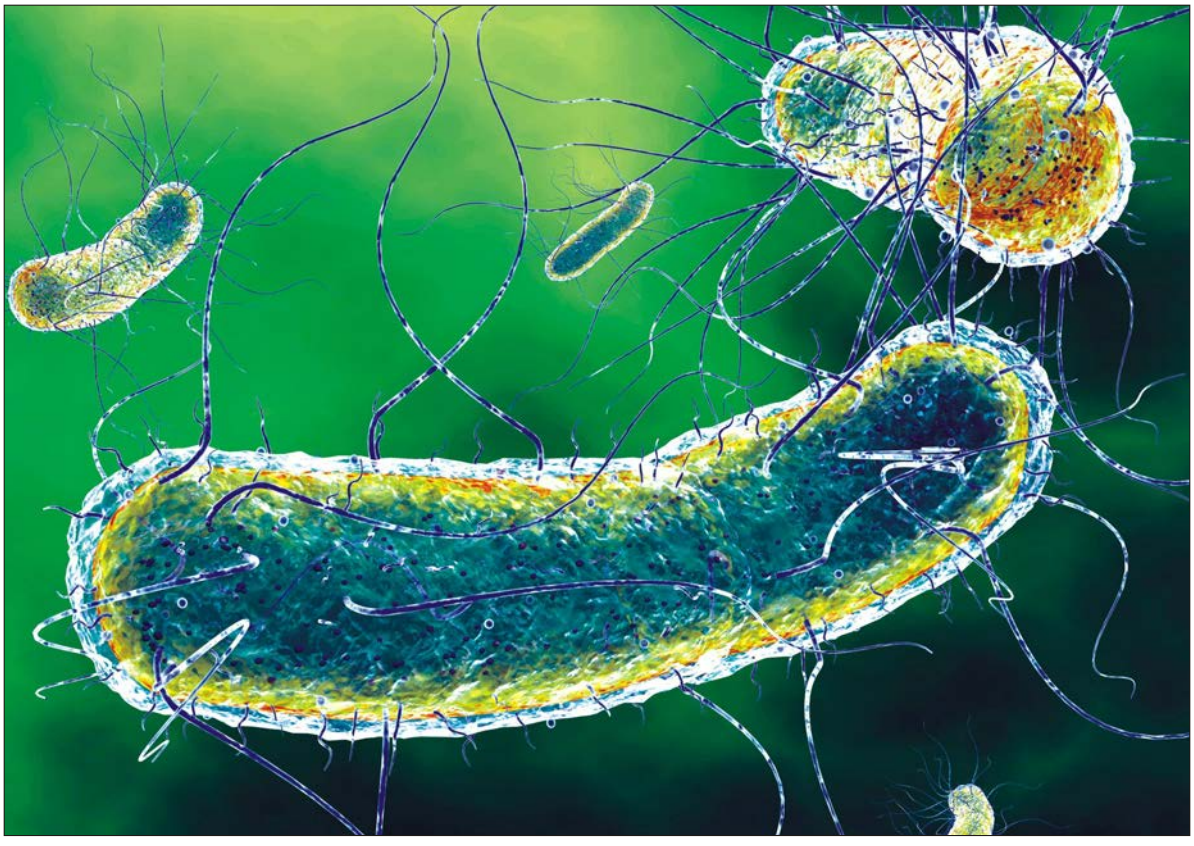

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Detection of *Esp* Enterococcal Surface Protein and *Bacteroides* Markers in Water Samples

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ABSTRACT

Finding the source of fecal pollution in various water resources has been becoming globally important. Molecular marker detection is the most reliable method for bacterial tracking. Since, rapidness is an important factor for the detection of water contamination, we have assessed the detection of water fecal contamination within a short period of time. In this study, twenty-two water samples, including 12 surface water from two rivers in East and West of Tehran (Iran) and 10 water samples from private wells were collected. In addition, five samples were collected from a Sewage Treatment Plant (STP) located in the West of Tehran. All of the samples were tested for the presence of fecal coliforms by Most Probable Number (MPN) method. Polymerase Chain Reaction (PCR) was performed for detection of enterococcal surface protein (Esp) and *Bacteroides* 16S rRNA. Direct extraction and colony extraction were simultaneously done for the detection of enterococcal contamination. The results showed that the coliform concentration for water samples ranged from >3->1100 MPN/100 mL. The PCR for *esp* was positive for 5 (23%) water samples. No samples were positive for 16S rRNA for *Bacteroides* with the exception of the samples obtained from the STP. Our results indicated that using PCR method directly following optimizing DNA extraction from environmental water sources is not suitable for the detection of human enterococcal contamination, while the use of colony extraction method is preferable.

