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METHODS AND SOFTWARE

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Recreational water monitoring: Nanofluidic qRT-PCR chip for assessing beach water safety

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Abstract

Improved recreational water monitoring using rapid molecular genetic methods would decrease both public health risks and unnecessary beach closures. Hence, we developed a novel nanofluidic quantitative real-time PCR (qPCR) in the OpenArray platform to (a) detect and quantify fecal indicator bacteria (FIBs; N = 2), (b) identify contaminant sources (microbial source tracking (MST); N = 7), and (c) detect human virulent pathogens (virulence gene markers; N = 15). Our water monitoring OpenArray plate reliably detects as few as two template copies/hole (OpenArray® well), with some marker sensitivities as low as single-copy detection. The OpenArray plate showed high target sequence specificity and returned expected patterns of contaminants for fecal and sewage samples. We found Canada geese and seagulls were the leading causes of contamination at beaches that tested positive for coliforms. When we incorporated robotic DNA extraction, we were able to process samples from water to FIBs, MST, and waterborne pathogen detection and quantification within four hours. Our monitoring TaqMan qPCR assays in the OpenArray platform is uniquely valuable for regulatory agencies charged with beach water safety as well as for researchers interested in human health implications of aquatic microbial community structure.

KEYWORDS
nanofluidic qRT-PCR, OpenArray, public health, source tracking, waterborne pathogens

1 | INTRODUCTION

Human fecal, and consequently pathogen, contamination is a serious environmental and public health problem that affects water bodies around the world. Fecal pollution has become more widespread due to population growth and climate change, and this is correlated with increases in waterborne disease (Levy, Woster, Goldstein, & Carlton, 2016). Recreational water is the second most common source of waterborne disease outbreaks worldwide, after contaminated drinking water (McClung et al., 2017). Degradation of freshwater quality also significantly reduces human recreational opportunities and results in economic loss (e.g., decreased tourism and reduced fishing activity.) (Vörösmarty et al., 2010). In the United States, for example, epidemiologic data indicate the risk for developing acute gastrointestinal illness symptoms is as high as 15 cases per 1,000 swimmers, with a total of 90 million recreational water use-related illnesses nationwide, which translates into $2.2-$3.7 billion in losses annually (DeFlorio-Barker, Wing, Jones, & Dorevitch, 2018). Consequently, improving beach monitoring and regulation technologies, coupled with beach remediation efforts guided by appropriate source tracking methods, would improve public health, recreation opportunities, and economic well-being.

Culturing and counting fecal indicator bacteria (FIBs), such as *Escherichia coli*, remains the most common approach for monitoring pathogenic water pollution (Soller et al., 2016). Although coliform culture has been the standard for water safety monitoring, it has a number of important limitations, including the following: (a) It is time consuming, (b) some FIBs can survive and grow in aquatic habitats (Ishii & Sadowsky, 2008), (c) there is generally a poor correlation between FIBs and the occurrence of pathogens (Jang et al., 2017), and (d) there is a lack of coliform host specificity (Pattis et al., 2017). Bacterial culturing is the gold standard for pathogen detection and has served us well for more than 150 years, but it is not applicable for routine water monitoring program due to cost and time of culture method. Only well-equipped laboratories with approved biosafety clearance are able to culture pathogenic bacteria (Fusco & Quero, 2014). Moreover, low sensitivity of culture methods has been reported for complex samples compared to new molecular methods (Jacovides et al., 2012). Improved molecular technologies will be able to evaluate water quality and safety rapidly, more precisely and less expensively, particularly when more than one pathogen is targeted (Wolk & Hayden, 2011).

Quantitative real-time PCR (qPCR) is an emerging tool for water pathogen monitoring and has been recommended by the US Environmental Protection Agency (EPA) for detection and quantification of *E. coli* as a FIB (Chern, Siefring, Paar, Doolittle, & Haugland, 2011). qPCR methods have been used to detect individual waterborne pathogens such as *E. coli* O157 (Wu, Hulme, & An, 2015) and *Yersinia enterocolitica* (Cheyne, Van Dyke, Anderson, & Huck, 2010).

Multiplex qPCR is widely applied to quantify groups of waterborne pathogens (Fan, Wu, & Kou, 2008; Nhung, Ohkusu, Miyasaka, Sun, & Ezaki, 2007). Some of those studies utilized microfluidic plates (Ishii, Segawa, & Okabe, 2013; Ramalingam et al., 2010). However, most studies do not validate and assess probe sensitivity, nor do they generally include source tracking markers. The inclusion of Microbial Source Tracking (MST) to determine the specific fecal source (e.g., humans, dog, cattle, and wildlife.) is critical as the source will influence human health risk (Harwood, Staley, Badgley, Borges, & Korajkic, 2014). However, the simultaneous detection and quantification of many water safety-related target loci are challenging due to the technical difficulties associated with multiplexed qPCR. High density, low volume qPCR (nanofluidic) platforms are becoming more readily available (Morrison et al., 2006), and they allow multiple target detection with very high sensitivity (Friedrich, Zec, & Wang, 2016).

A robust, high-throughput quantitative tool that targets FIBs, MST, and pathogen virulence markers is required for effective real-time monitoring of recreational water and subsequent risk management. Consequently, the objectives of this study were to (a) design and validate a rapid nanofluidic qPCR (TaqMan® assays in the OpenArray® platform) assays to monitor FIBs, MST markers, and waterborne pathogens simultaneously and (b) evaluate the application of the designed method for monitoring of FIBs, MST, and waterborne pathogens in different environmental samples. We combine the OpenArray platform with robotic DNA extraction to dramatically improve our ability to detect, monitor, and quantify key waterborne health risks in less than four hours. We apply our assay to a variety of samples (sewage, fecal control samples, environmental water samples, and beach sand samples) and show the wide utility of our approach for both regulatory and research applications.

