



Development of a culture-independent method for rapid monitoring of microbial indicators in water samples

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Abstract

Microbial indicators are routinely applied to test the efficacy of the drinking water treatment process. *Clostridium perfringens* spores and faecal coliforms are being suggested as indicators in order to evaluate the efficacy of the drinking water treatment process. Serial dilutions of *Escherichia coli* and extracted *C. perfringens* spores were prepared and filtered using 0.2- μm cellulose nitrate filter membranes. After filtration, elution and conventional cultivation methods were performed for *C. perfringens* and *E. coli*. Dark and pinkish colonies in the attributed media confirmed the presence of *C. perfringens* and *E. coli*, respectively. Cultivation method showed the limit of detection 10^2 and 10^4 CFU/mL for *C. perfringens* and *E. coli*, respectively. In addition, PCR assays showed the limit of detection 10^2 CFU/mL for both *C. perfringens* and *E. coli*. Elution of the spores/bacteria followed by the molecular method is a rapid and easy method that has similar results with traditional cultivation method, and decreases both cost and time for the assessment of the quality of water treatment process.

Keywords Drinking water · Microbial indicators · Water filtration · Molecular analysis

Introduction

Waterborne microbial infections are considered as one of the most important public health concerns in both developing and developed countries. However, it seems that approximately 4.0% of all deaths and 5.7% of the total disease burden all over the world can be caused by waterborne pathogens (Prüss et al. 2002). According the previously published studies, faecal coliforms like *E. coli* and *Enterococci* are the indicators that have been commonly used to evaluate the efficacy of water treatment process. Almost all faecal coliforms are more susceptible to the presence of chlorine in comparison with cysts/oocysts of parasites and enteric

human viruses, particularly in the aquatic conditions (Lipp et al. 2001; Tree et al. 2003; Wéry et al. 2008; Ryzinska-Paier et al. 2011). Nevertheless, despite the nonspecific faecal origin (Scott et al. 2002; Simpson et al. 2002), multiplying in environments (Solo-Gabriele et al. 2000; John et al. 2009), unknown contamination sources (Field et al. 2003) and low specificity of faecal coliforms as indicators for the assessment of the drinking water treatment process (Hörman et al. 2004; Byamukama et al. 2005; Savichtcheva and Okabe 2006; Girones et al. 2010; Parasidis et al. 2013), faecal coliforms, such as *E. coli*, together with *C. perfringens* spores could be good indicators to measure the efficacy of the water treatment process (Payment and Franco 1993). *C. perfringens* forms spores that increase the chance of prolonged detection in the environment in case of faecal contamination. Therefore, evaluation of the presence of spores of *C. perfringens* can help to assess the quality of the water treatment process (Araujo et al. 2004). Payment and colleague reported that *C. perfringens* spores are resistant facing routine chlorination and could be useful for the assessment of rough wall enteric microbes such as cysts/oocysts and ova of parasites and also viruses with faecal origin, in water resources (Payment and Franco 1993). Several studies have suggested different techniques to evaluate the efficacy of the drinking water treatment process (Araujo et al. 2004;

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Wohlsen et al. 2006; Girones et al. 2010; De Keuckelaere et al. 2013). Approximately, all studies have described the filtration of treated water followed by cultivation of microbes collected on top of the membrane on specific culture media. The necessity for specific culture media for distinct microorganisms and the time-consuming process of cultivation are known as the main limitations of conventional methods. Recently, an elution method has been described as sensitive method to recover enteric viruses from water samples (De Keuckelaere et al. 2013). However, this method has not been applied for evaluation of other microbial indicators from water samples. Therefore, it is hypothesized that an easy-to-use protocol can improve the pitfalls of the conventional methods, help simultaneous recovery of the faecal coliform and spore indicators, and save both time and cost of the water quality evaluation, as well. This study was performed in Foodborne and Waterborne Diseases Research Center, Shahid Beheshti University of Medical Sciences during June to December 2016. The current study aimed to evaluate the elution technique followed by the molecular method that was previously introduced to recover virus particles and compare it with the conventional culture-dependent method to recover microbial indicators from treated water.