Key words: *esp*, enterococci, water, fecal indicator, *Bacteroides*, MPN

INTRODUCTION

Two types of Bacterial Source Tracking (BST) including isolate-by-isolate typing of bacteria cultured from the water samples and the PCR detection of the bacterial genetic markers have been described (Stoeckel and Harwood, 2007). The development of various BST in water systems has received a great deal of attention lately to discriminate between the sources of fecal pollution in waters (Ahmed *et al*., 2008a).

Molecular techniques have been shown to be rapid, sensitive and a reliable replacement for bacterial culture for the detection of the source of fecal pollution in water samples (Griffith *et al*., 2003). *Escherichia coli* and members of the *Enterococcus* spp. have traditionally been used as water

quality indicators (Frahm and Obst, 2003). Their presence are used as a warning for fecal pollution in the drinking and recreational water (Scott *et al*., 2005).

Enterococcal surface protein (*Esp*) in *E. faecalis* has been associated with increased morbidity, colonization and biofilm formation (Van Wamel *et al*., 2007). A variant of *esp* gene has also been found in *E. faecium* isolated from humans (Hammerum and Jensen, 2002). The presence of this marker has been reported in the environmental water polluted with sewage (McQuaig *et al*., 2006). However, no correlation has been made between the concentration of the *esp* marker in polluted water and the degree of the fecal contamination. In addition, *Bacteroides* spp., the most common intestinal flora of warm blooded animals (Wexler, 2007), has been indicated as an indicator for the water fecal contamination and has been used as the BST in different countries (Bernhard *et al*., 2003; Gourmelon *et al*., 2007; Seurinck *et al*., 2006; Okabe *et al*., 2007).

In the present study, we assessed the use of *Esp* with both direct and colony extraction methods as well as 16S rRNA gene sequencing for immediate detection of fecal contamination in water samples including surface and well.

MATERIALS AND METHODS

Sample collection: Water samples were collected during July 2012 and December 2012. Twenty-two water samples were collected from private wells (10) and surface water (12) from two rivers located at the East and West of Tehran. Private well samples were collected from ten different wells which were used for drinking and agricultural irrigation in West of Tehran. Samples were collected from raw sewage (5) from urban STPs from West part of Tehran. All samples were kept refrigerated and transported to the microbiology laboratory for examinations. Bacteriological examination for the estimated MPN of coliforms was carried out using the multiple tube technique with nine tubes per dilution (Sutton, 2010).

For molecular and microbiological examination, the samples were diluted to1/10 and 1/100 with Phosphate-Buffered Saline (PBS) and then filtered on a 0.45 µm membrane (47 mm diameter) (Millipore Corporation, MA, USA).

Sample treatment prior to PCR: For direct DNA extraction from surface water, sewage and well samples, 250 mL of water samples were filtered through 0.45 µm nitrocellulose membranes. The filters were folded and immersed into PBS in 50 mL centrifuge tubes. The tubes were vortexed at high speed for 20 min. Then the filters were removed and the tube centrifuged at 8000 g for 15 min, after which 50 mL of the supernatant was discarded and the remaining sample was mixed by high speed vortexing. The pellet was then used for DNA extraction by High Pure PCR Template Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany) (Srinivasan *et al*., 2011).

Furthermore for colony extraction and Total Enterococcal Count (TEC) after water filtration, the filters incubated at 44°C on mEnterococcus (mE) agar (Difco, MI, USA) for 48 h. The filters were the transported to 10 mL normal saline and vortexed vigorously. After centrifugation, DNA was extracted by High Pure PCR Template Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany) (Scott *et al*., 2005).

PCR analysis: PCR was performed using the primers listed in Table 1. The PCR assay was carried out in a total volume of 25 µL containing 10 mM Tris- HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.5 U of Taq DNA polymerase (HT Biotechnology, Cambridge, UK) and each primer

