Application of quantitative real-time PCR compared to filtration methods for the enumeration of *Escherichia coli* in surface waters within Vietnam

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ABSTRACT

Surface water samples in Vietnam were collected from the Saigon River, rural and suburban canals, and urban runoff canals in Ho Chi Minh City, Vietnam, and were processed to enumerate *Escherichia coli*. Quantification was done through membrane filtration and quantitative real-time polymerase chain reaction (PCR). Mean log colony-forming unit (CFU)/100 ml *E. coli* counts in the dry season for river/suburban canals and urban canals were log 2.8 and 3.7, respectively, using a membrane filtration method, while using Taqman quantitative real-time PCR they were log 2.4 and 2.8 for river/suburban canals and urban canals, respectively. For the wet season, data determined by the membrane filtration method in river/suburban canals and urban canals samples had mean counts of log 3.7 and 4.1, respectively. While mean log CFU/100 ml counts in the wet season using quantitative PCR were log 3 and 2, respectively. Additionally, the urban canal samples were significantly lower than those determined by conventional culture methods for the wet season. These results show that while quantitative real-time PCR can be used to determine levels of fecal indicator bacteria in surface waters, there are some limitations to its application and it may be impacted by sources of runoff based on surveyed samples.

Key words | *Escherichia coli*, membrane filtration, quantitative PCR, surface water, tropical water quality

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INTRODUCTION

For rural populations in developing countries, surface waters are consistently used as sources of drinking water in some communities and may be susceptible to fecal contamination resulting in negative consequences to public health (Ibekwe *et al.* 2002). The use of fecal coliforms, and more specifically *Escherichia coli*, as an indicator of microbiological water quality dates from their first isolation from feces at the end of the 19th century (Rompre *et al.* 2002). The detection of fecal coliforms has been successfully used to serve as an indicator for fecal contamination, and as an index for the potential levels of pathogens, in water environments and have also helped in establishing total maximum daily loads (Rompre *et al.* 2002; Simpson *et al.* 2002).

E. coli is a Gram-negative, facultative anaerobic, lactose fermenting, non spore-forming bacterium. It is present in the gastrointestinal tract of humans and has good characteristics of a fecal indicator organism for surface waters (Scott *et al.* 2002). While *E. coli* is a commensal organism in the gut, there are some strains that are pathogenic, and currently there are five pathogenic classes of *E. coli* recognized, which are enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli*, enteroaggregative *E. coli*, and enterohemorrhagic *E. coli* (Nataro & Kaper 1998; Kaur *et al.* 2010). Although enumeration of this organism commonly uses simple culture techniques such as multiple-tube fermentation and membrane filtration plating, these

routine methods have limitations. The extended incubation times, contamination of natural microbial flora, and lack of specificity are some issues.

The use of molecular analysis methods with shorter reporting times such as quantitative polymerase chain reaction (qPCR) has been shown to be a technology that can be employed in environmental studies for the specific detection of a number of different fecal indicator organisms and water-borne pathogens (Haugland *et al.* 2005). A study by Rose *et al.* (2003) explored the use of molecular detection techniques using PCR primers that were specific for the *rrsB* gene of *E. coli* when used under stringent conditions in soil. This protocol, however, only provided information as to the presence or absence of *E. coli* DNA in the extracts. In addition, the potential existed for false negatives due to inhibition by contaminants co-extracted with the DNA (Rose *et al.* 2003). Another study described the application of three Taqman assays and qPCR to detect the *stx1*, *stx2*, and *rfbE* gene targets in surface water. The results from this study indicate that approximately 50 cells of *E. coli* O157:H7 could be consistently recovered from a 40-litre surface water sample (Mull & Hill 2009).

