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Original Research Paper

DNA extraction using FTA Elute cards for detection and enumeration of fecal indicator bacteria in the Chao Phraya River, Thailand

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Abstract:
Utilizing molecular methods for monitoring and determining water quality in developing countries can be problematic. Effective extraction and storage of DNA from environmental samples is especially difficult due to lack of access to sufficient laboratory facilities for molecular work. Use of a commercial product for the extraction of DNA, as well as its application in determining the microbial quality of surface waters, was evaluated and compared. Surface water samples collected from the Chao Phraya River in Thailand were processed to enumerate *Escherichia coli*. Quantification was done through culture methods (Colilert Quanti-tray) and quantitative real-time PCR (SYBR green and Taqman assays). Processing of surface water samples and culture enumeration of *E. coli* was done in Thailand, while DNA extraction and enumeration using real-time PCR was done in Korea. *E. coli* MPN counts were mean log 2.89/100ml. Quantitative real-time PCR assays counts ranged from 2.38 to 3.59 mean log CFU/100 ml. Taqman PCR assays had no significant difference in mean log counts compared to conventional culture methods. However mean log counts of SYBR green PCR assays were significantly higher than counts obtained by culture methods and Taqman PCR assays ($P<0.05$). Results demonstrated FTA Elute cards can be employed for the extraction of microbial DNA from surface waters and have potential use in detection of fecal indicator bacteria from environmental water samples.

Keywords: DNA Extraction; *E. coli*; Fecal Indicator Organisms; Surface Water; Quantitative PCR

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1. Introduction

Surface water quality is still reliant on the monitoring and enumeration of bacterial fecal indicators organisms based on cultivation methods and/or molecular analysis tools (Rochelle-Newall et al., 2015; Simpson et al., 2002). Certain bacteria can serve as indicators of fecal contamination from humans and warm-blooded animals, and classic examples include fecal coliforms such as *Escherichia coli* or fecal enterococci (Hunter, 2003; Maheux et al., 2009; Simpson et al., 2002). Their detection in surface waters may also signal the presence of pathogens, and some pathogenic strains of *E. coli* have been identified as causative agents in outbreaks (Saxena et al., 2015; Takahashi et al., 2009). However, there has been some evidence that a correlation of measured health outcomes and *E. coli* may have a weak association (Brown et al., 2008; Moe et al., 1991). Despite this, these organisms are

commonly listed as targets for many environmental regulatory agencies to infer fecal contamination of water sources (Boubetra et al., 2011; Douterelo et al., 2014; Jang et al., 2017).

Cultivation and enumeration techniques to detect fecal indicators in water commonly utilize selective and/or differential media, and one such presence/absence method uses a commercially available medium, Colilert (IDEXX US), which can be modified using the Quantity-Tray (QT) system to allow for quantification by Most Probable Number (Douterelo et al., 2014). Polymerase chain reaction methods and real-time PCR have also been described for detection and identification of microorganisms in food, sediment, and water samples (Allende et al., 2017; Mendes Silva & Domingues, 2015). Extraction and purification of nucleic acids from environmental samples are commonly done prior to PCR analysis (Mendes Silva & Domingues, 2015), and can be combined with real-time, fluorescence-based detection methods to quantify microorganisms present in the environmental samples. Additionally, real-time PCR techniques have the advantages of confirming amplicons without post-PCR processing and generally have shorter assay times (Amini & Kraatz, 2015; Penders et al., 2005; Simpson et al., 2002).

However, coupled with quantification of fecal bacteria through molecular means are the challenges of effective DNA extraction and preservation from environmental samples. Free DNA lacks the presence of enzymes for repair and is susceptible to chemical as well as physical conditions which lead to degradation, potentially limiting detection or quantification by molecular methods (Douterelo et al., 2014; Mendes Silva & Domingues, 2015). This is compounded in developing countries where lack of sustained access to power may be an issue for the long-term storage of DNA. Maintaining extracted DNA, even in buffers to reduce nuclease activity, is problematic as common methods of cold storage at -20 °C and lower temperatures can be difficult to maintain due to brownouts.

One possible solution could be to utilize commercial products which can stabilize DNA at higher temperatures such as FTA filter cards (Gómez et al., 2019). FTA filter cards are incorporated with a proprietary composition of chemicals that lyse cells and stabilize nucleic acids, where environmental samples can be spotted in low volumes on the cards allowing for future DNA extraction. Studies of washed food samples spiked with *Shigella spp*. cultures and raw enriched beef samples spiked with STEC *E. coli* strains were detected by PCR in equivalent levels to that of pure culture dilutions, indicating DNA extraction and PCR inhibitors from complex matrixes could be overcome using FTA cards (Kim et al., 2017; Lampel et al., 2000).