Table 1: Primers used for detection of water contamination

Genes	Primer sequence (5'¬3')	Product length (bp)	References
Esp-F	5-AGATTTCATCTTTGATTCTTGG	510	Vankerckhoven *et al*. (2004)
Esp-R	5-AATTGATTCTTTAGCATCTGG		
E. faecium-F	5-TTGAGGCAGACCAGATTGACG	658	Kariyama *et al*. (2000)
E. faecium-R	5-TATGACAGCGACTCCGATTCC		
HF183-F	5-ATCATGAGTTCACATGTCCG	570	Bernhard *et al*. (2000)
Bac708-R	5-CAATCGGAGTTCTTCGTG		
Enterococci/23S rRNA-F	5-ATCTACCCATGTCCAGGTTGAAG	223	Srinivasan *et al*. (2011)
Enterococci/23S rRNA-R	5-CCATCTCGGGTTACCGAATTCAG		

F: Forward (upstream) primer, R: Reverse (downstream) primer. Esp: Enterococcal surface protein, Bac: *Bacteroides-Prevotella*, HF: Human-specific

(40 pmol). The PCR assay was carried out with 10 µL DNA volume for direct extraction method and 2 µL for colony extraction method. An initial activation step at 95°C for 15 min and 30 cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min) and extension (72°C for 1 min), followed by one cycle consisting of 10 min at 72°C for *esp*. HF183 markers were amplified in the cycling parameters as follows: An initial activation step at 95°C for 15 min and 30 cycles of denaturation (94°C for 30 sec), annealing (59°C for 1 min) and extension (72°C for 1 min) followed by final 6 min extension at 72°C. *Enterococcus faecium* were amplified in the cycling parameters as follows: An initial activation step at 94°C for 5 min and 30 cycles of denaturation (94°C for 1 min), for annealing (54°C for 1 min) and extension (72°C for 1 min) followed by one cycle consisting of 10 min at 72°C. Enterococci/23S rRNA were amplified in the cycling parameters follows: An initial activation step at 95°C for 10 min and 35 cycles of denaturation (95°C for 30s), annealing (57°C for 30 sec) and extension (72°C for 1 min) and terminated by a final extension cycle at 72°C for 8 min. The PCR products were analyzed by gel electrophoresis in 1.5% (w/v) agarose gel. Positive controls in the PCR reactions were *E. faecalis* ATCC29212 and *E. faecium* BM4147.

RESULTS

Most probable number: Fecal coliforms were detected in 95% (21/22) of the water samples and all five (100%) of the STP samples. Six of the water samples (27%) and the five STP samples had MPN of >1100/100 mL. Coliform concentration for the remaining water samples ranged from <3 to 460 MPN/100 mL (Table 2). Total Enterococcal Count (TEC) was performed for 22 water samples. Enterococcal densities ranged from 4 to >1000/100 mL for 20 of the 22 water samples. Enterococci were not detected in 100 mL of the water analyzed for two of the samples. The STP samples contained >1000 enterococci per 100 mL.

PCR analysis: The PCR analysis showed that in all STP samples, enterococcal and *esp* specific genes were detected either through direct filter extraction or bacteria colonies. Direct filter extraction exhibited 10 (45%) water samples including 9 surface water and one sample from wells which were positive for enterococcal gene. In comparison, 20 water samples (90%) were positive for enterococci by direct colony extraction of the enterococcal DNA. Subsequent PCR with species specific primers were showed the presence of *E. faecium* in all positive samples. The *esp* was detected in 1 and 5 surface water samples, which were extracted directly and from colonies, respectively (Table 2). Comparison of PCR detection with colony counts indicated that all samples with at least 37 CFU/100 mL were positive for enterococci by PCR. The *esp* gene was detected by colony extraction in six water samples. Bacteroides 16S rRNA marker was not detected in any samples by PCR.

Table 2: PCR results of the enterococci, human-specific *esp* markers and *Bacteroides* markers in samples from raw sewage and surface water and wells

