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Applicability of universal *Bacteroidales* genetic marker for microbial monitoring of drinking water sources in comparison to conventional indicators

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Abstract Water quality monitoring is essential for the provision of safe drinking water. In this study, we compared a selection of fecal indicators with universal *Bacteroidales* genetic marker to identify fecal pollution of a variety of drinking water sources. A total of 60 samples were collected from water sources. The microbiological parameters included total coliforms, fecal coliforms, *Escherichia coli* and fecal streptococci as the fecal indicator bacteria (FIB), *Clostridium perfringens* and H_2S bacteria as alternative indicators, universal *Bacteroidales* genetic marker as a promising alternative fecal indicator, and *Salmonella* spp., *Shigella* spp., and *E. coli* O157 as pathogenic bacteria. From 60 samples analyzed, *Bacteroidales* was the most frequently detected indicator followed by total coliforms. However, the *Bacteroidales* assay failed to detect the marker in nine samples positive for FIB and other alternative indicators. The results of our study showed that the absence of *Bacteroidales* is not necessarily an evidence of fecal and pathogenic bacteria absence and may be unable to ensure the safety of the water. Further research, however, is required for a better understanding of the use of a *Bacteroidales* genetic marker as an indicator in water quality monitoring programs.

Keywords Monitoring · Water sources · *Bacteroidales* · Fecal indicator bacteria

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Introduction

The microbial contamination of drinking water poses a serious public health threat worldwide (Cabral 2010), and waterborne pathogens are still a major source of infection (Craun et al. 2010). About one third of the world population suffer from waterborne diseases, and 2 million children die each year because of exposure to enteric pathogens associated with contaminated water in developing countries (OECD 2003; Figueras & Borreg 2010). Water quality monitoring is, therefore, essential for the provision of safe drinking water. It is also the cornerstone of any water safety plan to protect people from water-related diseases.

Conventionally, cultivation-based methods for fecal indicator bacteria (FIB) including total coliforms (TC), fecal coliforms (FC), *Escherichia coli*, and enterococci (Schriewer et al. 2010) have long been used to indicate

fecal pollution of water samples (Fremaux et al. 2009). FIB can be easily cultivated and have been widely used as indicators of public health risks associated with water polluted by human and animal feces (EPA 2000). However, some studies have demonstrated that these conventional fecal indicators do not adequately correlate with the presence of pathogenic microorganisms. In other words, the absence of FIB is not necessarily an evidence of enteric pathogen absence (Walters et al. 2009; Schriewer et al. 2010). Furthermore, the fact that FIB can proliferate in the environment (Savichtcheva & Okabe 2006) challenges the usefulness of FIB as ideal indicators of fecal contamination. Therefore, a number of alternative indicators of fecal contamination have been proposed, evaluated, and applied (Figueras & Borreg 2010).

Clostridium perfringens has been specified as a suitable indicator for the presence of pathogens of fecal origin (Ryzinska-Paier et al. 2011). *C. perfringens* is able to form spores and therefore is a better choice as an indicator to predict protozoan cysts and viruses (Araujo et al. 2004). The use of this indicator was proposed for monitoring of the drinking water quality, and the new European Union regulations consider more specifically *C. perfringens* as the indicator of choice (European Union 1998).

Detection of hydrogen sulfide-producing bacteria (H_2S test) is also considered as an alternative indicator of fecal contamination of water samples (Manja et al. 1982). It offers advantages including low cost, simplicity, and ease of application to environmental samples (Sobsey & Pfaender 2002).

In recent years, *Bacteroides* species are increasingly used as promising alternative fecal indicators to FIB (Bernhard & Field 2000; Ahmed et al. 2008; Stapleton et al. 2009). These bacteria have distinct advantages over FIB. They can be relatively easily detected in aquatic environments because they make up a significant portion of the intestinal microflora and have little potential for growth in the environment (Savichtcheva & Okabe 2006). Furthermore, the development of nucleic acid-based methods for *Bacteroidales* detection has provided a fast, reliable, and relatively inexpensive mean (Bernhard et al. 2003; Ahmed et al. 2008). However, *Bacteroidales* are obligate anaerobes and may not persist in aerobic environments in the same way as FIB (Kreader 1995). Potential difference in their persistence could lead to different risk assessment interpretations (Walters et al. 2009). Therefore, extensive field testing is required to assess the presence of these fecal contamination genetic markers against the commonly used indicators, before these markers can be used for routine water quality monitoring.