2 | MATERIALS AND METHODS

2.1 | Target selection and designing of the primers

Fifteen key waterborne pathogenic species (primary causes of bacterial waterborne disease (Ramírez-Castillo et al., 2015)), two FIBs including *E. coli* (two independent genes; 23S rRNA and *uidA* gene) and *Enterococci* spp., one general fecal pollution MST marker, and six host-specific MST markers were selected for inclusion on our OpenArray® plate (Table S1). We used existing qPCR primers and probes for the detection and quantification of *E. coli* (targeting 23S rRNA) (Chern, Siefring, Paar, Doolittle, & Haugland, 2011) as a FIB, along with new primers and probe for the *uidA* gene; another universal gene conserved among different strains of *E. coli*. To assess the occurrence of inhibition during qPCRs, we also used previously suggested marker (amplification of 70 bp fragment of NH8B_3641 gene encoding NosZ-like protein of *Pseudogulbenkiania* spp. NH8B) as an internal positive control (Ishii et al., 2013).

Single-copy virulence genes, which are present in all pathogenic strains of each waterborne pathogen, were selected as targets for primers and probe design (Table S1). For MST of fecal pollution, 16S rRNA sequence from host-specific *Bacteroides* for Canada goose (*Branta canadensis*) (Vogt et al., 2018), dog (*Canis lupus* familiaris), and pig (*Sus scrofa domesticus*) (Okabe, Okayama, Savichtcheva, & Ito, 2007) and 16S rRNA sequence from *Methanobrevibacter smithii* (human MST marker) (Ufnar et al., 2006), *Catellicoccus marimammalium* (seagull MST marker) (Lee, Marion, & Lee, 2013), and C40 mitochondria gene (human MST marker) (Caldwell, Payment, & Villemur, 2011) were used to design specific primers for qPCR. A universal primer set targeting 16S rRNA gene of *Bacteroides* spp. was designed as a general MST marker. We used published sequence data of each target to design specific primers and probes targeting virulence genes using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). We used the following parameters of Primer3Plus software to design the primers: no CG clamp, max poly-X (score: 3), max self-complementarity (score: 3), and max 3' self-complementarity (score: 0). Other parameters kept as default.

3 | PRIMER VALIDATION

The specificity of the designed primers was checked in silico using NCBI BLAST tool (Altschul et al., 1997) against the GenBank database. We used primer specificity stringency of at least 2 nucleotide mismatches to unintended targets, including at least 2 nucleotide mismatches within the last 5 nucleotides at the 3' end of the primers as default parameters.

Further, each designed primer set was tested in the laboratory against the host-specific and pooled nonspecific fecal matter DNA (Canada goose, dog, humans, pig, seagull, and sewage) and the DNA of target and nontarget species of bacteria. For each marker (Table S1), PCR master mix was prepared as described previously and each target was amplified with 59°C annealing temperature in 35 cycles (Shahraki, Chaganti, & Heath, 2019). The amplicon for each marker was visualized on agarose gel, and the amplicon size was estimated along with 50 bp DNA Ladder (Invitrogen™). To test the sensitivity, we used purified PCR product—PCR product of each primer was purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer instruction. The concentration of each PCR product was measured using Agilent 2100 Bioanalyzer with a High Sensitive DNA chip (Agilent Technologies). The PCR product for each marker, with estimated copy numbers, was combined and then diluted (10-fold) to make seven known concentrations of all targets (2,000,000, 200,000, 20,000, 2,000, 200, 20, 2, and 1 copies of each target/reaction) for SYBR green qPCR assays to determine the sensitivity of the designed primers.

3.1 | Sample preparation and DNA extraction

We collected a variety of environmental samples, including beach water (6 × 250 ml), beach pore water (6 × 250 ml), and sand (6 × 20 g) from six public beaches (Windsor-Essex county). We also collected stream and pond water samples (3 × 250 ml, each) near the sampled beaches. We collected sewage samples (3 × 250 ml) and fecal samples (N = 5 from Canada goose, dog, pig, and seagull as well as two healthy human fecal samples) for use as control samples. The collected water and sewage samples were delivered to the laboratory on ice within four hours of collection. Water and sewage samples were filtered with 0.22-micron pore size, 47-mm-diameter polycarbonate filters (IsoporeTM, Millipore, MA). After filtration, each filter was placed in a 2-mL sterile tube containing 0.5 g glass beads (0.1 mm diameter, Bio-Spec Products). For sand samples, 20 g of sand from each location was added to a tube containing 250 ml distilled sterile water and the mixture was vigorously shaken for 2 min. The supernatant was transferred into a sterile bottle, and the sample was filtered as described above. For fecal samples, 2 g of each sample was added to a 2-ml sterile tube with 0.5 g glass beads.

For cell lysis, 400 µl of sucrose lysis buffer (400 mmol/L NaCl, 750 mmol/L, 20 mmol/L ethylenediaminetetraacetic acid (EDTA), 50 mmol/L Tris–HCl pH 9.0) was added to each tube. After bead beating (1 min, 3 times), each sample was treated with lysozyme (Sigma-Aldrich); 10 mg/ml, 37°C for 20 min, and proteinase K (Thermo Scientific); 0.2 mg/ml, 50°C for 20 min. Proteinase K was deactivated in 95°C for 5 min, and then, DNA was purified from the samples using the solid-phase reversible immobilization (SPRI) paramagnetic bead-based method on an automated liquid handling platform (Tecan Freedom Evo150 Liquid Handling Platform, Perkin Elmer) (Shahraki, Chaganti, & Heath, 2019). The purity and concentration of the extracted DNA from samples were checked (ND-1000, NanoDrop) (Shahraki, Chaganti, & Heath, 2019).