Materials and methods

Samples preparation and serial dilution

Clostridium perfringens: briefly, a previously proven *C. perfringens* strains (RIGLD-2) (Ganji et al. 2017) was cultivated on egg yolk agar medium (Merck, Germany) supplemented with 7% sheep blood and 0.04% neomycin. The cultured plate was incubated at 37 °C for at least 48–96 h under anaerobic conditions (Anoxomat, MART Microbiology, The Netherlands). The plate of egg yolk agar medium with pure colonies was placed at room temperature for 10 days to induce sporulation process. In order to confirm sporulation of *C. perfringens*, gram staining was performed on pure colonies before and after incubation of the colonies at room temperature. *C. perfringens* colonies were washed three times by phosphate-buffered saline (PBS) with pH 8, and then the plate was treated with buffer consisted of lysozyme (Sigma, USA) 50 µg/mL and 1 mM Tris-HCl, pH = 7.5 at 37 °C for 30 min. Afterwards, extracted spores were washed five times using sterile PBS and centrifuging at 12,000 rpm for 10 min. Finally, the extracted spores were diluted in defined volumes of sterile PBS.

E. coli: 100 µL of the stock of *E. coli* (ATCC25923) was cultured on MacConkey Agar (Merck, Germany) and incubated for 24 h. The bacterial colonies were suspended in sterile PBS, and then the turbidity of bacterial suspension was adjusted equivalent to a 0.5 McFarland standard.

Serial dilutions for *C. perfringens* spores, *E. coli* colonies and a mixture of bacteria and spores were freshly prepared at defined amounts of sterile PBS to give final concentrations, 10^1 , 10^2 , 10^4 , 10^6 and 10^8 colony-forming units (CFU)/mL.

Analysis of samples

All serially diluted mixtures, which were prepared as mentioned in the sample preparation section, were filtered using sterile 47-mm cellulose nitrate membrane (Sartorius, Goettingen, Germany) with pore size 0.2 µm and polysulfone in-line filter holder for 50-mm filters (Whatman). After that, one of the following procedures was performed for each of the filtered membranes.

Cultivation

Filtration of the diluted samples (separate suspensions of spores and bacteria) was done using sterile 0.2-µm cellulose nitrate filter membranes. Then, the filtered membrane was placed on the specific media (egg yolk agar and MacConkey agar for *C. perfringens* and *E. coli*, respectively). Cultivated egg yolk agar for each dilution was incubated in anaerobic condition, and all the cultivated media were examined for growth of bacteria every 48 h for a week. Furthermore, cultivated MacConkey agar media were incubated in aerobic condition at 37 °C for 24 h.

Elution

After filtration of each of the serially diluted mixtures (suspensions of spores and bacteria), filtered spores/bacteria were eluted as mentioned elsewhere (De Keuckelaere et al. 2013). Briefly, the membrane was rinsed in 300 mL 0.5 mM H₂SO₄ (pH 3.4). Then, 50 mL elution buffer (0.05 M KH₂PO₄, 1 M NaCl, 0.1% (v/v) Triton X-100, pH 9.2) was added to the membrane and remained on the filter membrane without pressure to soak the membranes for 10 min. Afterwards, the elution was filtered through the membranes to gather spores and bacteria. Then, the filtered elution was immediately counteracted using 1 M HCl. The eluted samples were centrifuged for 15 min at 3000 rpm, supernatant was discarded, and the pellet was collected in sterile tubes. Acquired pellets were introduced to DNA extraction kit. Figure 1 shows the schematic overview of the applied methods.