Sources	MPN/ 100 mL	Enterococcal colony count CFU/100 mL	Enterococci		*esp*		*Bacteroides*
			Direct extraction	Colony extraction	Direct extraction	Colony extraction	
SW1	460	91	Positive	Positive	Negative	Negative	Negative
SW2	75	22	Negative	Positive	Negative	Negative	Negative
SW3	1100	151	Positive	Positive	Negative	Negative	Negative
SW4	460	201	Positive	Positive	Negative	Negative	Negative
SW5	460	19	Negative	Positive	Negative	Negative	Negative
SW6	1100	37	Positive	Positive	Negative	Positive	Negative
SW7	210	220	Positive	Positive	Negative	Negative	Negative
SW8	93	119	Positive	Positive	Negative	Positive	Negative
SW9	>1100	250	Positive	Positive	Negative	Negative	Negative
SW10	1100	250	Positive	Positive	Negative	Positive	Negative
SW11	1100	44	Positive	Positive	Positive	Positive	Negative
SW12	1100	26	Negative	Positive	Negative	Negative	Negative
Well 1	1100	>1000	Positive	Positive	Negative	Negative	Negative
Well 2	93	4	Negative	Positive	Negative	Positive	Negative
Well 3	9	<2	Negative	Negative	Negative	Negative	Negative
Well 4	4	<2	Negative	Negative	Negative	Negative	Negative
Well 5	460	24	Negative	Positive	Negative	Negative	Negative
Well 6	26	8	Negative	Positive	Negative	Negative	Negative
Well 7	<3	5	Negative	Positive	Negative	Negative	Negative
Well 8	23	8	Negative	Positive	Negative	Negative	Negative
Well 9	43	5	Negative	Positive	Negative	Negative	Negative
Well 10	21	7	Negative	Positive	Negative	Negative	Negative
Sewage 1	>1100	>1100	Positive	Positive	Positive	Positive	Positive
Sewage 2	>1100	>1100	Positive	Positive	Positive	Positive	Positive
Sewage 3	>1100	>1100	Positive	Positive	Positive	Positive	Positive
Sewage 4	>1100	>1100	Positive	Positive	Positive	Positive	Positive
Sewage 5	>1100	>1100	Positive	Positive	Positive	Positive	Positive

Repetitions of sampling were once

DISCUSSION

The most useful BST method should be specific and applicable over a broad geographic region (Scott *et al*., 2005). Finding the source of fecal pollution in various water resources has globally become important. Some of the current water quality guidelines are based on the total number of enterococci. While, these guidelines have been shown to be reliable as the general surveillance of water quality, molecular methods are more sensitive techniques for better characterization of the species composition and recognition of pollution in the BST (Scott *et al*., 2005).

Presently, accurate speciation of enterococci is ambiguous and difficult by the biochemical tests (Pourcher *et al*., 1991). Molecular tests for targeting the bacterial contamination of water sources could help rapid identification of suitability of water usage. Genes encoding for *esp* in *E. faecium* and HF183 16S rRNA in bacteroides are good examples of such markers (Scott *et al*., 2005; Jenkins *et al*., 2009).

Real time PCR has been used for detection of *esp* in environmental water (Ahmed *et al*., 2008b). However, an initial cultural enrichment step is required as the prerequisite to the application of real time PCR, making this process cumbersome and time-consuming. Similar to other studies (Scott *et al*., 2005; Ahmed *et al*., 2008a; LaGier *et al*., 2007), we could find *esp* directly from STP and one surface water samples, suggesting contamination by enterococcal species.

On the other hand, performing PCR by *E. faecium* and enterococcal 23S rRNA and esp primers have shown that the fifteen samples were positive for *E. faecium* and enterococcal

23S rRNA but they were negative for *esp*. This result indicated that some samples were contaminated by other sources beside human fecal.

Since, rapidness is an important factor for the detection of water contamination, direct DNA extraction from samples was performed. Only one sample, however, was positive for *esp* gene, suggesting *esp* is more frequently detected by PCR from colonies.

As expected, the data showed that direct DNA extraction from the water sources resulted in 45% positive, comparing to 90% from colony extraction. Although, the direct PCR detection from water was not as sensitive as PCR detection from the bacterial colony, the short time that needed to perform PCR direction detection was a great advantage. It can, therefore, be concluded that when PCR detection of a specific gene is negative, bacterial cultivation can be considered. In addition, contrary to the report by others who have indicated that 104 CFU mL^{-1} of enterococci were needed (Griffin *et al*., 2001) for PCR gene detection from bacterial colonies, our results showed that the PCR sensitivity of enterococci detection requires at least 37 CFU/100 mL, using lesser number of colonies in the present study may be due to primers used, DNA extraction method or PCR condition.

Previous studies have used cultural method for the detection of *esp* and bacteroides markers in sewage and environmental waters (Ahmed *et al*., 2008a, b) with time taken to detect these genes in 3-7 days. In the present study, all water samples were screened for HF183 and CF128 markers using conventional PCR. We, however, could not find these markers which could be due to the fact that we performed conventional PCR insist of real-time PCR.

Overall, our results indicated that using PCR method directly following optimizing DNA extraction from environmental water sources is not suitable for the detection of human enterococcal contamination, while the use of colony extraction method is preferable. Moreover, the direct extraction for detection of human enterococcal contamination were suitable only for STP and for the samples with high concentration of enterococcal contamination.

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