3.2 | SYBR green qPCR assay

We used SYBR green qPCR to test the efficiency of the primers prior to designing the OpenArray® plate. Furthermore, our validation of the SYBR green qPCR allows applications of our markers where the OpenArray® plate is not feasible due to lack of access to the OpenArray® equipment, or where single marker vs. multiple markers are interested. We applied SYBR green qPCR to the control (fecal and sewage) and environmental samples and validated the assays using dilutions of positive controls. SYBR green qPCRs were carried out on environmental and fecal sample DNA and all known concentration target template DNAs in 20 µl including 10 µl SYBR green master mix (Applied Biosystems™), 1 µl primers (combined forward and reverse primers, final concentration 10 pmol for each), 1 µl/ reaction DNA, and 8 µl ddH_2O. We also used SYBR green assays of two pathogen virulence genes (*ctxA* and *manC*) and NH8B_3641 gene (internal positive control) to evaluate the presence of PCR inhibitors over qPCR assays. Extracted DNA of water samples was negative in SYBR green qPCR assays for *ctxA*, *manC*, and NH8B_3641 genes. For this experiment, extracted DNA from each water sample was inoculated with known concentration of *ctxA*, *manC*, and NH8B_3641 genes (2,000,000, 200,000, 20,000, 2,000, 200, 20, 2, and 1 copies of each gene/µl) in fresh tubes and was subjected to SYBR green qPCR. No template control (NTC) reaction including SYBR green master mix, forward, and reverse primers of each marker and ddH_2O but not DNA template was run for each assay. Real-time program consisted of 95°C for 1 min followed by 40 cycles 95°C for 10 s, 60°C for 60 s. All reactions were duplicated in MicroAmp Fast 96-well reaction plates and were run on QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems).

3.3 | Nanofluid OpenArray®

The goal of this research was to develop a high-throughput panel of qPCR assays in 33-nl reactions to monitor recreational water safety. We chose the nanofluidic TaqMan® assays in the OpenArray® platform (gene expression platform, Thermo Fisher Scientific), as it is capable of performing duplicate qPCR assays for 25 marker loci, including 3 FIB markers (two assays for *E. coli* (23S rRNA and *uidA* gene), and one for *Enterococcus* spp.), 7 MST markers (two human specific markers, one Canada goose marker, one dog marker, one pig marker, one seagull marker and one general MST marker), and 15 waterborne pathogen virulence gene markers simultaneously. The OpenArray® plate was preloaded with primers and probes labeled with carboxyfluorescein (FAM) at the 5′-end and Black Hole Quencher® 1 (BHQ1) at the 3′-end. The efficiency of our TaqMan® assays in the OpenArray® platform was evaluated using DNA from our selected control (sewage and fecal) and environmental samples. The extracted DNA from each sample (2.5 µl) was mixed with 2.5 µl TaqMan® OpenArray® Real-Time PCR Master Mix (Applied Biosystems™) for loading on the OpenArray® instrument. Also, seven combined known concentrations (2,000,000, 200,000, 20,000, 2,000, 200, 20, 2, and 1 copies of each target/

hole (OpenArray® well) were loaded on the OpenArray® plate. The mixture was loaded using the QuantStudio 12K Flex Accufill System (Applied Biosystems™), and the OpenArray® plate was run on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Two no template controls (NTCs) were run along with fecal and environmental samples. After the initial denaturation at 95°C for 3 min, 40 cycles of the following program were used for amplification, denaturation at 95°C for 10 s and annealing/extension at 60°C for 10 s.

3.4 | Data analysis

The most common method of quantitation associated with qPCR assays is based on measurement of threshold cycle (C_T) and is the C_T (baseline threshold) method (Bustin et al., 2009). Relative threshold (Crt) is an alternative method which is more robust for analyzing data generated on the Applied Biosystems™ QuantStudio™ 12K Flex Real. As these two methodologies give comparable quantitation cycle (Cq) values and fold change (FC) values in large data sets (Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System, Application Note), we used C_T method to analysis our data. The assay was classified as a true positive when two replicates were positive. The standard curve for each marker was generated by linear regression analysis of the C_T value vs. the amounts of the template DNA using log copies/µl for SYBR green assays and log copies/hole for TaqMan® assays. The efficiency of each assay was calculated using standard curve with the equation: Eff = 10(−1/slope) − 1, where slope corresponds to the slope of the standard curve (Bustin et al., 2009). If all target sequences doubled during each replication cycle of qPCR, amplification efficiency considered as 100% (Bustin et al., 2009).

We estimated template copy number of the target locus in environmental and fecal samples for each assay using the TaqMan® assays C_T value based on the standard curve. The lowest concentration of each target which generated a C_T value in all replicates was identified as the detection limit. We converted gene copies/250 ml water or gene copies/2 g of fecal matters, assuming that the cell recovery and DNA extraction efficiency were 100% in all samples, and that only one gene copy was present per cell. Zero was given to nondetect assays for statistical analysis. Pearson's correlation was used to correlate the concentration of *Bacteroides* spp. (general MST marker), *E. coli*, and host-specific markers (Canada goose and seagull) individually, with detected waterborne pathogens (combined as single variable). Also, we correlated combined host-specific MST markers' concentration as single variable with combined waterborne pathogens as another variable in all environmental samples. Then, we excluded the environmental samples which were negative for waterborne pathogens from the data and the correlation was repeated between individual MST markers, *E. coli*, and the combined host-specific marker with combined waterborne pathogens as single variable. Heatmaps were generated in R version 3.5.3 with ggplot2 on $\log_{10}$ transformed gene copies measured by the OpenArray® plate (Warnes et al., 2009).

4 | RESULTS

4.1 | DNA extraction

All the samples had more than 50 ng/µl DNA concentration with the optimum ration of 260/280 (~1.8) and 260/230 (~2.0–2.2) (Lucena-Aguilar et al., 2016) (Table S2).

