Evaluation of real-time PCR for quantitative detection of *Escherichia coli* in beach water

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ABSTRACT

The current investigation evaluated the use of real-time polymerase chain reaction (PCR) for quantitative detection of *Escherichia coli* in marine beach water. Densities of *E. coli* in 263 beach water samples collected from 13 bathing beaches in Hong Kong between November 2008 and December 2009 were determined using both real-time PCR and culture-based methods. Regression analysis showed that these two methods had a significant positive linear relationship with a correlation coefficient (*r*) of 0.64. Serial dilution of spiked samples indicated that the real-time PCR had a limit of quantification of 25 *E. coli* colonies in 100 mL water sample. This study showed that the rapid real-time PCR has potential to complement the traditional culture method of assessing fecal pollution in marine beach water.

Key words | *cyd*, *Escherichia coli*, hybridization probes, marine beach water, real-time PCR

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INTRODUCTION

Fecal indicator organisms, which reside in the gastrointestinal tracts of humans and animals, are used globally to assess microbial water quality of bathing beaches. In Hong Kong, densities of *Escherichia coli* in marine beach water showed the best correlation with swimming-associated illnesses when compared with other sewage-related, water-borne pathogens, as well as fecal indicators including enterococci (Cheung *et al.* 1990, 1991a, b). Quantitative detection of *E. coli* using traditional culture-based methods, however, cannot provide same-day notification of beach conditions to the public. Before overnight culture followed by bacterial identification, swimmers are still being exposed to deteriorated beach water. A reliable quantitative detection method with a shorter turnaround time is, therefore, in demand.

Real-time polymerase chain reaction (PCR) is possibly a quicker alternative to the traditional culture-based method. Its usability in detecting fecal indicator organisms in different aquatic ecosystems, such as recreational lakes (Haugland *et al.* 2005), agriculture watersheds (Khan *et al.* 2007), and beaches (Noble *et al.* 2010), has been evaluated. The real-time PCR amplifies and simultaneously quantifies a specific DNA sequence of an organism. In each cycle of amplification, a double-stranded DNA molecule is separated, hybridized with fluorescent probes, and replicated if the specific DNA sequence is present. By real-time monitoring of the fluorescence emitted by hybridization probes (HybProbes), the quantity of targeted sequences in samples can be determined in an hour. The real-time PCR gives an opportunity for same-day evaluation of water quality. The

doi: 10.2166/wh.2013.038

current investigation evaluated the use of real-time PCR for quantitative detection of *E. coli* in marine beach water from Hong Kong.

Different gene candidates have been suggested for *E. coli* detection, such as *lacZ*, *uidA*, and *cyd* (Bej *et al.* 1991; Horakova *et al.* 2006). The *lacZ* and *uidA* genes encode beta-D-galactosidase and beta-D-glucuronidase, respectively, which are common detection targets of biochemical assays for *E. coli* (Bej *et al.* 1991). The *cyd* gene, which encodes cytochrome *d* terminal oxidase complex, was subsequently included in the detection panel to increase the specificity of *E. coli* detection (Horakova *et al.* 2006). The current investigation examined the specificity of *lacZ*, *uidA* and *cyd* genes for *E. coli* detection. The most specific gene candidate was later selected to quantify the densities of *E. coli* in 263 samples collected from 13 bathing beaches in Hong Kong using the real-time PCR platform. The results of real-time PCR were compared with the densities enumerated using the traditional culture-based method in parallel. The limit of quantification of real-time PCR was also determined using serially diluted spiked samples.