Other studies demonstrated FTA cards are applicable to extract DNA from a variety of tissue sample types, and recovery of DNA from blood samples stabilized at room temperature could be detected by PCR up to 44 months (Smith and Burgoyne, 2004), while other studies demonstrated successful recovery of bacterial cultures from FTA cards stored at ambient temperatures after 3 years (Rajendram et al., 2006). A more recent survey study of cystic *Echinococcus* from slaughter facilities amplified DNA from field samples after storage at room temperature for 1 week (Boué et al., 2017). FTA cards have also been utilized for recovery of viruses and some studies have shown effective recovery could be attained for periods of 2 weeks or more (Davis et al., 2022; Elnagar et al., 2021).

The Chao Phraya River, or King River, was selected for this study because it passes through various land use patterns, namely, agricultural, residential, urban, and industrial areas. The origin of the Chao Phraya River is the confluence of the Ping, Wang, Yom and Nan rivers about 200 km north of Bangkok. The Upper Chao Phraya river basin starts from Nakhon Sawan and has a drainage area of 21,725 km2 (Tachikawa et al., 2004). The river's total length is 380 km with 15 provinces located along the river. Nakhon Sawan province covers the largest catchment area, followed by Bangkok and Pathum Thani. The river is subdivided into three sections: upper (RKM 7 to 62), middle (RKM 62 to 142) and lower (RKM 142 to 379). From Nakhon Sawan, the river flows through the central plain passing Bangkok toward the gulf of Thailand. The river basin covers 160,000 km2, representing 30 percent of Thailand's total area. The river basin is mountainous with productive agriculture valleys in the upper region, while lower region contains alluvial plains that are also highly productive for agriculture.

This study aimed to enumerate *E. coli* in surface water samples collected from the Chao Phraya River, Thailand, using both traditional culture methods (Colilert system) and through employing quantitative real-time PCR (qPCR) assays.

2. Materials and Methods

Surface water sample collection

Fifteen sample locations were used to collect surface water samples along the Chao Phraya River system, Thailand, for the dry season (April) and collection points were recorded using a portable GPS unit. Two sampling collection points were used to characterize each location except for the Ping and Nan River site locations, where only one site was used for collection. Grab samples of surface water (100 ml volumes) were collected at 20 cm depths for each sampling point using sterile, plastic polypropylene bottles. Water samples were stored on blue ice until laboratory processing (within 18 hours). A general map of the river and region are presented in Figure 1. Sample locations for the Chao Phraya River were characterized as either upper and middle river locations, more impacted by agricultural runoff, aquaculture, and rural land use, and lower river locations which were considered impacted more by urban and industrial runoff within the greater Bangkok metropolitan area.

Water Sample Processing

For duplicate collection point locations, 50 ml of each sample were aseptically pooled into a single, composite 100 ml volume resulting in two composite 100 ml samples. In the cases of the Ping and Nan River locations, non-composite 100 ml volumes were processed (as there was only one sample point for these two locations). One 100 ml volume of surface water was processed using the Colilert Quanti-tray system (IDEXX US) and *E. coli* MPN numbers per 100 ml enumerated as per manufacturer's recommendations. The second 100 ml water sample was filtered through a sterile 0.45 μm membrane. The filter membranes were transferred to sterile 50 mm petri dishes and 0.2 ml of sterile filter wash solution (0.002 g peptone weight/volume, and 0.5 ml Tween 80 per 100 ml filter solution) was overlaid on the surface of the membrane. The plates were gently shaken (less than 100 rpm/min) using a rotary shaker for 10 minutes.

Afterwards 40 μl of the elutant was removed and placed onto a Whatman non-indicating FTA Elute card (GE Health Sciences, USA). This was done in quadruplicate for a total of 120 μl filter wash solution per FTA Elute card (four spots per card). The FTA Elute cards were carefully closed, covers of the filter pads sealed with tape, and kept out of direct sunlight at room temperature until processing remotely in Korea.