4.2 | Assay validation

All the designed amplicons had a length of 66–90 bp (Table S1) and melting temperature (Tm) of 59 ± 2°C. We used the NCBI BLAST tool to predict the specificity of the primers and possible nonspecific amplification. All the designed probes and primers were specific for their intended targets with a few exceptions; the designed probe for *Enterococcus* spp. showed possible annealing with *Sphingobacterium psychroaquaticum*; however, due to specificity of the forward and reverse primers for the *Enterococcus* genus, we used the designed probe in the OpenArray® plate. Also, the designed probe and primers for *Bacteroides* spp. as a general MST marker showed possible annealing with *Prevotella* spp. As genera *Bacteroides* and *Prevotella* belongs to the same phyla (*Bacteroidetes*) and both have high sequence similarity of 16S rRNA and have fecal source (Bernhard & Field, 2000), the designed primer set was *Bacteroides-Prevotella* taxon specific; however, in the manuscript we considered *Bacteroides* spp. (general MST marker). Also, as *E. coli* and *Shigella* spp. are sister species and consequently have high similarity in their genome sequence (Zuo, Xu, & Hao, 2013), the designed probe and primers for *uidA* gene of *E. coli* also showed possible annealing with *Shigella* spp. based on our "in silico" sequence-based analysis.

In PCR trials, the virulence gene and FIBs primers amplified the appropriate target (Table S1) in the target bacterial species and no false positives or false negatives were detected by conventional PCR (Figure S1–S2). The virulence genes and FIBs also were amplified in sewage samples. FIBs amplicons were amplified in different fecal matters (Figure S1-S2). Some virulence genes were amplified by their corresponding specific primers in pooled fecal samples of different animals and humans. For example, both *C. coli* and *C. jejuni* were amplified in pooled fecal samples of Canada goose and seagull (Figure S2). General MST marker (*Bacteroides* spp.) was detected in all fecal and sewage samples (Figure S2). No PCR amplification was detected for any of the host-specific MST markers (i.e., no amplification of nontarget host fecal sample DNA), while all host-specific MST assays detected their specified host fecal matter DNA (Figure S1-S2). As *E. coli* (different strains) and *Shigella* spp. are closely related, detection of *Shigella* spp. by *uidA* and 23S rRNA gene PCRs was considered as true positive.

While the goal of this study was to develop a TaqMan® qPCR assays in the OpenArray® platform, we also tested SYBR green qPCR performance for applications where the OpenArray® plate is not feasible. The mean SYBR green qPCR C_T values across all 25 marker loci with known template DNA concentrations of 2,000,000, 200,000, 20,000, 2,000, 200, and 20 copies/reaction were 17.42 ± 0.36, 21.17 ± 0.45, 24.12 ± 0.56, 27.4 ± 0.50, 30.6 ± 0.59, and 33.8 ± 0.57 qPCR (Table S3), a pattern of

TABLE 1 Performance of the SYBR green qPCR and TaqMan® assays in the OpenArray® platform

		TaqMan® assays in the OpenArray® plate			SYBR green qPCR		
Species/Target	**Genes**	**Slope**	**r^2**	**Efficiency (%)**	**Slope**	**r^2**	**Efficiency (%)**
Acinetobacter baumannii	*gltA*	−3.1687	.98	106	−3.0267	.99	114
Aeromonas hydrophila	*Lip*	−3.3741	.99	97	−3.1509	.99	107
Bacterioides spp.	16S rRNA	−3.0304	.98	113	−3.182	.99	106
Bacterioides sp. clone CGOF52 (goose marker)	16S rRNA	−3.1662	.99	106	−3.1183	.98	109
Bacteriodes A3 (dog marker)	16S rRNA	−3.3384	.99	99	−3.1981	.99	105
Bacteriodes Cluster 1, PigA4 (pig marker)	16S rRNA	−3.0957	.99	110	−3.0081	.98	115
Campylobacter coli	*glyA*	−3.2852	.99	101	−3.0625	.99	112
Campylobacter jejuni	*hipo*	−3.1499	.99	107	−3.1499	.99	107
Catellicoccus marimammalium (seagull marker)	16S rRNA	−3.1158	.99	109	−3.3625	.99	98
Enterococcus spp.	23S rRNA	−3.5028	.99	92	−3.3171	.98	100
Escherichia coli	23S rRNA	−3.3234	.99	99	−3.1791	.99	106
Escherichia coli	*uidA*	−3.0039	.99	115	−3.4497	.99	94
Escherichia coil O157:H7	*manC*	−3.0807	.99	111	−3.2806	.99	101
Escherichia coli O111	*manC*	−3.2033	.99	105	−3.359	.99	98
Escherichia coli O26	*manC*	−3.1909	.99	105	−2.9999	.98	115
Human C40 mitochondria (human marker)	MT-ND2	−3.2345	.99	103	−3.0903	.99	110
Klebsiella pneumoniae	*phoE*	−3.1186	.99	109	−3.0449	.99	113
Legionella pneumophila	*mipA*	−3.1292	.99	108	−3.4601	.99	94
Listeria monocytogenes	*Hly*	−3.3589	.98	98	−3.4888	.99	93
Methanobrevibacter smithii (human marker)	*nifH*	−3.1292	.99	108	−3.4705	.99	94
Pseudomonas aeruginosa	*regA*	−3.1515	.99	107	−3.0936	.97	110
Salmonella typhimurium	*invA*	−3.2793	.99	101	−3.3565	.97	98
Shigella spp.	*ipaH*	−3.1852	.99	106	−3.1458	.99	107
Staphylococcus aureus	*gyrA*	−3.2261	.99	104	−3.2081	.98	104
Vibrio cholerae	*ctxA*	−3.1595	.99	107	−3.3796	.99	97

increasing C_T consistent with the dilutions. Two copies/reaction were detected in 6 (24%) of the assays, but none of the assays generated a C_T value for one copy/reaction (Table S3). Mean amplification efficiency (across all 25 assays) was 93%–115% for the SYBR green qPCR (Table 1). No C_T value was detected for NTC for each SYBR green assay. Also, SYBR green qPCR assays of three genes (*ctxA*, *manC*, and NH8B_364) with known concentration of each gene in water samples show a liner relationship between the inoculated gene copies of each marker and the those quantified by qPCR assays (Figure S4).