Previous studies regarding the behavior of *Bacteroidales* genetic markers against culturable FIB have focused largely on relatively heavily polluted waters (Bernhard & Field 2000; Ahmed et al. 2009; Fremaux et al. 2009; Gourmelon et al. 2010) and there are a few studies about drinking water sources (Saunders et al. 2009; Agudelo et al. 2010; van der Wielen & Medema 2010). In our knowledge, this is the first study which compares a selection of fecal indicators with universal *Bacteroidales* genetic marker to identify fecal pollution of a variety of drinking water sources. Furthermore, the study was also conducted to determine any predictive relationship between fecal indicators and enteric pathogens.

Methods

A total of 60 water samples were collected from various drinking water sources including wells, springs, aqueducts, and a river in the Isfahan province located in central part of Iran. All samples were collected in sterile glass bottles, transferred to the laboratory in an insulated box, and processed immediately after arrival at the laboratory. The microbiological parameters included total and fecal coliforms, *E. coli* and fecal streptococci (FS) as the fecal indicator bacteria, *C. perfringens* and H_2S bacteria as alternative pollution indicators, universal *Bacteroidales* 16S ribosomal RNA (rRNA) genetic marker as a promising alternative fecal indicator, and *Salmonella* spp., *Shigella* spp., and *E. coli* O157 as pathogenic indicators.

Fecal indicator bacteria

Total and fecal coliforms, *E. coli*, and FS were determined by multiple-tube techniques as described in *Standard Methods* (APHA 2005). Each test was performed with 100 ml of water samples. It was formatted in a 10-tube MPN arrangement. The number of bacteria per 100 ml was estimated from a 10-tube MPN table, and results were reported based on the most probable number (MPN) of detected microorganisms per 100 ml.

Alternative indicators

Detection of alternative pollution indicators including *C. perfringens* and H_2S bacteria was also performed with 100 ml aliquots of water samples which was formatted in a 10-tube MPN arrangement.

For detection of *C. perfringens*, water samples were incubated at 37 °C for 24–48 h in thioglycolate broth in an atmosphere containing 9–13 % carbon dioxide. The positive tubes were then sub-cultured anaerobically on Tryptose Sulphite Cycloserine (TSC) agar. Black, gray, or yellow brown colonies were considered as presumptive *C. perfringens*. The confirmation procedure consisting of Gram staining, motility, and nitrate reduction was performed on suspected colonies (Environment Agency 2010). Suspected colonies were also further verified by CP ChromoSelect agar.

The H_2S test was carried out as described by Manja et al. (1982) except that L-cysteine was added as a medium component (Manja et al. 1982).

Pathogenic microorganisms

For isolation and identification of pathogenic microorganisms, the *Standard Methods* procedure was followed for the detection of *E. coli* O157, *Salmonell*a spp., and *Shigella* spp.

A loopful from each EC tube showing growth was spread plated onto sorbitol-MacConkey agar. Plates were incubated at 35 °C for 18 to 24 h. Colorless colonies were subsequently transferred into lauryl tryptose broth containing 4-methylumbelliferyl-*B*-D-glucuronide (LTB-MUG) and incubated at 35 °C for 18 to 24 h. Following incubation, test tubes were exposed to UV light and non-fluorescent tubes were considered as *E. coli* O157.

For detection of *Salmonella* and *Shigella* spp., 1,000 ml of water samples were concentrated by membrane filtration (0.22 μm, 47 mm in diameter, Millipore). Membrane filters were washed in 100 ml of peptone water and incubated for 6 h at 37 °C for pre-enrichment. Aliquots of peptone water (10 ml) were inoculated into 10-ml double strength Selenite F medium tubes and subsequently streaked from each positive tube to plates of xylose lysine deoxycholate (XLD) agar. Following incubation, suspected colonies were confirmed and identified with cultural and biochemical tests using triple sugar iron (TSI) agar, urea agar base, and SIM medium (APHA 2005).