DNA extraction for water samples and creation of real-time PCR standards

FTA Elute cards were processed approximately 2 months after the sampling event as per manufacturer's recommendations with minor modifications. Briefly, in quadruplicate, 3 mm diameter portions of the filter cards were removed and placed in sterile microfuge tubes. The filter disks were washed with 0.5 ml volume of sterile water, and finally suspended in a 50 μl volume of TE buffer (pH 8.0) which was heated for 30 minutes at 95 °C. After eluting DNA from the filters, the filter material was removed, and the four DNA samples were pooled into a single volume. DNA samples were then stored at -20 °C until PCR analysis could be conducted.

For qPCR standards, an overnight culture of *E. coli* (ATCC strain 15597) was grown in Tryptic Soy Broth and 40 μl added directly to each spot pad on a FTA Elute card (for a total of 120 μl per card). The cards were stored in the dark for 24 hours and then processed for DNA extraction in a similar manner to the water samples as previously described. The culture was also serially diluted in sterile DDI water blanks and select dilutions spread plated in duplicate on tryptic soy agar plates for CFU/ml enumeration. The extracted DNA was pooled, 10-fold serial dilutions made in pH 8 TE buffer, and the DNA standards were maintained at -20 °C.

Confirmation of DNA extraction from FTA Elute cards

To establish DNA extracted from FTA Elute cards could be used as template for PCR reactions, PCR experiments with primers designed to detect prokaryotic universal rDNA (Takai and Horikoshi, 2000) were

done. PCR reactions using Maxime PCR Premix (Intron Biotechnology, Korea) consisted of 1 µl of DNA sample template, 0.5 µM each of forward and reverse primers, Bac349F (5′-AGGCAGCAGTDRGGAAT-3′) and Bac806R (5′-GGACTACYVGGGTATCTAAT-3′), and additional DDI water for a final 20 µl reaction volume.