The mean C_T values across all assays for the known template DNA concentrations 2,000,000, 200,000, 20,000, 2,000, 200, 20, and 2 copies/hole (OpenArray® well) were 16.63 ± 0.5, 19.85 ± 0.64, 22.93 ± 0.66, 25.91 ± 0.69, 29.06 ± 0.5, 32.68 ± 0.77, and 35.62 ± 0.79, respectively, for the TaqMan® assays (Table S3). All assays generated C_T values for 2 copies/hole; however, only 6 (24%) of the assays generated C_T values for one copy/hole (Table S3). Mean amplification efficiency (across all 25 assays) was 92%–115% for TaqMan® assays (Table 1). No C_T value was detected for NTC for each TaqMan® assay.

4.3 | TaqMan® assays in the OpenArray® plate applications

Five different types of environmental samples (beach water, beach sand, beach pore water, stream water, and pond water), sewage samples (known as contaminated environmental samples), and fecal samples from various sources (Canada goose, dog, humans, pig, and seagull) were used to evaluate the effectiveness of the designed OpenArray® plate. We successfully quantified FIBs and MST markers in all fecal samples and sewage samples (as known contaminated environmental samples) using our newly developed TaqMan® assays. Virulence genes of waterborne pathogens were detected in sewage

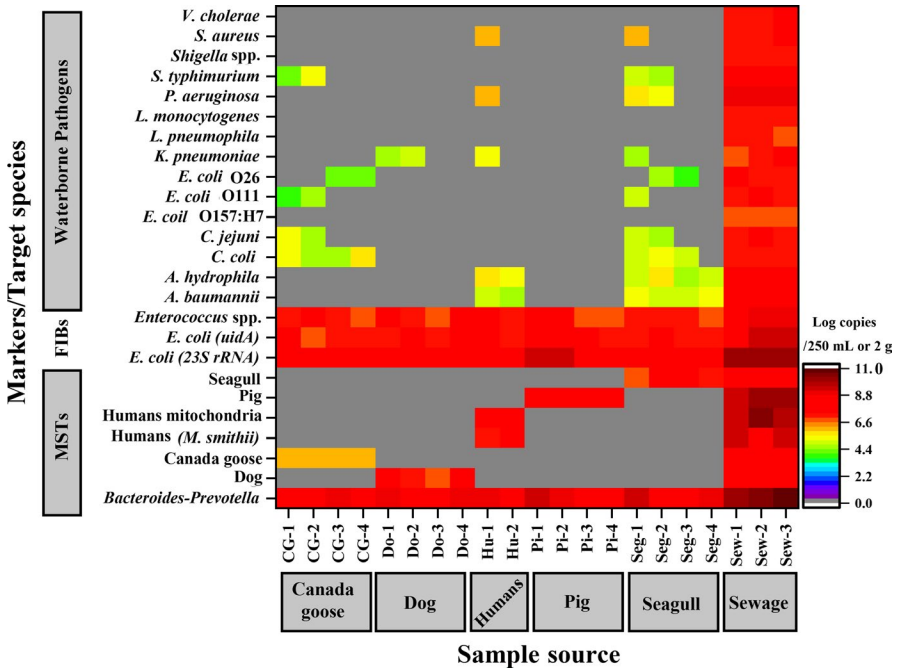

FIGURE 1 Heatmap of qPCR amplification (based on $\log_{10}$ [C_T]) using TaqMan® assays in the OpenArray® platform to detect targets in fecal samples. Seven MST, 2 FIBs, and 15 waterborne pathogen markers are presented on the Y-axis. Nondetection shown by gray color in heatmap

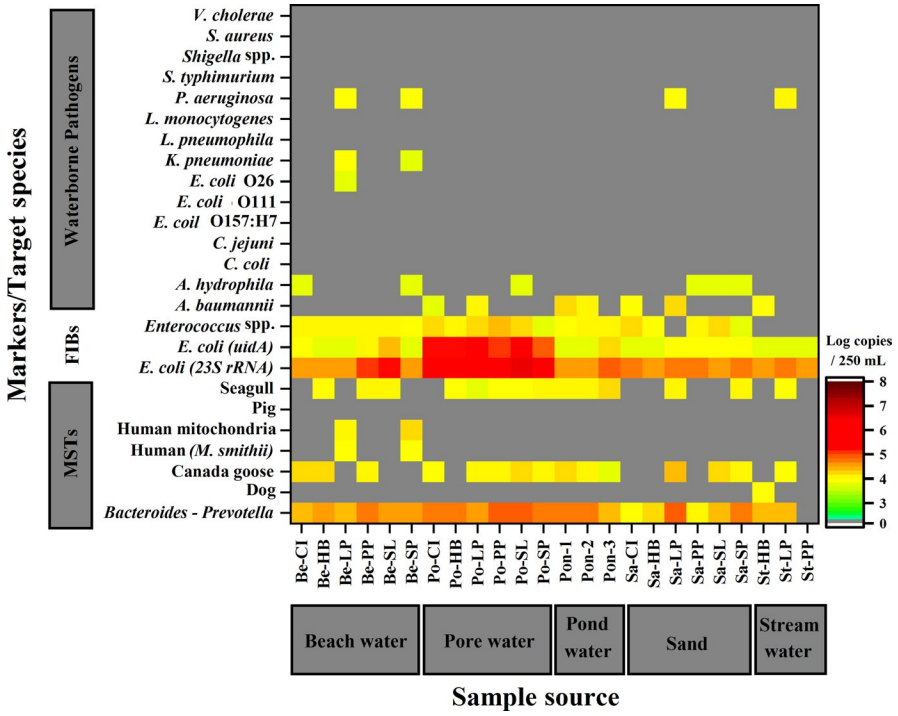

FIGURE 2 Heatmap of qPCR amplification (based on $\log_{10}$ [C_T]) using TaqMan® assays to detect targets in the environmental samples. Seven MST, 2 FIBs, and 15 waterborne pathogen markers are presented on the Y-axis. Environmental samples were collected from six public beaches: CI, cedar island beach; HB, holiday beach; LP, lakeview park beach; PP, point pelee beach; SL, seacliff beach; SP, sand point beach. Nondetection shown by gray color in heatmap