Total *Bacteroidales* assay

Of water samples, 500-ml volumes were concentrated by membrane filtration (0.22 μm, 47 mm in diameter, Millipore). Membrane filters were washed in sterile phosphate buffer solution, shaken for 15–30 min, placed in an ultrasonic bath for 5 min for optimum clean-up of the filters, and then concentrated by centrifugation.

To extract DNA, the resuspended pellets were frozen in liquid nitrogen and heated in boiling water three times. The DNA was further extracted and purified using Promega DNA Extraction Kit (Promega Wizard® Genomic DNA Purification Kit, Madison, USA) according to manufacturer's instruction. The purified DNA was finally recovered in 25 μl of distilled water and used in nested PCR assay for detection of *Bacteroidales*.

In the first PCR step, a ~1,420-bp fragment of 16S rRNA gene region of bacteria was amplified using the bacterial primer set Eubac27F and 1492R (Lane 1991) to check the nucleic acid extraction as well as the presence of inhibitors. For the detection of *Bacteroidales*, a nested PCR technique was applied in order to increase the sensitivity. In the second PCR step, the universal *Bacteroidales* 16S rRNA genetic marker was amplified by using the Bac32F and Bac708R (Bernhard & Field 2000). The PCR amplification was conducted in a final volume of 25 μl consisting of 2.5 μl of 10× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTPs, 2 units of Taq DNA polymerase, and 2 μl of DNA. All PCR assays contained a positive and a negative control. PCR was performed with an initial denaturation for 5 min at 94 °C followed by 30 cycles of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1.30 min, and a final extension at 72 °C for 5 min. The PCR products were analyzed by agarose gel electrophoresis using 1.5 % gels containing ethidium bromide together with a DNA molecular weight marker. Gels were viewed on a UV transilluminator (UV Tech, France), and DNA fragment sizes were compared with the 100-bp ladder DNA.

DNA sequencing of PCR fragment of universal *Bacteroidales* 16S rRNA gene was also carried out to confirm the identity of the gene.

Sensitivity analysis

The sensitivity of the entire assay for total coliforms and *Bacteroidales* was tested by preparing 10-fold serial

dilutions of raw wastewater. Of serial dilutions, 2 ml was added to 1 l of drinking water. Of seeded water samples, 100- and 500-ml volumes were analyzed for total coliforms and *Bacteroidales*, respectively, as described above.

Statistical analysis

Statistical analyses were performed with SPSS 20.0. The chi-square test was performed to test the relationship between the various groups of analyzed indicator microorganisms. A P value of <0.05 was considered significant.

Results and discussion

The current waterborne disease problem stems in part from the absence of completely reliable methods to identify the fecal pollution of water samples (Figueras & Borreg 2010). Therefore, the ability to identify fecal contamination is crucial for effective management of water sources and protection of the public from risks associated with fecal-dwelling pathogenic microorganisms (Fremaux et al. 2009). In this study, we compared a selection of conventional fecal indicators with a universal *Bacteroidales* genetic marker for detection of fecal pollution of a variety of drinking water sources. This study was also performed to determine any predictive relationship between indicator and pathogenic bacteria in low-polluted waters. From 60 water samples analyzed, *Bacteroidales* was the most frequently detected indicator (68.3 %) followed by total coliforms (51.7 %) as indicated in Table 1. Total coliforms were detected with concentration ranging from <1 MPN 100 ml^{-1} to >23 MPN 100 ml^{-1}. Other indicator microorganisms were detected at the following frequencies: *C. perfringens* and H_2S bacteria > FS > FC > *E. coli* (Table 1). Out of all water samples tested for pathogens, only *E. coli* O157 was detected in 5 % (3/60) of samples, while *Salmonella* and *Shigella* spp. were not detected in any water samples.