Real-time PCR has several advantages including enhanced speed and the absence of post-PCR processing steps. It has been demonstrated that qPCR assays can not only be specific, but also have amplification kinetics suitable for the specific quantification and detection of *E. coli*, irrespective of strain (Takahashi *et al.* 2009). One particular target for PCR amplification is the *uidA* gene, which encodes for β-D *glucuronidase*, and is found in almost all strains including the pathogenic, *E. coli* O157:H7 (Takahashi *et al.* 2009).

This study was intended to detect and quantify *E. coli* through real-time PCR in environmental surface waters of Vietnam, particularly within the Saigon River and neighboring canals impacted by suburban, agricultural, and urban runoff. The Saigon River is the second most important source of water supply for Ho Chi Minh City (HCMC) and Binh Phuoc Province. Agriculture and husbandry are main activities in the middle of the Saigon River, while the downstream is mainly affected by urban sewage. Their discharges combined contribute significant amounts of bacteria into the Saigon River water. Two techniques for qPCR were utilized, one method using SYBR green, a fluorogenic minor groove DNA binding dye, and the second method a Taqman qPCR

assay, involving a dual-labeled specific probe designed to bind to a target amplicon sequence. Additionally, this study intended to demonstrate the accuracy of this molecular technique being comparable to that of traditional plate count methods, and to assess the potential application of qPCR for monitoring water surface quality in Southeast Asian countries.

METHODS

Preparation of DNA standards and DNA extraction

An overnight culture of *E. coli* ATCC® strain 15597 was grown in simple enrichment medium and 10-fold serial dilutions were prepared in sterile distilled, deionized (DI) water, in duplicate. Serial dilutions of the *E. coli* culture were also spread plated onto tryptic soy agar plates to enumerate the DNA standards as colony-forming units (CFU)/100 ml. Each dilution had 1 ml filtered through a sterile 0.45 μm membrane, filters were transferred to sterile 50 mm petri dishes, and the surface of each membrane filter was washed with 0.75 ml of DNA extraction buffer (250 mM NaCl, 100 mM Ethylenediaminetetraacetic acid (EDTA), pH8, 2% Sodium dodecyl sulfate (SDS)). After 5 minutes, the DNA extraction buffer was carefully aspirated and transferred into a sterile microcentrifuge tube.

DNA from the dilution standards was extracted following a protocol modified from Porteous *et al.* (1994). Briefly, the DNA extraction buffer was sonicated for 3 min, and 25 μl of 5M guanidine thiocyanate was added, followed by an incubation step at 68 °C for 1 h. Afterwards, samples were centrifuged at 10,000 × g for 10 min and supernatant transferred to fresh microfuge tubes. The supernatant was mixed with isopropanol and incubated at −20 °C for 24 h. After incubation, samples were centrifuged at 10,000 × g for 10 min, the pellet was washed with 70% ethanol, and centrifuged again. Finally, the samples were re-suspended in 100 μl Tris-EDTA buffer. Extracted DNA from equivalent dilutions were pooled together and maintained at −20 °C.

Survival study

Initial microcosm studies were done to evaluate the application of qPCR for enumeration of *E. coli* in surface

waters under more controlled conditions. Samples were pre-pared in 500 ml volumes as either autoclaved DI water, or filtered surface water. Surface water was collected from Youngsan River, located in Gwangju, Korea. The surface water samples were filter sterilized using 0.45 µm membrane filters and placed in sterile polypropylene bottles. All water samples were spiked with approximately 6 log *E. coli* (ATCC strain 15597) washed cells from an overnight culture in tri-plicate (unless noted otherwise). Additionally, non-spiked samples were prepared to serve as negative controls. All water samples were maintained at 4 °C. Survival microcosm studies for SYBR green real-time PCR quantification were prepared both in DI water (in triplicate) and with filter ster-ilized surface water (prepared in duplicate) with samplings done at 1 and 10 days. For Taqman qPCR, only filter steri-lized surface water samples were prepared (in triplicate) for survival studies with samplings conducted at day 1, 10, and 110.