MATERIALS AND METHODS

Bacterial strains

Ten different bacteria were used to examine the specificity of *lacZ*, *uidA* and *cyd* genes for *E. coli* detection. They included *E. coli*, *Enterococcus faecalis*, *Salmonella typhimurium*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Aeromonas hydrophila*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Citrobacter freundii* and *Acinetobacter lwoffii*. *E. coli*, *E. faecalis*, *S. typhimurium*, *S. flexneri* and *K. pneumonia* may be found in the human intestine, whereas *A. hydrophila*, *P. mirabilis*, *P. aeruginosa*, *C. freundii* and *A. lwoffii* are present in the natural environment. *E. coli* (ATCC 25922), *E. faecalis* (ATCC 29212) and *S. typhimurium* (ATCC 14028) cultures were purchased from American Type Culture Collection (ATCC). The seven other bacterial species were clinical isolates collected by the Department of Microbiology, Queen Mary Hospital of Hong Kong, as identified using the Vitek 2 system (bioMérieux, France).

Water sampling

A total of 263 water samples were collected from 13 bathing beaches in Hong Kong between November 2008 and December 2009. The beaches covered a wide range of water quality (ranked from 'Good' to 'Very poor'), with salinity in the range 0.1–34.1 parts per thousand and temperature in the range 12.4–31.9 °C. The beach ranking system in Hong Kong classifies beaches into four different ranks according the *E. coli* colonies per 100 mL beach water (Hong Kong Environmental Protection Department 2006). A beach with an *E. coli* count of less than 25 *E. coli* colonies per 100 mL water is classified as 'good'. The 'fair', 'poor' and 'very poor' ranks are equivalent to 25–180, 181–610 and more than 610 *E. coli* colonies per 100 mL water, respectively. Sterilized 8 L polypropylene bottles were filled at 1 m depth after removing the caps and rinsing thoroughly while submerged. The filled bottles were emptied slightly to allow a 2.5 cm headspace. The capped bottles were then stored in a cooler packed with ice and transported to the laboratory for further processing.

Culture methods

In the laboratory, bacteria in 1, 10 and 100 mL beach water samples were concentrated by filtration using 0.45 μm membrane filters (Advantec, Japan). The filters were then placed on absorbent pads (AP10; Millipore, USA) soaked with 2 mL CHROMagar Liquid ECC (CHROMagar, France) and incubated at 44.5 °C for 24 h. After incubation, *E. coli* colonies on filters were enumerated in accordance with Ho & Tam (1997). *E. coli* appeared as blue colonies whereas other fecal coliform bacteria and other gram-negative bacteria appeared as purple and colorless respectively. The Vitek 2 system was used to confirm the identification of *E. coli* on membrane filters. *S. flexneri* in water samples were also detected using the American Public Health Association Method 9260E (American Public Health Association 1992).

DNA extraction

Water samples (2 L) were filtered using a 0.45 μm membrane filter. The filter was then transferred into a 50 mL centrifuge tube containing 10 mL STE buffer solution

[0.1 M sodium chloride, 10 mM tris(hydroxymethyl)aminomethane (Tris) and 1 M methylenediaminetetraacetic acid (EDTA) at pH 7.6]. The centrifuge tube with the filter inside was vigorously shaken for 10 min using the Vortex-Genie 2 vortex mixer (Scientific Industries, USA). After removing the membrane filter from the tube, suspended bacterial cells in the STE buffer solution were pelleted at 10,000 rpm for 30 min at 4 °C. The bacterial pellet was resuspended in 1 mL distilled water. Bacterial DNA was extracted using the NucliSenseasyMAG system (bioMérieux, France) according to manufacturer's instructions.

DNA of the 10 species of bacteria used to assess the specificity of *lacZ*, *uidA* and *cyd* genes was also extracted using the NucliSenseasyMAG system. Samples of these bacteria were individually spiked into 2 L artificial seawater followed by membrane filtration and DNA extraction. Similarly, external standards ranging from 10^1 to 10^7 *E. coli* colonies in 100 mL water samples were incorporated into 2 L artificial seawater followed by membrane filtration and DNA extraction. These external standards were used to calculate the densities of *E. coli* in marine beach water samples during real-time PCR. In addition, a blank control was prepared by membrane filtration and DNA extraction of the artificial seawater without *E. coli* addition.