The fact that *Bacteroidales* were detected more frequently than FIB could be due to differences in detection methods (Lee et al. 2010; Schriewer et al. 2010). Our results are comparable with the results of Bernhard & Field (2000) which detected *Bacteroidales* markers in samples that contained no detectable coliforms. They detected *Bacteroidales* markers in all (8/8) river and estuarine water samples, but the coliforms were detected in seven of eight samples (Bernhard & Field 2000). The presence of *Bacteroidales* in samples that contain no detectable coliforms or other alternative indicators is probably the result of more sensitivity of PCR-based methods than cultural methods (Dick & Field 2004; Agudelo et al. 2010). Our sensitivity results also demonstrated that nested PCR method for detection of universal *Bacteroidales* genetic marker was 10-fold more sensitive than culture method for coliform bacteria. Higher sample volume for *Bacteroidales* assay could also result in more sensitive detection limits than coliforms. Furthermore, *Bacteroides* spp. make up a significant portion of the intestinal microflora and were found in greater abundance than coliforms in animal feces (Kildare et al. 2007). However, the study of Van der Wielen & Medema (2010) showed that general *Bacteroidales* 16S rRNA gene assay could detect environmental rather than fecal *Bacteroidales* species in water samples (van der Wielen & Medema 2010).

Although universal *Bacteroidales* genetic marker was detected in more samples than coliform bacteria, the *Bacteroidales* assay failed to detect the marker in

Table 1 Percentage of samples with detectable indicator microorganisms in different drinking water sources

Sample type	No. of samples	Percent (%) of water samples positive for:						
		TC	FC	*E. coli*	FS	*C.perfringens*	H_2S bacteria	*Bacteroidales*
Well	34	29.4	2.9	2.9	8.8	14.7	8.8	58.8
Spring	8	62.5	12.5	0.0	37.5	37.5	37.5	75.0
Aqueduct	15	86.7	86.7	80.0	80.0	66.7	80.0	80.0
River	3	100.0	66.7	66.7	66.7	100.0	100.0	100.0
Total	60	51.7	28.3	25.0	33.3	35.0	35.0	68.3

Table 2 Microbial characteristics of *Bacteroidales* negative samples which were known to be polluted because of the presence of other indicators

Type of water sample	Presence or absence of indicator and pathogenic bacteria						
	TC	FC	E.coli	FS	*C.perfringens*	H_2S bacteria	*E .coli* O157
Spring	+	−	−	+	+	−	−
Well	+	−	−	−	−	−	−
Well	+	−	−	−	−	−	−
Well	+	−	−	−	−	+	−
Well	−	−	−	−	+	−	−
Aqueduct	+	+	+	−	−	−	+
Well	+	+	+	+	+	−	−
Aqueduct	+	+	+	+	+	+	−
Aqueduct	+	+	−	+	−	+	−

nine samples which were known to be polluted as measured by FIB and other alternative indicators (Table 2). The microbial characteristics of the samples are presented in Table 2. The observed difference could be described by the following three possible explanations: first, when PCR-based methods are used to environmental samples, the potential for PCR inhibition by compounds readily found in environmental samples is of concern (Layton et al. 2006). However, this explanation is not acceptable because out of the 60 water samples tested, none of the samples showed PCR inhibition. Second, the discrepancy could have resulted from the difference in environmental persistence of fecal indicator bacteria and *Bacteroidales* (Lee et al. 2010). Some studies have demonstrated that *Bacteroidales* are affected differently from FIB by biotic and abiotic factors such as light, salinity, dissolved oxygen, and temperature (Balleste & Blanch 2010; Okabe & Shimazu 2007).