For all water sample microcosms (both filtered surface water and DI water), at selected day intervals, 10 ml portions were removed and after membrane filtration, enumerated by colony counts after incubating membrane filters on modified mTEC agar plates (Difco BBL, NJ, USA). At the same day intervals for plate count enumeration, 10 ml portions were also filtered through sterile 0.45 µm membranes, transferred to sterile 50 mm petri dishes, and 0.75 ml of DNA extraction buffer (250 mM NaCl, 100 mM EDTA, pH8, 2% SDS) was added to each membrane filter. The filters were maintained at −20 °C for 5 days until further DNA extraction processing, employing a similar method as described previously. Main-taining the filters for DNA extraction at −20 °C for 5 days was done to somewhat mimic processing delays expected for remote water sampling in Vietnam, where samples would be held for extended periods until they could be shipped to Korea for extraction and qPCR analysis. Quantitat-ive PCR using either SYBR green or Taqman real time assay was conducted on the DNA extraction samples using similar methods described later in the text. Counts from both spread plating and qPCR were calculated as $\log_{10}$ CFU/100 ml.

Collection and processing of surface water samples

Surface water samples were collected at 44 locations, from the Saigon River (10 locations), six suburban canals (24 locations), and five urban canals (10 locations) within HCMC, Vietnam over a 2-month period for both the dry (April 2011) and wet seasons (August 2011). Sampling locations are indicated in Figure 1. Grab samples were col-lected in 200 ml volumes within sterile polypropylene plastic bottles during both high and low tide on the same date for all locations except three suburban canals for the dry season. Samples were kept at 8 °C in an improvised ice box until processing in the laboratory (within 8 hours of collection).

For water sample processing, 100 ml of sample was fil-tered through a 0.45 µm membrane, filters were transferred to sterile 50 mm petri dishes, and 0.75 ml of DNA extraction buffer (250 mM NaCl, 100 mM EDTA, pH8, 2% SDS) was added to each filter. After 5 minutes, the DNA extraction buffer was carefully aspirated and transferred into sterile, screw cap polypropylene tubes, and then kept at −20 °C until shipping to Korea. Additionally, between 25 and 50 ml were filtered through a 0.45 µm membrane filter and trans-ferred onto modified mTEC agar plates. Sample plates were incubated as per the manufacturer's instructions. After 24 hours, colonies showing atypical morphology (red-magenta

Figure 1 │ Surface water sampling locations. Sites indicated by a gray circle (river) or triangle (canal) were sampling locations employed in this study.

colonies), were enumerated and recorded as colony forming units per 100 ml for each surface water sample.

Real-time PCR and analysis of surface water samples

Two techniques for qPCR were utilized, one using SYBR green and another using a Taqman qPCR assay. All amplification of DNA targets was performed using a Rotor-Gene™ 3000 (Corbett Research) real time PCR instrument. The *E. coli* specific primers used were adapted from Takahashi *et al.* (2009). The forward primer ECN1254F (5′-GCA AGG TGC ACG GGAATA TT-3′) and reverse primer ECN1328R (5′-CAG GTG ATC GGA CGC GT-3′) employed in this study amplify the *uidA* gene. For the Taqman real time PCR assay the inclusion of a dual labeled (FAM-TAMRA) probe, ECL1277p (5′-CGCCACTGGCGGAAG-CAACG-3′), was added to the reaction.

For SYBR green qPCR, reaction volumes of 20 µl consisted of QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.5 µM of forward and reverse primers, 0.1 µl bovine serum albumin (50 mg/ml), and 2 µl of DNA template. Each set of samples were assayed in triplicate and included a non-template control. Bovine serum albumin was included to alleviate PCR inhibition (Artz *et al.* 2006). The qPCR conditions for these reactions 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 the acquisition of the fluorescent signal was measured. All SYBR green qPCR reactions included a final melting curve analysis. Three-point standard curves of select dilutions for DNA standards were run in duplicate with each reaction. SYBR green qPCR reactions were only done for the dry season water samples.