samples (Figure 1). FIBs and MST markers (except the pig marker), and the virulence genes of some waterborne pathogens, including *A. baumannii*, *A. hydrophila*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli* O26, were quantified in environmental samples (Figure 2).

E. coli as a FIB was detected in all sewage samples, in the appropriate fecal samples, and in the environmental samples. The estimated concentration of *E. coli* was high in our study using the 23S rRNA marker compared to the single-locus *uidA* gene marker due to the presence of multiple rRNA loci in *E. coli* cells (Brosius, Dull, Sleeter, & Noller, 1981) and potentially low taxonomic specificity for environmental samples (Figures 1 and 2) (Chern et al., 2011). We thus focus on *E. coli* levels based on the single-copy *uidA* gene as all virulence genes targeted in this study are single-copy genes. However, the inclusion of the 23S rRNA marker allows comparisons to other studies that use that marker. Based on *uidA* qPCR data among 24 environmental samples, 9 (37.5%) had *E. coli* levels > 4 log copies/250 ml, including 2 beach water samples and 1 pond water sample and 6 pore water samples. The remaining 15 (62.5%) environmental samples had *E. coli* levels < 4 log copies/250 ml level. *Enterococcus* spp., the other tested FIB, was present in most of the

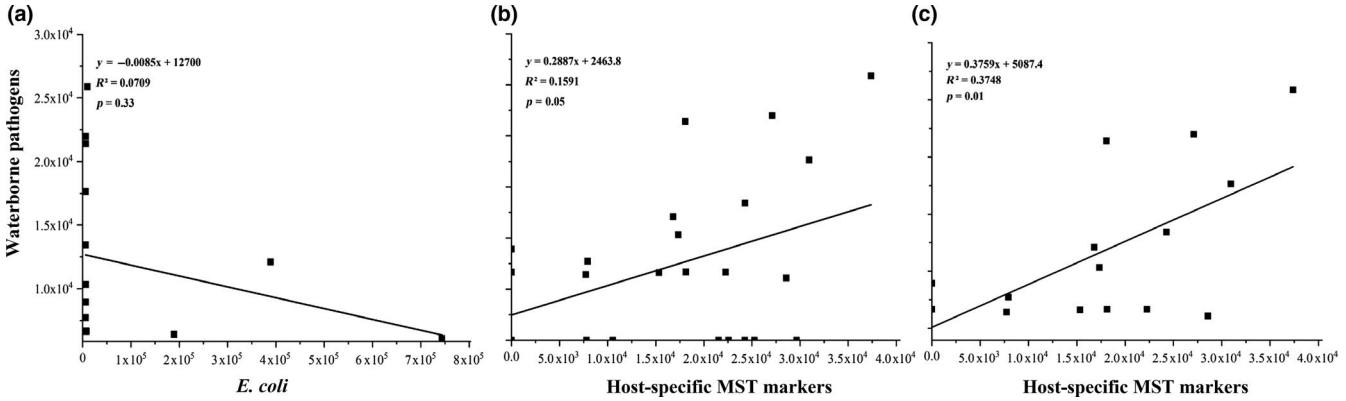

FIGURE 3 Correlations between combined gene concentrations (copies/250 ml) of host-specific MST markers (Canada goose, dog, humans, and seagull), *E. coli*, and waterborne pathogens among the environmental samples measured by TaqMan® assays in the OpenArray® plate. (a) correlation between waterborne pathogens (combined concentration of pathogens as single variable) with *E. coli* (*uidA* gene) among 15 environmental samples positive for pathogens. (b) correlation between host-specific markers (combined concentration of markers as single variable) with waterborne pathogens (combined concentration of detected pathogens as single variable) among all 24 environmental samples. (c) correlation between host-specific markers (combined concentration of markers as single variable) with waterborne pathogens (combined concentration of detected pathogens as single variable) among 15 environmental samples positive for pathogens

tested samples except four environmental samples (one sand and three stream samples)—despite *E. coli* being detected in those samples (Figure 2). *E. coli* was not correlated with waterborne pathogens across all 24 environmental samples (R^2 = .005, p = .87), nor was it correlated in the samples which were positive for waterborne pathogens (15 samples, R^2 = .07, p = .33; Figure 3a).

All seven MST markers were detected in sewage samples and in their appropriate host fecal samples (Figure 1). Out of 24 environmental samples, two beach water samples (8%) were positive for human MST markers (C40 mitochondria and *M. smithii*). Canada goose and seagull MST markers were detected individually in 15 (62.5%) and 14 (58%) of the environmental samples, respectively. Canada goose and seagull MST markers amplified simultaneously in 12 (54%) of the environmental samples. The dog MST marker amplified in one environmental sample (stream), while the pig marker did not amplify in any of the environmental samples. The samples with *E. coli* levels > 4 log *uidA* copies/250 ml (6 pore water samples, 2 beach water samples, and 1 pond water sample) were positive for one of the bird species (Canada goose and seagull) MST markers. Out of 15 environmental samples with *E. coli* levels < 4 log *uidA* gene copies/250 ml), 11 samples (73%) were positive for general and host-specific MST markers (humans and birds) and 4 (27%) samples showed none of the specific MST marker signatures but were positive for general MST marker (*Bacteroides* spp.).