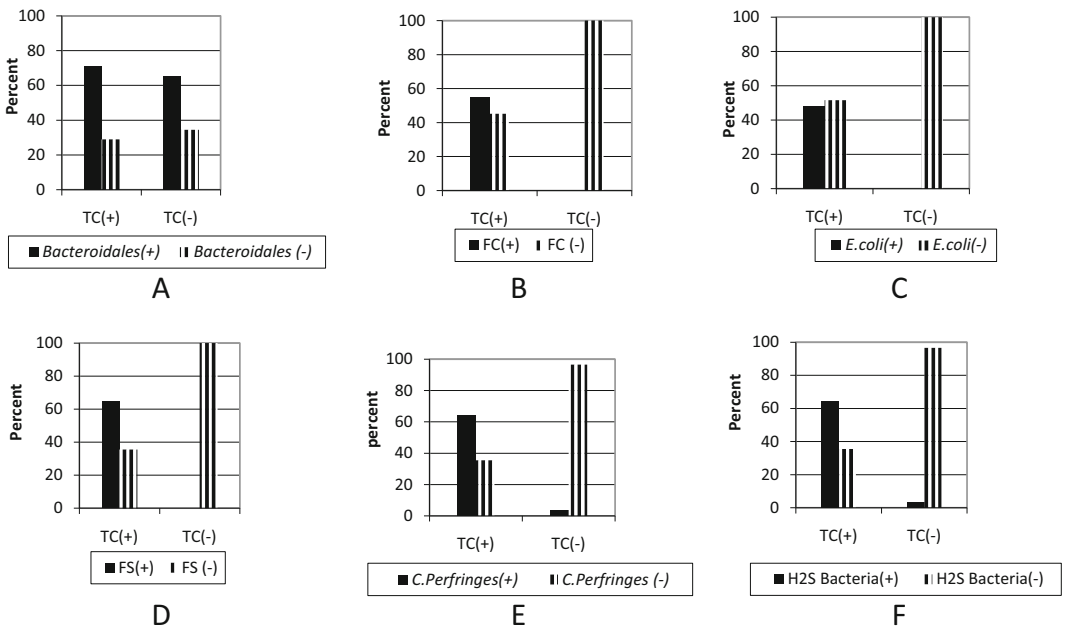

Fig. 1 Relationship between detection of total coliforms and presence-absence of other indicators

The study of Balleste & Blanch (2010) showed that the survival period of *Bacteroides* spp. was shorter than that observed for fecal coliforms and enterococci. They also indicated that temperature was the main factor affecting DNA degradation (Balleste & Blanch 2010). Hence, *Bacteroidales* have been considered as an indicator of recent fecal pollution (Balleste & Blanch 2010; Schriewer et al. 2010), and ability of these markers may be limited to the point source or recent non-point source contamination of water environments (Rogers et al. 2011). Similar results are found in the study of Agudelo et al. (2010) who compared multiplex PCR assay for *Bacteroidales* and fecal enterococci with conventional microbiological method. Their results revealed 89.2 % of the samples being positive with real-time PCR and 75.7 % with plate culture. They found two real-time PCR negative samples and culture positive (Agudelo et al. 2010). However, there is controversy over the persistence of *Bacteroidales* in the environment. The study of Saunders et al. (2009), in a simulated fecal contamination of unchlorinated drinking water, showed that the persistence of fecal *Bacteroides* 16S rRNA gene copies was similar to the survival of fecal indicators (Saunders et al. 2009). Seurinck et al. (2005) also reported that the human-specific *Bacteroides* marker persisted in fresh water in a pattern similar to FIB. However, they indicated that the persistence of low concentrations of fecal bacteria may differ from their observations (Seurinck et al. 2005).

The third possible explanation would be that some analyzed indicators especially total coliforms are not restricted to fecal sources but are commonly found naturally in the environment. Therefore, the presence of these bacteria in samples that contained no detectable *Bacteroidales* was probably the result of non-fecal pollution. However, this difference is not certainly expected for three *E. coli* positive samples (Table 2). In addition, the presence of *C. perfringens* in samples that contained no detectable *Bacteroidales* genetic marker could confirm the hypothesis of the presence of *Bacteroidales* as recent fecal pollution indicators. *C. perfringens* is able to form spores which could persist a long time in the environment. Similarly, most previous studies have reported no correlation between the number of FIB and *Bacteroidales* markers. Whereas, they detected *Bacteroidales* markers and total coliforms simultaneously on polluted waters (Okabe & Shimazu 2007; Lee et al. 2010).