For the Taqman qPCR reactions, 20 µl consisted of 1x Taqman Universal PCR Mastermix (Applied Biosystems), 0.5 µM of ECL1277p probe, 0.5 µM of forward and reverse primers, 0.1 µl bovine serum albumin, and 2 µl of DNA template. Each set of samples were assayed in triplicate and included a non-template control, with three-point standard curves of select dilutions for DNA standards run in duplicate with each reaction. Taqman qPCR was performed with an initial denaturation at 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 sec, and a 63 °C extension step for 60 sec where the fluorescent signal was acquired.

Taqman qPCR analysis was done for all collected water samples. Determined cycle threshold (Ct) values for the samples were compared to the linear function of standard curves and corresponding log CFU/100 ml values of the standards (Gueimonde *et al.* 2004; Noble *et al.* 2010).

Statistical analysis

Log_{10}/100 ml values for plate counts and real-time PCR data underwent statistical analysis to determine normal distributions and either non-parametric tests (Wilcoxon W ranked) or t-tests were employed, where appropriate, using a commercial statistical software package (SPSS 14.0, Chicago, USA).

RESULTS

Survival study results

Plate counts and qPCR results were compared to determine if stressed *E. coli* cells could be detected with the selected primers under controlled conditions. Initial results for the SYBR green studies looked very promising as average mean counts after 10 days were 5.67 log CFU/100 ml and 6.98 log CFU/100 ml in the DI water survival studies for plating and qPCR, respectively. Additionally, the standard curves for the DNA standards indicated a linear relationship ($R^2 = 0.9651$). Mean counts for specific days from the filtered surface water survival study experiments are summarized in Figure 2. For filtered surface water, the average mean counts after 10 days for SYBR green qPCR and plate counts were 6.31 log CFU/100 ml and 4.84 log CFU/100 ml, respectively, while mean counts determined by Taqman qPCR was 7.24 log CFU/100 ml. For the Taqman qPCR assay, a longer study was conducted in filtered surface water and resulted in a more pronounced difference in average mean log CFU/100 ml values, with averages being 3.71 log CFU/100 ml for plate counts and 6.49 log CFU/100 ml for qPCR. Overall the results indicated that real-time PCR and extended DNA processing times could recover and detect sufficient numbers of spiked *E. coli* under controlled conditions within water, and even filter-sterilized surface water samples for up to 110 days.
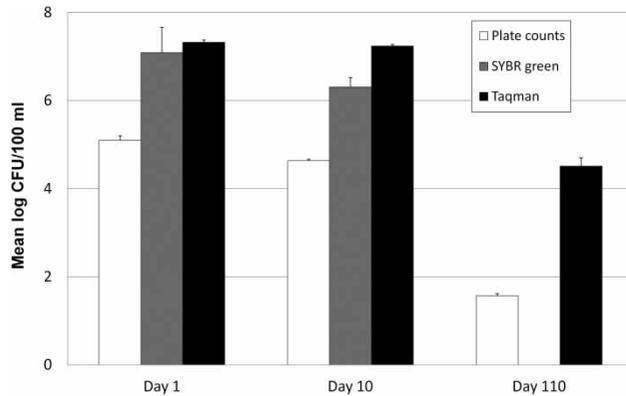
Figure 2 | Survival study results for filtered surface water. Bars represent the mean log CFU/100 ml counts determined by spread plating (open bars), SYBR green real-time PCR (gray bars), or Taqman real-time PCR (black bars) at 1 and 10 days, respectively. For day 110 samplings, only spread plating and Taqman real-time PCR assays were conducted. Error bars are standard error of the mean.