All 15 waterborne pathogens were detected in sewage samples (Figure 1). Interestingly, virulence genes of *C. coli*, *C. jejuni*, *E. coli* O26 and O111, and *S. typhimurium* were detected in Canada goose and seagull fecal samples, while seagull samples additionally contained *A. baumannii*, *A. hydrophila*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* (Figure 1). *A. baumannii*, *A. hydrophila*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli* O26 were detected in 29%, 25%, 16%, 8%, and 4% among 24 environmental samples, respectively. The *A. baumannii* virulence gene (*gltA*) was detected in 7 environmental samples (pond (2), pore (2), sand (2), stream (1); Figure 2). Also, five environmental samples (beach (1), pore (1), sand (3)) were positive for the *A. hydrophila* virulence gene (*lip*). *P. aeruginosa* virulence gene (*regA*) was positive in 4 environmental samples (beach (2), sand (1) and stream (1)). Two beach water samples were positive for *K. pneumoniae* virulence gene (*phoE*), and one of those samples was also positive for *E. coli* O26 virulence gene (*manC*). Virulence genes of *C. coli*, *C. jejuni*, *E. coil* O157:H7, *E. coli* O111, *L. pneumophila*, *L. monocytogenes*, *S. typhimurium*, *Shigella* spp., *S. aureus*, and *V. cholerae* were not detected in any of the collected environmental samples (Figure 2).

Out of 24 tested environmental samples, 20 (83%) samples were positive for host-specific MST markers (either of birds, humans, or dog markers) of which 13 (63%) were positive for 1–3 virulence genes of waterborne pathogens. Out of 24 samples, 2 (8%) samples with pathogens were negative for host-specific MST markers but were positive for general MST marker (*Bacteroides* spp.). Out of 24 samples, 2 samples were negative for host-specific MST markers and pathogens but one of them was positive for general MST marker. Among all 24 environmental samples, there was no significant correlation between any MST markers with waterborne pathogens in this study (Canada goose marker: R^2 = .02, p = .62; seagull marker: R^2 = .008, p = .72; general MST marker: R^2 = .027, p = .33). However, there was better correlation (R^2 = .159, p = .05) between combined host-specific MST markers (Canada goose, dog, humans, and seagull) with waterborne pathogens (Figure 3b).

Out of the 15 environmental samples positive for waterborne pathogens, 3 (20%) samples were pore water with the highest *E. coli* level (>4 log *uidA* copies/250 ml) but 12 (80%) samples had *E. coli* levels < 4 log *uidA* copies/250 ml. Out of 15 environmental samples which were positive for waterborne virulence genes, 13 (87%) samples were positive for host-specific MST markers (10 positive for

the two bird markers, 2 for the human marker, and 1 for the dog marker); however, 2 (sand) samples were negative for all host-specific MST markers but still were positive for the general MST marker (*Bacteroides* spp.). For the environmental samples which were positive for pathogens (15 samples), there was no significant correlation between any MST markers individually with waterborne pathogens (Canada goose marker: R^2 = .009, p = .56; seagull marker: R^2 = .07, p = .38; general MST marker: R^2 = .049, p = .47) but there was a significant correlation (R^2 = .37, p = .01) between the combined host-specific MST markers with waterborne pathogens (Figure 3c).

5 | DISCUSSION

In serially diluted positive controls, our TaqMan® assays in the OpenArray® platform were sensitive to the detection of 2 copies/hole (OpenArray® well) for all assays and to 1 copy/hole (OpenArray® well) for 24% of the assays. This compares well to SYBR green qPCR (positive in 24% of assays with 2 copies/reaction and negative in all 1 copies/reaction). However, due to the much larger input volume of DNA (~60 times) in SYBR green qPCR (1 µl) compared to TaqMan® assays (16.5 nl), SYBR green qPCRs would be more efficient for detection. This sensitivity challenge for the TaqMan® assays in the OpenArray® platform was due to the small volume of input DNA. It could be overcome by increasing water sample volume, or by concentrating DNA using DNA precipitation protocols or commercially available filters (up to 100X concentration using Microcon® Centrifugal Filters; Millipore, MA) or by specific target amplification (Ishii et al., 2013) before loading the DNA on the OpenArray® plate. Courdray-Meunier et al. found SYBR green qPCR to be more sensitive than TaqMan® assays in the OpenArray® platform for quantifying human pathogenic viruses (Coudray-Meunier et al., 2016). We found that 2 copies/hole were the robust detection limit across all 25 of our target loci using TaqMan® assay qPCR. Detection limits ranging from 2.0 to 2.0 × 10^6 copies/µl were previously reported for microfluidic qPCR assays designed for FIBs and waterborne pathogens (Ishii et al., 2013). High sensitivity of assays reported by Ishii et al. (2013) may be a result of preamplification step of input temples used for microfluidic qPCR assays (Ishii et al., 2013).

Evaluation of presence of PCR inhibitors in qPCR experiments is crucial (Schrader, Schielke, Ellerbroek, & Johne, 2012). High-quality extracted DNA from the samples (Table S2) as well as presence of liner relationship between the inoculated know concentration of *ctxA*, *manC*, and NH8B_3641 (internal control) to the DNA of water samples in SYBR green qPCR assays (Figure S4) indirectly confirmed accurate quantification of genes in TaqMan assays.