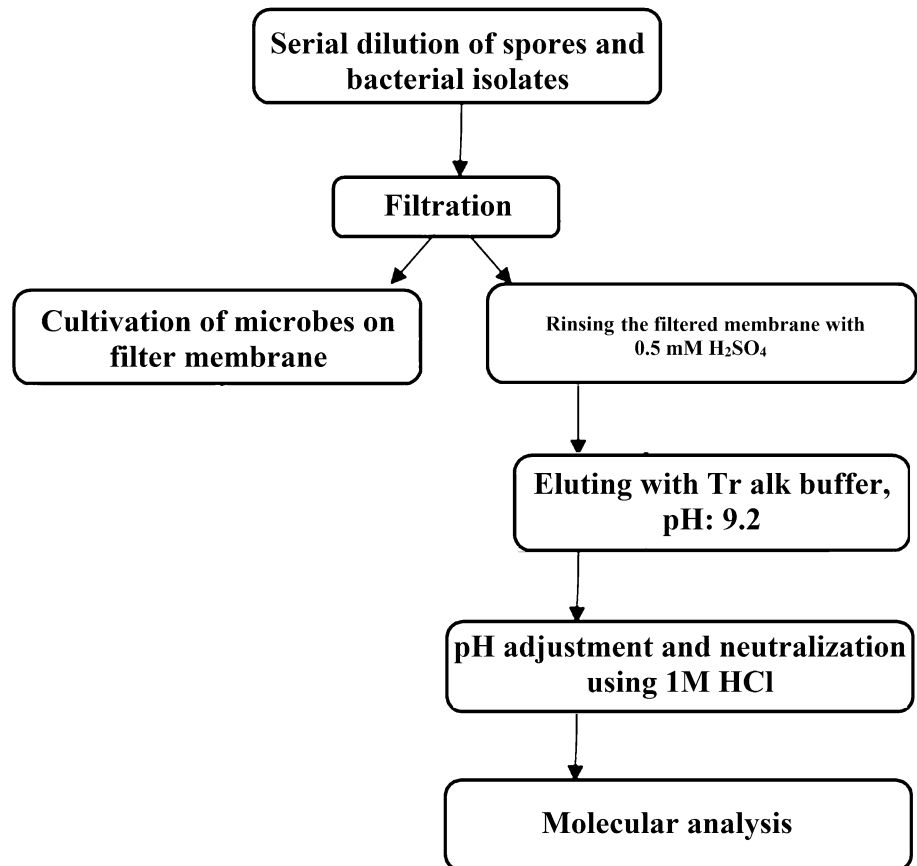
Molecular identification

DNA extraction

DNA of the pellets was extracted using Instagen kit (InstaGene™ Matrix, Bio-Rad, USA) according to



Fig. 1 Schematic overview of applied techniques for detection of *C. perfringens* spores and *E. coli* from water samples



manufacturer's procedure. Extracted DNA was stored at $-20\text{ }^{\circ}\text{C}$ for further investigations.

Polymerase chain reaction

For identification of *C. perfringens*, amplification of about 279-bp fragment of 16S ribosomal RNA (rRNA) gene was carried out in a final volume of 25 μL consisted of 2.5 μL of 10X PCR buffer, 0.5 μL of each primer (20 picomol), 1 μL of MgCl_2 (50 mM), 0.5 μL of each deoxy ribonucleotide triphosphate (dNTP) (10 mM), 0.3 U of Taq DNA polymerase (5 U/mL) and 3 μL of DNA. PCRs were accomplished using primers forward, 5'-AAAGATGGCATCATCATTCAAC-3', and reverse, 5'-TACCGTCATTATCTTCCC AAA-3', which were mentioned elsewhere (Wu et al. 2009) in a thermocycler (AG 22331; Eppendorf, Hamburg, Germany) as the following: initial denaturation for 10 min at $95\text{ }^{\circ}\text{C}$ and then 30 cycles of $95\text{ }^{\circ}\text{C}$ for 10 s, $52\text{ }^{\circ}\text{C}$ for 1 min and $72\text{ }^{\circ}\text{C}$ for 1 min, and final extension at $72\text{ }^{\circ}\text{C}$ for 10 min.

Moreover, 95-bp fragment of 16S rRNA gene was amplified using primers forward, 5'-CATGCCGCGTGTATGAAGAA-3', and reverse, 5'-CGGGTAACGTCAATGAGCAA-3', which were introduced previously (Huijsdens et al. 2002), to detect *E. coli*. The 25 μL PCR mixture

consisted of 10X buffer, 1 U Taq polymerase, and 1.5 mM of MgCl_2 , 0.5 mM dNTPs and 0.5 μL 20 μmol of each primer and 5 μL of DNA sample. PCR conditions were as follows: initial denaturation at 5 min at $95\text{ }^{\circ}\text{C}$, followed by 35 cycles of 1 min at $95\text{ }^{\circ}\text{C}$, 1 min at $58\text{ }^{\circ}\text{C}$ and 1.5 min at $72\text{ }^{\circ}\text{C}$, and final extension at $72\text{ }^{\circ}\text{C}$ for 10 min.

Ten microlitres of amplified products was electrophoresed on 1.2% agarose gel, and then the PCR products were stained with ethidium bromide and visualized under UV light (Cleaver scientific Ltd, Warwickshire, UK).

Results and discussion

In order to motivate the sporulation of *C. perfringens*, cultured media were placed at room temperature for 10 days. The presence of *C. perfringens* spores was confirmed using gram staining before and after the sporulation period. Accordingly, microscopic examination showed gram-positive spore-forming bacilli of *C. perfringens* at the beginning of the sporulation process that after 72 h, spore production was started and after 10 days almost all vegetative forms were changed to the spore formation.