Comparison of plate counts and quantitative PCR analysis for *E. coli* enumeration in surface water samples

Results of Taqman and SYBR green qPCR dry season water samples were compared. Mean *E. coli* counts were more pronounced in Taqman assays of 2.50 log CFU/100 ml as compared to SYBR green qPCR assays resulting in 1.51 log CFU/100 ml, with the difference deemed statistically significant ($p = 0.003$). Standard curves for the assays indicated a linear relationship, for both the Taqman ($R^2 = 0.9391$) and SYBR green qPCR assays ($R^2 = 0.9634$). However, because of this notable observed difference in mean log CFU/100 ml counts, SYBR green qPCR was not used for wet season water sample analysis and all the remaining PCR analysis was done with Taqman PCR reagents only.

For the dry season, water samples collected from both river and suburban sites had mean log CFU/100 ml counts of 2.79 determined by spread plate counting ($n = 64$) and 2.42 determined by Taqman qPCR ($n = 65$), which were not significantly different ($p = 0.99$). Likewise for urban water samples collected in the dry season ($n = 20$), despite higher observed mean plate counts of 3.68 log CFU/100 ml, they were deemed not significantly different ($p = 0.093$) from the mean counts of 2.76 log CFU/100 ml determined by Taqman qPCR. For the wet season, observed differences in mean counts were not statistically significant in both the river and suburban sites, as mean log CFU/100 ml plate counts of 3.73 ($n = 58$) and Taqman real-time

PCR mean log CFU/100 ml values ($n = 57$) of 3.08 were shown to be not significant ($p = 0.11$). However, when comparing urban water sampling locations ($n = 20$) in the wet season, the mean counts were noticeably higher for spread plating (log 4.61 CFU/100 ml) compared to Taqman qPCR assays (log 2.07 CFU/100 ml) and were significantly different ($p = 0.001$). Additionally, for the wet season, standard curves for Taqman assays indicated a linear relationship ($R^2 = 0.968$). Mean counts for the different seasons and water sample types are summarized in Table 1 and box plots of the enumeration data are presented in Figure 3.

Additional statistical analysis was conducted to determine if there was any overall difference in plate count data for high or low tide sampling events. All water samples were compared for both high tide and low tide with no statistically significant difference noted ($p = 0.524$). This trend was also observed comparing just the urban water sampling locations ($p = 0.318$) and the suburban water sampling locations ($p = 0.824$) among each other.

DISCUSSION

The preliminary survival experiments had indicated that greater enumeration could be obtained through real-time PCR as opposed to plate culture methods over longer periods of time. Additional experiments determined that using the mentioned methods for DNA extraction, qPCR had a detection limit of 2 log CFU/100 ml (data not shown). Although higher mean counts from qPCR were observed in filtered surface water treatments, these samples would not accurately model degradation of DNA under true environmental conditions

Table 1 | Mean log CFU/100 ml *E. coli* counts

Water sample type	Plate counts	Taqman qPCR	SYBR green qPCR
Suburban/river (dry season)	2.79 (± 0.26)	2.4 (± 0.2)	1.24 (± 0.27)
Urban (dry season)	3.68 (± 0.41)	2.76 (± 0.42)	2.3 (± 0.6)
Suburban/River (wet season)	3.73 (± 0.19)	3.08 (± 0.25)	–
Urban (wet season)	4.61 (± 0.2)	2.07 (± 0.49)*	–