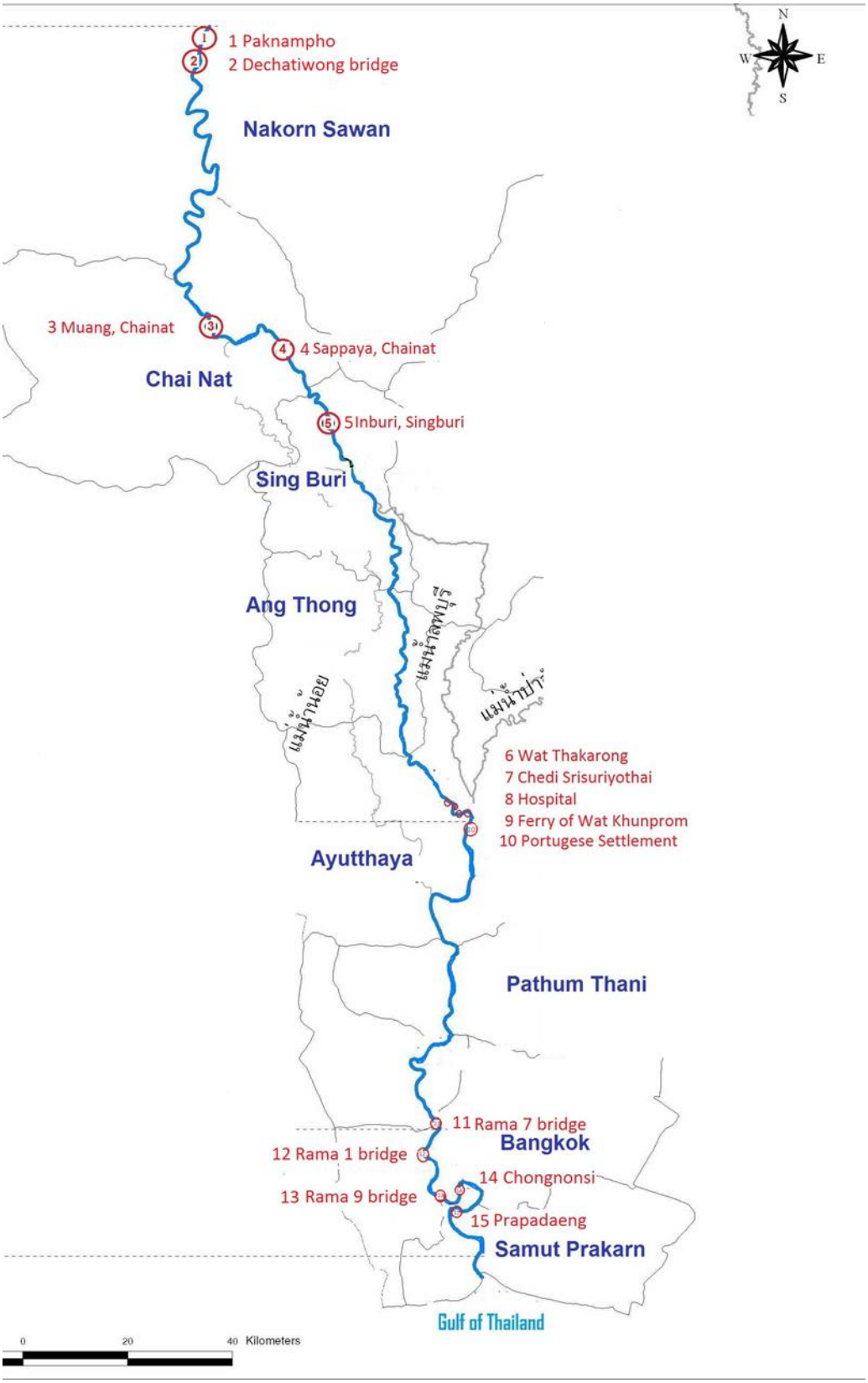

Figure 1. Map of Chao Phraya River

PCR reaction conditions were as follows: an initial denaturation step at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 sec, at 63 °C for 30 sec, and 72 °C for 30 sec, with a final extension step of 72 °C for 5 sec. A non-template control consisting of sterile DI water was also run with the reaction samples.

PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Digital images were obtained after UV transillumination and the products were compared to a commercial molecular weight standard. Amplicons of the appropriate size (~450 BP) were considered a positive indication of successful DNA extraction, in addition to the lack of an observed amplified product in the non-template control sample.

Quantitative Real-time PCR

Two assays were employed for qPCR. One assay method used Taqman reagents, while the other used SYBR green reagents, with amplification and quantification for both types of assays conducted using a step-one plus real-time PCR instrument (Applied Biosystems, USA). The *E. coli* specific primers used in this study targeted a portion of the *uidA* gene (Takahashi et al., 2009). Forward primer, ECN1254F (5′-GCA AGG TGC ACG GGAATA TT-3′) and reverse primer ECN1328R (5′-CAG GTG ATC GGA CGC GT-3′) were used as well as a dual labeled (FAM-TAMRA) probe, ECL1277p (5′-CGCCACTGGCGGAAGCAACG-3′) for the Taqman assays. All oligonucleotide primers and probes were synthesized using a commercial service.

SYBR green qPCR, 20 µl reaction volumes consisted of Power Green PCR Master Mix (AB Biosystems, USA), 0.5 µM of forward and reverse primers, and 2 µl of DNA template. Each set of samples were assayed in triplicate and included a non-template control. SYBR green PCR conditions were as follows: an initial denaturation step at 95 °C for 15 min, followed by 35 cycles at 95 °C for 30 sec, at 63 °C for 60 sec, and 72 °C for 60 sec, at which point acquisition of fluorescent signals were measured. All SYBR green qPCR reaction assays included a final melting curve analysis. Select dilutions of DNA standards were run in duplicate, creating three-point standard curves for each reaction run.

For Taqman qPCR reactions, 20 µl reaction volumes comprised of 1x Taqman Universal PCR Mastermix (Applied Biosystems), 0.5 µM of ECL1277p probe, 0.5 µM each of forward and reverse primers, and 2 µl of DNA template. Each set of samples were assayed in duplicate and included a non-template control, as well as duplicates for select 10-fold dilutions of DNA standards used for creating three-point standard curves. Taqman qPCR reactions were performed with an initial denaturation at 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 sec, and a final 1 minute 63 °C extension step sec for fluorescent signal acquisition. Ct values for the sample assays were determined using the step-one instrument software with cell numbers of the DNA standards used to estimate log CFU/100 ml values (Noble et al., 2006). Linear regression analysis was done to generate standard curves for the diluted DNA standards resulting in R2 values of 0.974 and 0.998 for SYBR green and Taqman qPCR experiments, respectively.

Statistical analysis

Statistical analysis for MPN counts and qPCR data was done using R, an open-source statistical program (R Foundation for Statistical Computing, Vienna, Austria). Initial Shapiro-Wilk analysis of the data indicated it was not normally distributed. Hence, Wilcoxon rank sum non-parametric statistical tests were used to determine any statistically significant differences in mean log/100 ml values based on the enumeration methods employed.