Real-time PCR

The real-time PCR was performed using the LightCycler 2.0 Instrument and the LightCyclerFastStart DNA Master HybProbe kit (Roche, Germany). Each 20 μL real-time PCR reaction was kept in a LightCycler capillary tube (Roche, Germany) and included 1 × LightCyclerFastStart DNA Master HybProbe, 3 mM magnesium chloride, 1 μM of each forward and reverse primers, 0.2 μM of each HybProbe and 10.4 μL extracted DNA. The primers and HybProbes used in this study are shown in Table 1. Cycling profile was as follows: 10 min 95 °C followed by 50 cycles of 95 °C for 10 s, 50 °C for 20 s and 72 °C for 15 s. After amplification, the instrument was cooled at 40 °C for 30 s. The ramp rate was 20 °C/s except for the annealing step, which had a ramp rate of 5 °C/s. The fluorescence signal emitted from the HybProbes was acquired once at each annealing step. Fluorescence channels used for monitoring amplification of *lacZ*, *uidA* and *cyd* genes were 640/530, 670/530 and 705/530 nm, respectively. Densities of *E. coli* in beach water samples were determined using the absolute quantification mode in the LightCycler Software 4.0. The limit of quantification of real-time PCR was estimated from assays of the serially diluted external standards.

RESULTS AND DISCUSSION

Specificity

The specificity of *lacZ*, *uidA* and *cyd* genes for *E. coli* detection was evaluated by performing real-time PCR on 10 different bacterial species (see Table 2). The *cyd* gene was the most specific gene for *E. coli* detection among the three genes tested, although it also exhibited a positive result for *S. flexneri*. However, no *S. flexneri* was detected in any of the 263 beach water samples collected during the study period. Therefore, the *cyd* gene was selected to quantify the densities of *E. coli* subsequently.

Table 1 | Primers and HybProbes used in this study

Gene	Primers	HybProbes[a]	Reference
lacZ	5′-GGGTTGTTACTCGCTCACATT-3′	5′-GCGCCCGTTGCACCACAG-FL	Bej *et al.* (1991)
	5′-CGGTTTATGCAGCAACGAG-3′	5′-LC640-TGAAACGCCGAGTTAACGCCATCAA-PH	
uidA	5′-GGAATGGTGATTACCGACGA-3′	5′-TCCATGATTTCTTTAACTATGCCGGG-FL	Bej *et al.* (1991)
	5′-CGTCCACCCAGGTGTTC-3′	5′-LC670-TCCATCGCAGCGTAATGCTCTACACC-PH	
cyd	5′-GCGCTCTCTTTCTGGAGTGT-3′	5′-GGCGAACTCGTCACTGACCGC-FL	Horakova *et al.* (2006)
	5′-CCACCAGGAACAGGGTATACA-3′	5′-LC705-GGCGATCTCATCTTCTCAATGGTGCTGA-PH	

[a]FL, fluorescein; LC640, LightCycler Red 640; LC670, LightCycler Red 670; LC705, LightCycler Red 705; PH, phosphate.

Table 2 | Specificity of real-time PCR

Bacterium	Real-time PCR result[a]		
	lacZ	*uidA*	*cyd*
Escherichia coli ATCC 25922	+	+	+
Enterococcus faecalis ATCC 29212	–	–	–
Salmonella typhimurium ATCC 14028	–	–	–
Shigella flexneri	–	+	+
Klebsiella pneumoniae	+	–	–
Aeromonas hydrophila	+	+	–
Proteus mirabilis	–	–	–
Pseudomonas aeruginosa	–	+	–
Citrobacter freundii	+	–	–
Acinetobacter lwoffii	–	–	–

[a] +, detected; –, not detected.