An asterisk denotes that means from Taqman qPCR assays were significantly different from means determined by plate count assays ($p < 0.05$).
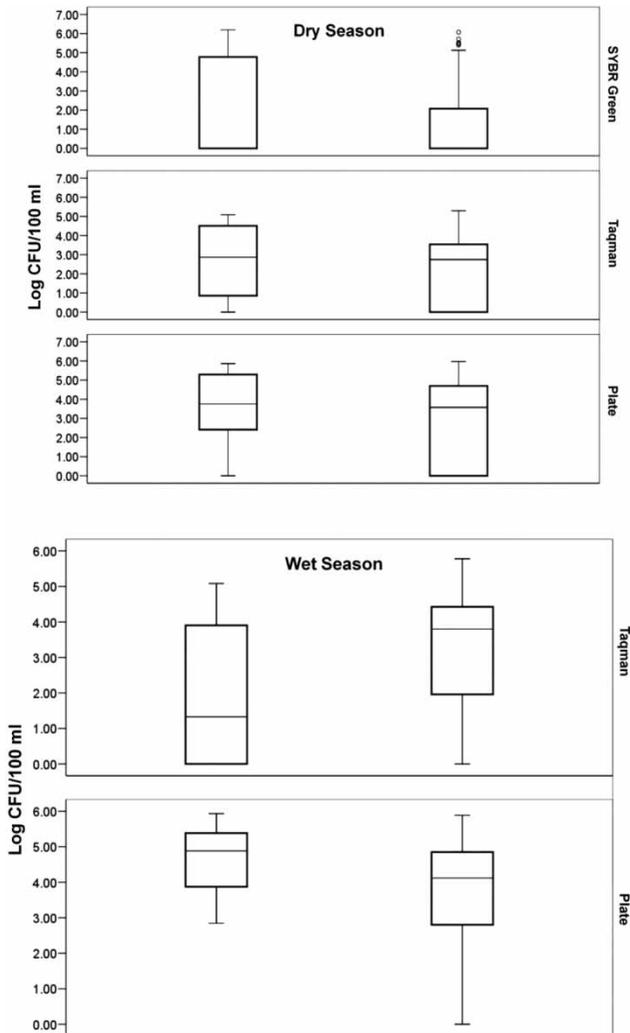
Figure 3 | Log CFU/100 ml of surface water samples. Box plots are either the dry season (top section) or the wet season (bottom section) and split into rows based on enumeration method (SYBR green or Taqman qPCR, or plate counts). The bars in the left column are from urban surface water samples and the bars in the right column are suburban/river surface water samples.

which incorporate other factors such as UV light exposure (Häder & Sinha 2005), particularly after storm events (Chan *et al.* 2015). However, the initial experiments with the survival studies demonstrated that DNA extraction methods and PCR assays employed were capable of detecting spiked *E. coli* DNA in some cases up to 110 days, demonstrating the method could have some application in field studies. Additionally, as samples were kept at $-20\,^{\circ}\mathrm{C}$ for 5 days before processing, this further indicated the potential of using this method for DNA extraction and PCR analysis at different laboratory facilities from those utilized for sample collection.

Not all real-time PCR methods were robust enough to accurately quantify bacterial numbers. For samples analyzed during the dry season, mean values from the Taqman assay were significantly higher than those determined by using SYBR green qPCR. It is interesting to note that this trend was also observed in the preliminary survival studies using filtered surface water, as day 10 counts for Taqman qPCR were approximately 1 log higher than mean counts determined using SYBR green qPCR. Primarily due to this difference in mean counts between PCR methods, SYBR green qPCR was not attempted on further samples and Taqman assays were employed for the sampling events in the wet season.

Sampling was done over the course of the study collecting at locations during low and high tide. Comparing average mean plate counts for the water samples indicated no statistical difference showing that tidal conditions did not significantly influence *E. coli* microbial populations in these waters over daily sampling events. Quantification of *E. coli* using Taqman qPCR had mean counts which were slightly lower than enumeration conducted using membrane filtration plate count methods but were not significantly different among most of the water samples. Exceptions to this were the urban water samples collected during the wet season as these samples has substantially higher numbers determined by membrane filtration plate counts as opposed to quantitative PCR.