3. Results and Discussions

All PCR products indicated the expected size amplicon using Bac349F/Bac806R primers, while an absence of a product in the non-template control was observed (data not shown). The results demonstrated that bacterial DNA could be extracted from the environmental samples, and more importantly, be of sufficient quality to serve as template for PCR experiments.

Mean log/100 ml MPN values for all river samples were 2.89 while for qPCR assays, values were 3.59 and 2.38 for SYBR green and Taqman qPCR experiments, respectively (Table 2). Shapiro-Wilk tests for normality

indicated non-parametric tests were appropriate for statistical analysis (data not shown). Comparisons of different PCR enumeration methods were made against counts established through the Colilert Quanti-tray MPN method. Nonparametric tests indicated a significant statistical difference between mean log/100 ml counts determined by SYBR green qPCR compared to that of MPN methods (P=0.004). Additionally mean counts obtained by SYBR green qPCR were also significantly higher than that of Taqman qPCR (P=0.000019). However, while mean log/100 ml counts were lower for Taqman qPCR when compared to MPN enumeration methods, this difference in means was not statistically significant (P=0.9699). For PCR reactions, non-template control samples indicated no observable signal, and results for melting curve analysis of SYBR green qPCR experiments showed that observed signals were not due to non-specific amplification (no data shown).

Table 2. Mean Log/100 ml Values of Surface Waters

Enumeration method	Mean (± standard deviation)
SYBR green real-time PCR	3.59 (±0.45)*
Taqman real-time PCR	2.38 (±1.17)
Colilert MPN	2.89 (±0.65)

Asterisk indicates mean value was significantly different (P<0.05) compared to other enumeration methods.

Observed log/100 ml counts established from MPN methods were in agreement with previous studies quantifying *E. coli* from surface waters in Southeast Asian cities using culture techniques (Widmer et al., 2013). Traditional microbiological techniques for detection and quantification of bacterial pathogens are time-consuming and faster alternative methods such as PCR and real-time PCR have been utilized over traditional culture techniques (Douterelo et al., 2014; Ibekwe et al., 2002; Walker et al., 2017). Also, these methods have demonstrated the ability to rapidly identify fecal contamination in environmental samples (Dorevitch et al., 2017; Gensberger et al., 2014; Layton et al., 2006).

However, these methods are sensitive to PCR inhibition (Loge et al., 2002; Stults et al., 2001), where humic acids and metal ions can serve as common PCR inhibitors for environmental samples (Wilson, 1997). Long term persistence of DNA in freshwater is possible especially if adsorbed by environmental particulates, however predation from other microorganisms as well as degradation from environmental conditions such as ultraviolet light have a significant impact on the fate of extracellular DNA (Lorenz & Wackernagel, 1994; Nielsen, Johnsen, Bensasson, & Daffonchio, 2007). This is further supported by individual studies where seeded DNA in surface waters degraded within 24 hours as opposed to being stable up to 5 days in distilled and tap water samples (Alvarez et al., 1996) and a similar study investigating the persistence of plasmid DNA in lake water samples indicated that complete degradation was observed within 170 h (Matsui et al., 2001). Employing PCR for the detection of bacteria DNA in seawater indicated similar declines, as one study observed that the detection of *Legionella spp*. in ocean water microcosms maintained at 16 °C declined after 4 days (Palmer et al., 1993). Temperature can impact the stability of DNA in aqueous environments, as microcosms of seawater spiked with both free DNA and heat-killed *Salmonella* culture suspensions had DNA detectable through PCR up to 55 days when samples were maintained at 10 °C, while similar microcosms varied from 2 to 10 days if held at 20 °C (Dupray et al., 1997). These studies highlight that effective extraction along with preservation of DNA is critical in the application of molecular methods for identification and quantification in environmental samples.

Given that average log/100 ml counts quantified from SYBR green qPCR were significantly higher than means counts obtained from MPN culture methods, these results indicate that qPCR can serve as a comparable, if not improved, enumeration technique for quantifying fecal indicator organisms from surface waters (Figure 2). It is of note that qPCR assays employed in this study were not comparable with each other, with the SYBR green qPCR assays performing significantly better than Taqman qPCR. It is possible that the probes and/or primers for the Taqman qPCR assays might be more sensitive to inhibition from the water matrixes, interfering with the amplification and detection efficiency, including DNA from other non-target organisms and humic acid from sediment (Green & Field, 2012; Sidstedt et al., 2020). Some studies have observed that after being spiked with mixed bacterial cultures as a pseudo-microbial community, FTA and FTA Elute cards had issues with efficient recovery of all strains, especially Gram positive organisms (Gray et al., 2013). However, the average log/100 ml

counts quantified from Taqman qPCR, while lower than mean counts obtained from MPN culture methods, were not significantly different.