Cultivation method

Clostridium perfringens: All the cultured egg yolk media supplemented by Neomycin and 7% sheep blood were kept out at 37 °C and checked every 48 h for a week. Specific dark colonies of *C. perfringens* were considered as positive. The results showed that the limit of detection for *C. perfringens* spores filtered by 0.2- μ m cellulose nitrate membrane was 10^2 spores/mL.

E. coli: Cultivation of the filtered membrane of *E. coli* was carried out on MacConkey agar media. The results showed that only dilution $\geq 10^4$ CFU/mL successfully grew in MacConkey agar after 24 h. For further confirmation, expected fragments of 16S rRNA gene were amplified for both *C. perfringens*- and *E. coli*-positive colonies.

Elution method

C. perfringens: Amplification of 16S rRNA gene for the spores resulted from the elution process showed that the expected fragment of *C. perfringens* was amplified in dilutions with 10^2 – 10^8 spores/mL. The sample with 10 spores/mL was positive for neither cultivation method nor concentration method (Figs. 2a, b, 3a, b).

E. coli: The results showed that the 95-bp fragment of 16S rRNA gene of *E. coli* was only amplified for the eluted bacteria with bacterial concentration $> 10^2$ CFU/mL (Fig. 2a, b).

Numerous approaches are available to assess the microbial quality of water resources to reduce the risk of waterborne outbreaks (Tyagi et al. 2007). Evaluation of microbial indicators such as coliforms, *E. coli*, as well as *C. perfringens* spores have been introduced as a reliable measurement tool to monitor the efficacy of water treatment process, so far. It seems that these indicators with their morphology, ability to persist in the environment and resistance during treatment processes, are similar to harmful pathogens (Napier et al. 2006). Moreover, because of this fact that coliform bacteria are easily detectable in water samples, they are useful indicators to assess the treatment process. The presence of faecal coliform bacteria as well as *C. perfringens* spores in treated water samples in more numbers than the allowed range (Lee et al. 2002) indicates both/either the possibility of pollution of the water supply system with the faeces and/or an insufficient disinfection process (Rompré et al. 2002).

In the current study, the conventional cultivation was evaluated and compared with elution followed by the molecular method for the detection of *C. perfringens* spores and *E. coli* in water samples in order to introduce a

Fig. 2 PCR amplification of serially diluted mixtures attributed to **a** *E. coli* and **b** *C. perfringens*. M: 100-bp DNA ladder; NC: negative control

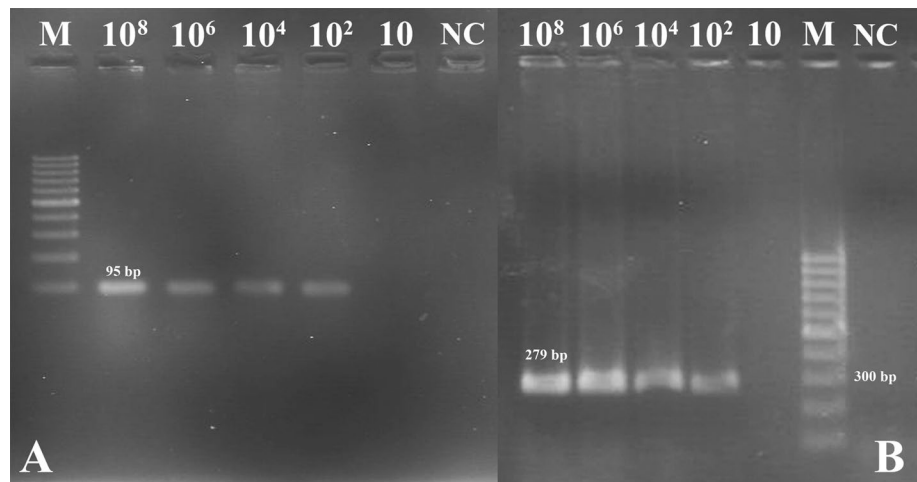
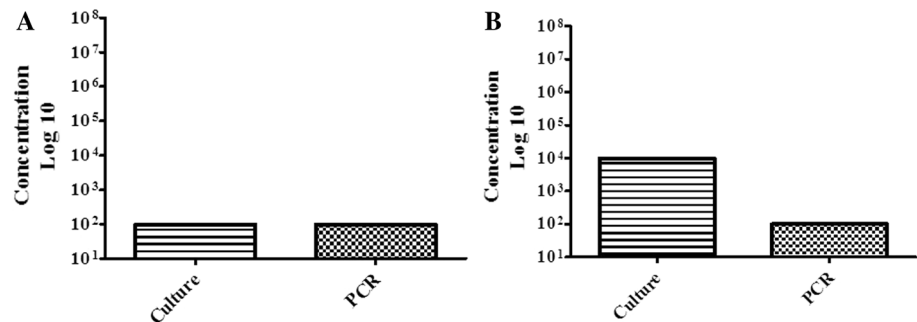


Fig. 3 Comparison of the limit of detection of cultivation and molecular method applied after water filtration spiked with **a** *C. perfringens* and **b** *E. coli*



rapid and reliable technique to evaluate the efficacy of the water treatment process.