The absence of universal *Bacteroidales* genetic marker in the presence of *E. coli* O157 (Table 2) also

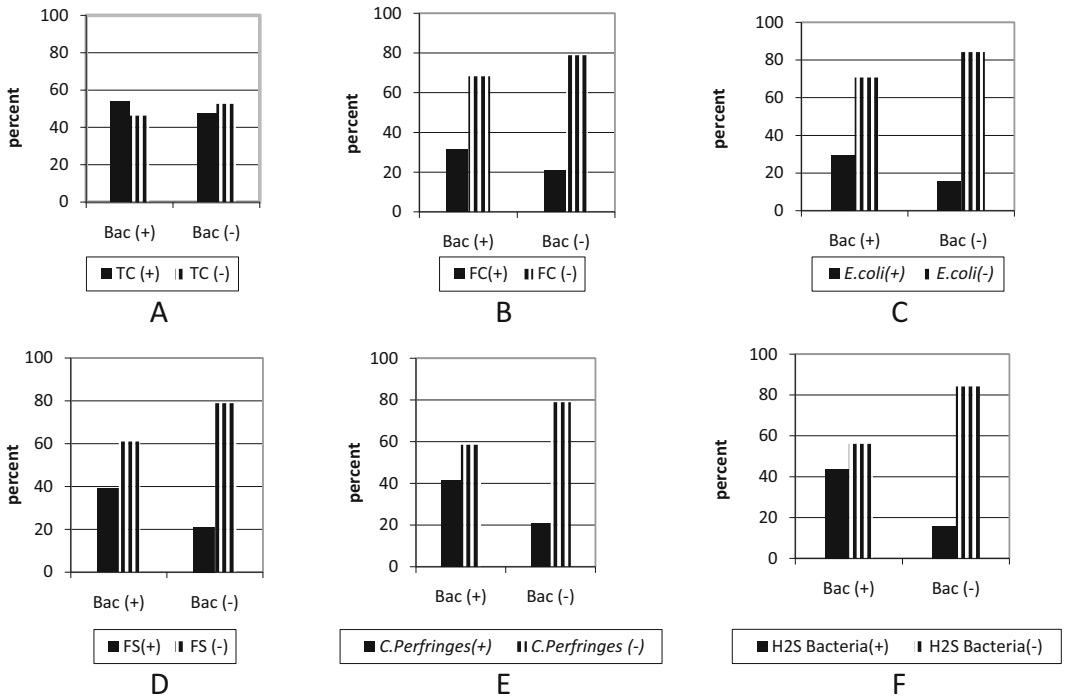

Fig. 2 Relationship between detection of universal *Bacteroidales* genetic marker and presence-absence of other indicators

demonstrated that *Bacteroidales* could not adequately reflect the occurrence of pathogens. However, due to a very low detection of analyzed pathogenic bacteria, further data is required to certainly assay the predictive value of *Bacteroidales* genetic marker.

The statistical analysis showed no association between the presence of universal *Bacteroidales* genetic marker and occurrence of the other indicator microorganisms, whereas there was a significant relationship between the presence of total coliforms and occurrence of other indicator bacteria including FC, FS, *E .coli*, *C. perfringens*, and H_2S-producing bacteria. As shown in Figs. 1 and 2, total coliforms are more predictive of the presence or absence of other indicators than *Bacteroidales*. Among water samples that tested positive for total coliforms, 83.9 % of samples also contained at least one other indicator, but this percentage dropped to 56.1 % when considering for *Bacteroidales*. Total coliforms also tended to be more absent (93.1 %) than *Bacteroidales* (47 %) when other indicators were absent.

Conclusions

The results of our study showed that *universal Bacteroidales* genetic marker alone was not capable of accurately detecting the presence of fecal pollution. In other words, the absence of *Bacteroidales* genetic marker is not necessarily an evidence of fecal and pathogenic bacteria absence, and may be unable to ensure the safety of the water. Further research, however, is required for a better understanding of the use of *Bacteroidales* genetic marker as an indicator in water quality monitoring programs.

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