Overall means of *E. coli* CFU/100 ml roughly increased by 1 log in the wet season compared to samples collected in the dry season. This trend is opposite to that of a larger study which surveyed primarily river surface waters in several Southeast Asian countries (Widmer *et al.* 2013). It is likely that the intense number of rainfall events due to the seasonal monsoon may have impacted *E. coli* populations in these waters. This may also explain the poor enumeration of urban water samples by Taqman qPCR. It may be that increased rainfall also led to an increased presence of compounds which could be inhibitory to PCR. Consistently, enumeration efforts using Taqman qPCR with urban water samples had mean CFU/100 ml values less than those determined by membrane filtration. However, it was only in the wet season that these differences were statistically significant. Further, even means for the urban water samples were approximately 1 log higher in the wet season compared to the dry season, if

determining counts based on membrane filtration. Although these were not significant, which may likely be due to the lower number of urban water samples, this may indicate a greater incidence of runoff within the urban canals which could impart greater amounts of fecal contamination compared to samples collected in rural canals or larger bodies of surface water such as the Saigon river. However, this increased urban runoff may also have resulted in a greater concentration of compounds which could impede PCR reactions in the collected water samples.

Other research studies have discussed the importance of sample inhibitors to PCR methods as being a critical issue to address when enumerating pathogens in water samples (Stults *et al.* 2001; Loge *et al.* 2002) and while humic acids are a common class of inhibitors, metal ions such as aluminum and iron can also act as PCR inhibitors (Shieh *et al.* 1995). Additionally, some have suggested that while *uidA* has a high specificity for the detection of *E. coli* through qPCR, this gene may not be an ideal target for monitoring environmental waters for fecal contamination (Chern *et al.* 2011). Despite these limitations there have been studies demonstrating the utilization of quantitative qPCR being comparable to other standard culture methods for enumerating *E. coli* in surface waters, yet DNA extraction efficiency was considered a critical factor in enumeration efficiency (Noble *et al.* 2010). The rapid processing and analysis times, along with greater specificity using qPCR does also have advantages over methods that employ traditional culture methods for detection and identification of fecal indicator bacteria and waterborne pathogens. The method described in this study allows for timelier sample analysis which could be useful if needed for rapid confirmation of monitoring results, and the fact that such analysis could be done at a central laboratory facility indicates that such techniques could be applicable in developing countries.

CONCLUSIONS

Enumeration data of *E. coli* in surface waters from suburban and urban canals as well as from the Saigon River in Vietnam were determined by both culture methods and by quantitative real-time PCR. Initial data indicated that Taqman qPCR was more applicable than SYBR green PCR

for quantification. For the dry season there were no significant differences in the mean *E. coli* counts by either membrane filtration counts or Taqman qPCR analysis. However, in the wet season, mean counts established by Taqman qPCR in urban canal water samples were significantly lower. This indicates that the application of molecular methods for enumeration of fecal indicator bacteria may have limitations, quite possibly due to the presence of PCR inhibitors.

Despite these limitations, this study supports the notion that molecular methods could be employed to enumerate fecal indicator bacteria in tropical surface waters. Moreover, the processing of samples can be done remotely. The methodology described here provides a proof of concept that a central laboratory facility could provide DNA extraction and quantitative PCR analysis while remote laboratories would only be responsible for sample collection and minimal processing. Because of this the application of molecular techniques for more thorough monitoring and analysis of surface waters could be more easily implemented and feasible as the requirements of trained technical staff and advanced equipment would be minimized. More advanced techniques for monitoring and investigative studies for source tracking are likely needed as there is a growing shift in land use for developing countries. Rapid urbanization is especially a pressing issue in Asia, since as of 2010 it was home to 12 out of the 23 megacities in the world, and it is projected that by 2050 65% of the population in Asia will live in urban cities (Singru 2015). Because of the unique environmental characteristics and seasonal meteorological events in tropical climates, current studies that investigate the prevalence, modeling, transport, and persistence of fecal indicator bacteria in temperate environments may have limited application in tropical surface waters (Rochelle-Newall *et al.* 2015). This may require more research to be done in tropical environments and potentially need the application of more advanced techniques such as the methods described in this study.

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