Pore water samples had the highest *E. coli* levels (4.9–5.8 log copies/250 ml), showing that the pore water may act as a reservoir and nonpoint source of *E. coli* for surface waters (Vogel, O'Carroll, Edge, & Robinson, 2016). Generally, in our environmental samples, *E. coli* was not consistently associated with waterborne pathogens both across all 24 environmental samples (R^2 = .005, p = .87) and in the 15 waterborne pathogen-positive samples (R^2 = .07, p = .33, Figure 3a), potentially indicating that *E. coli* is a poor health risk indicator for recreational waters—a pattern previously reported (Jang et al., 2017; Odonkor & Ampofo, 2013). However, Thoe et al. reported that *E. coli* is a suitable FIB in their weekly study of 37 Hong Kong beaches over four years (Thoe et al., 2018). This highlights the fact that *E. coli* as a FIB should be evaluated in the context of local environmental conditions—our OpenArray® plate will facilitate the efficient local assessment of FIBs.

Out of 24 environmental samples, 9 (37.5%) samples (6 pore water, 2 beach water, and 1 pond water sample) with *E. coli* levels > 4 log *uidA* gene copies/250 ml showed positive bird MST marker signatures. On the other hand, out of 15 (62.5%) environmental samples with *E. coli* levels < 4 log *uidA* gene copies/250 ml, 11 samples (73%) were positive for host-specific MST markers (birds, human, and dog markers). The presence of host-specific MST markers in 73% of the samples with *E. coli* level < 4 log *uidA* gene copies/250 ml, along the presence of general MST marker (*Bacteroides* spp.) in 93% those samples, indicates the likely presence of other sources of fecal pollution (Rogers, Shaffer, Langen, Jahne, & Welsh, 2018). Similar to our findings, inconsistent relationships between FIBs and MST markers have been reported previously (Bradshaw et al., 2016).

The presence of pathogen virulence genes in sewage samples in our study and others (García-Aljaro et al., 2018) indicates that sewage is potentially an important source of waterborne pathogens. Detection of *C. coli*, *C. jejuni*, *E. coli* O26 and O111, and *S. typhimurium* in Canada goose fecal samples and the presence of those pathogens plus *A. baumannii*, *A. hydrophila*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* (Figure 2) in seagull fecal samples highlight the potential role of birds as vectors for pathogenic bacteria at beaches (Vogt et al., 2018).

The detection of our host-specific MST markers in 87% of the environmental samples which were positive for pathogen virulence genes shows that in our study host-specific MST markers are much better (R^2 = .159, p = .05) than FIBs for determining recreational water safety, while also defining the source of the pollution. This correlation of the MST markers with the pathogen markers was significant for 15 environmental samples which were positive for waterborne pathogens (R^2 = .37, p = .01; Figure 3c), showing that combination of host-specific MST markers (in our setting; Canada goose, dog, humans, and seagull) is potentially better predictors of water safety. Replacing MST with FIBs would be an excellent solution based on our data. However, as FIBs are in practice for long time instead of eliminating FIBs completely, incorporating MSTs along with FIBs will help in making more accurate decisions. Our data also show that the combining the multiple host-specific MST markers would be the best tool for monitoring of recreational water quality and potentially could avoid false positive or false negatives.

Prior to this study, several approaches have been proposed for monitoring recreational water safety. Li et al. developed a microarray chip to detect waterborne pathogens and MST (bovine, pig, and humans but not birds such as Canada goose and seagull markers) (Li et al., 2015); however, their microarray sensitivity and specificity are relatively low compared to our TaqMan® assays, as well as previously published qPCR assays (Ishii et al., 2013). Additionally, Ishii et al. and Ramalingam et al. developed TaqMan assays (microfluidic platform) that allowed the simultaneous detection of 14 and 4 waterborne

pathogens, respectively (Ishii et al., 2013; Ramalingam et al., 2010); however, neither incorporated MST markers. To the best of our knowledge, this is first molecular genetic water safety tool that incorporates human and animal MST markers along with waterborne pathogens and FIBs in a high sensitivity parallel assay. While specificity and sensitivity are critical for recreational water safety monitoring, time lines are also important. We thus incorporated robotic DNA extraction (Shahraki et al., 2019) to minimize human error and assay run time. Using such a platform, we completed environmental sample preparation, DNA extraction, TaqMan® assays (OpenArray® platform) analyses, and data processing in less than four hours.

We used the TaqMan probe assays in our OpenArray® plate to achieve higher specificity than possible with SYBR green qPCR method. Because only sequence-specific amplification is measured in TaqMan assays, the detection of false positives is greatly reduced (Tajadini, Panjehpour, & Javanmard, 2014). Also, we ran duplicates for each sample and the results were highly consistent; however, false negatives are still possible due to the low concentration of pathogens expected in most environmental samples (Johnson, Nolan, & Bustin, 2013) coupled with stochastic sampling effects (Peccoud & Jacob, 1996). We detected few positive hits for waterborne pathogens in our environmental samples (Figure 2) potentially due to low concentration of the pathogens in our collected samples. For actual risk assessment, increasing the volume of water sampled would increase the likelihood of waterborne pathogen detection.

The running cost for TaqMan® assays in the OpenArray® platform (48 subarrays, 66 wells) for 25 markers (duplicate run in each subarray) for this study was $11/sample and $0.44/marker, including the reagent costs for the assays and the plate but excluding costs for labor and equipment, which is comparable to conventional qPCR ($0.45/assay/sample) previously estimated (Ishii et al., 2014).

Through rapid and simultaneous quantification of waterborne pathogens, MST markers and FIBs, our OpenArray® plate will contribute to determining the concentration of the target pathogens with significant health risks. In properly equipped laboratories, this OpenArray® plate would be able to quantify FIBs, MSTs to define the source of fecal pollution, and waterborne pathogens in less than four hours, which would allow close to real-time monitoring of water safety and hence facilitate effective risk management.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

All the authors have contributed to the manuscript. AHS has collected the samples, did all the laboratory work, data analysis, and wrote the first draft of Manuscript. AHS, DDH, and SRC made major contributions to the design of the study. DDH and SRC reviewed the interpretation of the data and revised the manuscript.

DATA AVAILABILITY STATEMENT

Supplementary information is provided.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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