Limit of quantification of real-time PCR method

Amplification of serially diluted spiked samples indicated that the real-time PCR had a limit of quantification of 25 *E. coli* colonies in a 100 mL water sample, which is similar to the study by Khan *et al.* (2007). The amplification efficiency was 1.803. Results showed that the real-time PCR was capable of differentiating between the 'good' (<25 *E. coli* colonies in 100 mL water sample) and the 'fair' (25–180 *E. coli* colonies in 100 mL water sample) ranks of beach ranking system, even when they are present in the lower detection range.

Comparison between culture and real-time PCR methods

Regression analysis in log-scale revealed that there was a significant positive linear relationship between the culture and the real-time PCR methods (Figure 1). The overall correlation coefficient (r) of these two methods was 0.64, which is similar to that reported in Haugland *et al.* (2005) but lower than that in Noble *et al.* (2010). It is uncertain whether the lower correlation coefficients are due to sample size, because the sample sizes in the current study and Haugland and colleagues' study were at least double that in Noble and colleagues' study. Nevertheless, all the three studies showed a significant positive linear relationship between the culture and the real-time PCR methods.

False-positive and false-negative rates of the real-time PCR were 5% (12 out of 263 samples) and 10% (27 out of 263 samples), respectively. The former may be due to the presence of dead or viable but non-cultivable *E. coli* cells, whereas the latter may be due to a low *E. coli* density or the presence of PCR inhibitors, which all complicate the interpretation of real-time PCR.

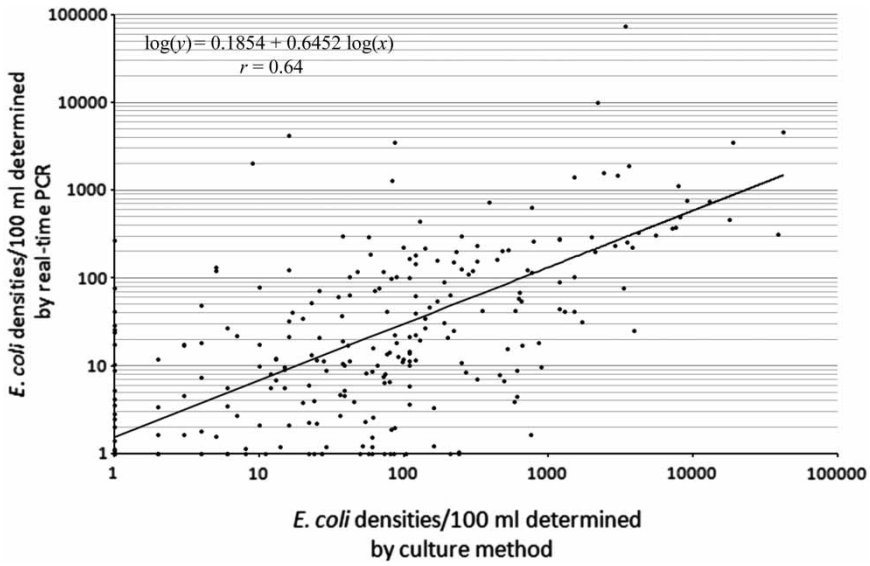

Figure 1 | Scatter plot and regression analysis of *E. coli* densities determined by culture and real-time PCR methods.