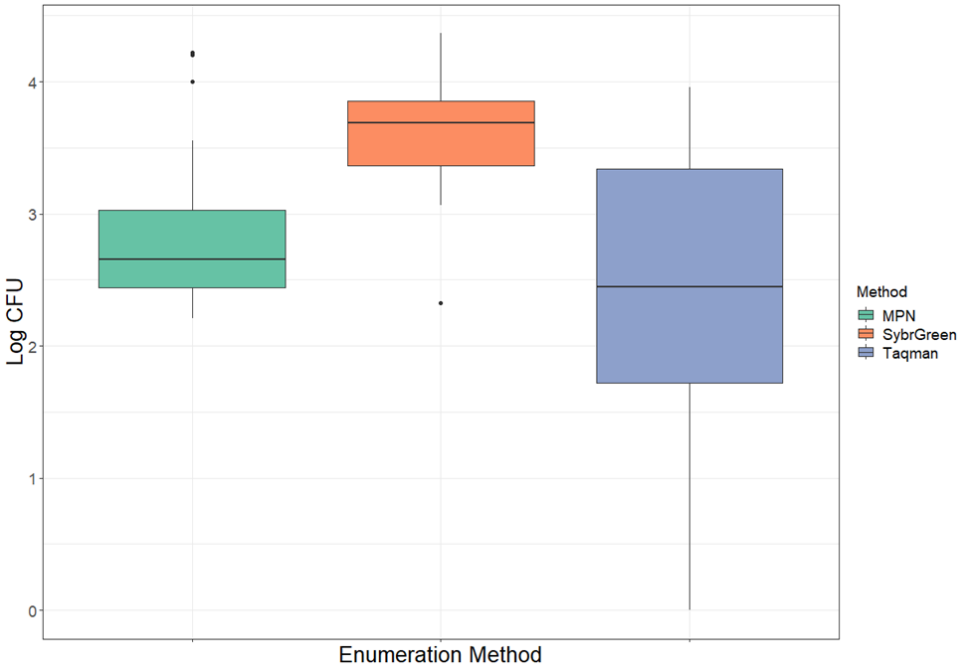

Figure 2. Y axis is log CFU/100ml. Solid lines within bar plots represent the median for each enumeration method

This study demonstrates proof of concept that Whatman FTA Elute cards can be utilized for microbial DNA extraction from environmental water samples. As the DNA samples could be maintained at room temperature, and the extracted bacterial DNA could serve as an effective template for qPCR, it demonstrates the potential of using FTA Elute cards for other molecular applications in developing countries. As the processing of water samples and DNA extraction is relatively easy and does not require atypical laboratory equipment, combined with the capability of storing processed DNA samples at room temperature, a single central facility for DNA extraction and post-processing methods could be employed for a larger geographical region.

Rapid advancements in molecular methods have incorporated more comprehensive means of characterizing the microbial communities of environmental samples (Acharya et al., 2020; Urban et al., 2021). While historically molecular methods have been considered costly compared to traditional culture methods, these costs have been reduced and have demonstrated that new technologies that can be conducted in the field (Batra et al., 2023; Manzanas et al., 2023). However, these methods still require effective means of extracting environmental DNA.

Utilization of FTA cards allow for DNA extraction from a simple bench laboratory, which could then be shipped to other facilities for more extensive testing and sample processing. It can be more economically feasible having a central laboratory facility capable of conducting more advanced microbial genome sequencing and molecular characterization, which then could provide service to several provinces or at the national level. This would allow for developing countries to utilize more sensitive and specific methods for routine monitoring of environmental samples, or in special cases where rapid identification of microbial pathogens might be needed. Additionally, they would not require advanced laboratory facilities to be on site or near points of collection, and instead samples could be analyzed remotely. The processing of water samples using Whatman FTA Elute cards highlights the potential of central laboratory analysis facilities which could service remote water monitoring and sampling stations in developing countries for water quality analysis, allowing for the use of more advanced and sensitive molecular-based methods.

Conflict of Interests

The authors declare no conflict of interest which could affect the representation or interpretation of the findings presented.

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