Recently, Keuckelaere and colleagues introduced and evaluated four different methods to concentrate the enteric viruses from water resources using filtration. This study showed that the elution method using electronegative cellulose nitrate filter membrane and Tr alk elution buffer was more effective to recover the enteric viruses from water sample (De Keuckelaere et al. 2013). In the current study, the aforementioned method with some modifications was used to recover the microbial indicators (*E. coli* and *C. perfringens*) from water samples. The findings were in agreement with the mentioned study and showed a consistency between *C. perfringens* spore counts in the filter membrane method followed by cultivation with those determined by the elution method followed by the molecular test. The analysis of culture and molecular method for detection of *C. perfringens* showed a sensitivity limit of 10^2 spores/mL. Additionally, the findings represented discordance between the results of the elution method followed by molecular methods and the conventional cultivation of filter membrane for *E. coli*. Interestingly, the limit of detection of elution followed by molecular methods was 10^2 CFU/mL, whereas the limit of detection of conventional cultivation of filter membrane was 10^4 CFU/mL.

There are several methods like pour plates and membrane filter methods for a quantitative detection of *C. perfringens* spores in water resources (Mead 1985; Araujo et al. 2004; Maheux et al. 2013). Sartory and colleagues proved the selective role of elevated temperatures for the detection of *C. perfringens* in water samples (Sartory 1986). All of the applied techniques have been based on the sulphite reduction and stormy fermentation after anaerobic incubation. Although sulphite reduction has been introduced as the best test for the detection of *C. perfringens*, this method is not specific enough for the identification of *C. perfringens* in water samples.

Furthermore, several techniques for enumeration of coliform bacteria and *E. coli* like multiple-tube fermentation (MTF), the membrane filter (MF) technique, enzymatic procedures and direct determination of enzymatic activity by fluorimetry have been approved as common procedures for testing of water quality in many countries (Rompré et al. 2002; Mesquita and Noble 2013; Hojris et al. 2016). The main disadvantages of the conventional cultivation method are: (1) subculturing of *C. perfringens* colonies is time-consuming, (2) no specificity of morphological criteria of *C. perfringens* colony and (3) complex cultivation and further confirmation steps that are needed for faecal coliforms (Rompré et al. 2002). Therefore, the molecular methods have been described as appropriate tests for identification of the faecal coliform, *E. coli*, as well as spores

of *C. perfringens* during water treatment process (Wohlsen et al. 2006; Deshmukh et al. 2016).

Signoretto showed that the PCR method due to its advantages is a valuable implement for identification of microbial indicators/pathogens in water resources that can generate abundant as well as important information about the common and emerging/re-emerging microbes (Signoretto and Canepari 2008). However, the filtration method is an acceptable method for detection of low counts of microbial indicators such as *C. perfringens* spores and *E. coli* in high volume of water samples. According the findings, elution followed by molecular method showed sufficient sensitivity, and specificity compared with the conventional cultivation. Therefore, concerning the reliable results, rapidity and the cost-effectiveness of the elution followed by molecular method, this approach could be introduced for evaluation of efficacy of water treatment process.

Conclusion

Elution of spore/bacteria followed by molecular method is a rapid and easy method that was able to detect 10^2 CFU/mL of both *C. perfringens* spores and *E. coli*. In addition, traditional cultivation method was only able to recover 10^2 and 10^4 CFU/mL from water samples for *C. perfringens* spores and *E. coli*, respectively. The current study suggested that elution method followed by molecular method is not only a sensitive technique for detection of virus particles in water samples as mentioned previously, but also has higher sensitivity for detection of microbial indicators during water treatment process in comparison with conventional methods. Furthermore, this method can decrease both cost and time (*E. coli* and spores of *C. perfringens* need at least 24 h and 3–4 days, respectively, to grow in specific media while elution followed by molecular technique needs only 3–4 h) in order to evaluate the efficacy of water treatment process.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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