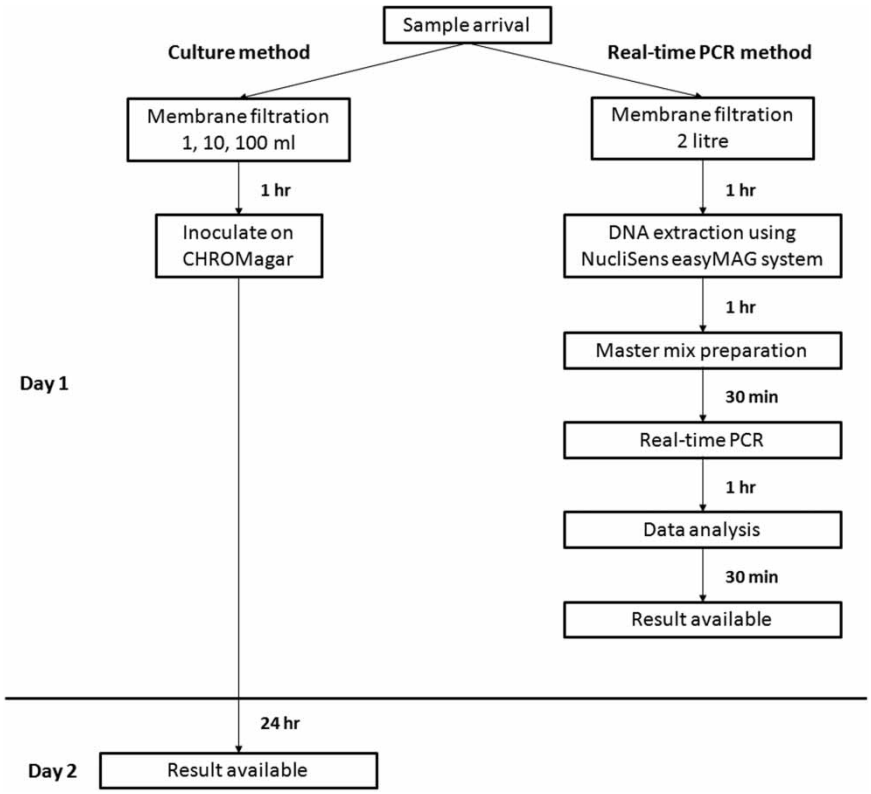

Figure 2 | Turn-around-time of culture and real-time PCR methods.

PCR inhibition was reported to be frequently encountered in real-time PCR by Haugland *et al.* (2005); in their study, bacteria were disrupted by beating with glass beads and the released DNA was then used directly for real-time PCR without purification (Haugland *et al.* 2005). The current study used the NucliSenseasyMAG system to extract and simultaneously purify DNA. The DNA released from bacteria was bound to magnetic silica particles and it was possibly separated from inhibitory substances that may be present in the sample. Nevertheless, the current protocol did not include an internal control to detect the presence of inhibitors. External DNA that is not normally present in the environment, such as salmon, may be incorporated in the PCR as an internal control (Haugland *et al.* 2005).

More importantly, turnaround time of the real-time PCR was remarkably shorter than that of the culture-based method (Figure 2). The results of real-time PCR were available within four hours after sample arrival, whereas the results of culture-based method were available a day after sample collection. The real-time PCR opens up the opportunity for same-day evaluation of water quality. The shorter turnaround time could speed up closure or opening of beaches, thus reducing health risks to many swimmers. The use of a mobile laboratory, which analyzes water quality *in situ*, could even further reduce sample delivery time, particularly for the laboratories that are far from sampling sites (Lane *et al.* 2003).

Nevertheless, a larger sample volume than for culture is required for this real-time PCR protocol, which may reduce the overall number of samples that can be collected. Apart from a higher cost of DNA extraction and real-time PCR, the real-time PCR instrument may not be easily available in many water quality laboratories. However, this protocol is especially suitable for the laboratories which perform real-time PCR on a daily basis.

CONCLUSIONS

The current investigation demonstrated that the real-time PCR method had the potential to complement the traditional

culture method of assessing fecal pollution in marine beach water. Regression analysis showed that a significant positive linear relationship existed between the culture and the real-time PCR methods. Using HybProbes targeting the *cyd* gene, the real-time PCR was capable of quantifying as few as 25 *E. coli* colonies in a 100 mL beach water sample within four hours. The rapid real-time PCR makes the same-day evaluation of beach water quality possible. This shorter turnaround time could speed up closure or opening of beaches and thereby reducing health risks to swimmers.

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First received 7 March 2013; accepted in revised form 28 August 2013. Available online 18 October 2013