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Detection of Shiga toxin-encoding genes in small community water supplies

Melitza Crespo-Medina, Isabel Greaves, Paul R. Hunter, Harvey Minnigh and Graciela Ramírez-Toro

ABSTRACT

Shiga toxin (Stx), one of the most potent bacterial toxins known, can cause bloody diarrhea, hemolytic uremic syndrome, kidney failure and death. The aim of this pilot was to investigate the occurrence of Shiga toxin-encoding genes, *stx* (*stx*1 and *stx*2) from total coliform (TC) and *E. coli* positive samples from small community water systems. After aliquots for TC and *E. coli* analyses were removed, the remnant volume of the samples was enriched, following a protocol developed for this study. Fifty-two per cent of the samples tested by multiplex PCR were positive for the presence of the *stx* genes; this percentage was higher in raw water samples. The *stx2* gene was more abundant. Testing larger volumes of the samples increase the sensitivity of our assay, providing an alternative protocol for the detection of Shiga toxin-producing *E. coli* (STEC) that might be missed by the TC assay. This study confirms the presence of Stx encoding genes in source and distributed water for all systems sampled and suggests STEC as a potential health risk in small systems.

Key words | diarrhea, *E. coli*, Puerto Rico, Shiga toxin, small systems, STEC

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HIGHLIGHTS

- An assay to detect Shiga toxin-encoding genes in water supplies was developed.
- An enrichment protocol increased the sensitivity of the assay.
- Shiga toxin-encoding genes were detected.
- Genes encoding *stx2* were more abundant.
- The potential presence of Shiga toxin-producing *E. coli* in small potable water supplies represents a health risk for their consumers.

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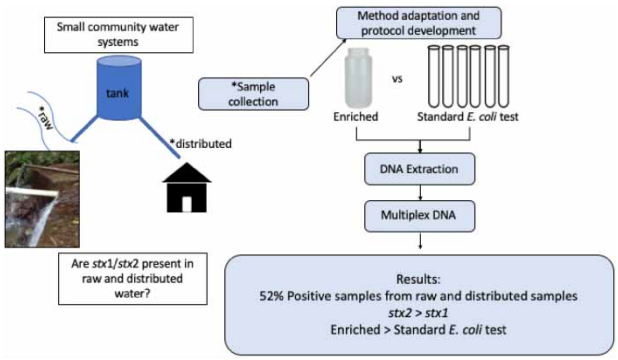
GRAPHICAL ABSTRACT



INTRODUCTION

Shiga toxin (Stx), produced by some serotypes of *E. coli* belonging to the enterohemorrhagic *E. coli* (EHEC) pathotype (Nataro & Kaper 1998), is one of the most potent bacterial toxins known. In the United States (USA) alone, it is estimated that Shiga toxin-producing *E. coli* (STEC) causes more than 265,000 infections, 3,600 hospitalizations and 30 deaths annually (CDC 2012). Hemolytic uremic syndrome (HUS) and bloody diarrhea are the most recognized symptoms; however, the toxin can lead to kidney and multi-organ failure, long-term illness and death (Garg *et al.* 2003; Marshall *et al.* 2004). The virulence of STEC strains is associated with the activity of the toxins encoded by Stx-converting phages (Schmidt 2001), which are mobile elements involved in the transfer of genes from one host to another.

STEC is mostly associated with foodborne illness but STEC has also been documented as the causative agent of important waterborne outbreaks in Japan, in Fife, Scotland and in Canada. In the USA, major outbreaks have been reported in Missouri, Wyoming and New York.

Even though most potable water regulations use *E. coli* as the bacterial water quality indicator of fecal contamination (USEPA 2013), very little information is available about the occurrence and prevalence of STEC in water. Most research on STEC has focused on clinical samples, environmental samples following an outbreak or river and drinking water samples impacted by either sewage, slaughterhouses or agricultural processes (Ram *et al.* 2011; Cooley *et al.* 2013; Kabiru *et al.* 2015 and others). The few studies in water mostly center on its detection in surface waters (Heijnen & Medema 2006; Shelton *et al.* 2006; Mull & Hill 2009; Lascowski *et al.* 2013; Ndlovu *et al.* 2015).

The increase in the number of infections associated with STEC in drinking water has exposed the need to improve methods for its detection in environmental samples. Moreover, available methods detect only one serotype of *E. coli*, which can produce false negative results (Hamner *et al.* 2007; Yang *et al.* 2007). Recent developments for the molecular identification of diarrheagenic *E. coli* are expensive or time consuming and include quantitative PCR or multiplex PCR (mPCR) methods (Ram *et al.* 2011; Omar & Barnard 2014).

This pilot study attempts to adapt methods currently used in the monitoring of *E. coli* in potable water to develop a protocol for the detection of Shiga toxin-encoding genes in drinking water focusing on small potable water supply systems serving fewer than 3,300 people. Even though small systems comprise the largest number of potable water supplies around the world, they are often excluded from comprehensive sampling campaigns to evaluate the prevalence and occurrence of emerging pathogens and other contaminants. Small systems around the world, as in the USA, typically serve remote, underserved populations, those most at risk from waterborne illness due to the lack of capacity to operate, improper treatment and disinfection (Minnigh & Ramirez-Toro 2004; US EPA 2006; Hunter *et al.* 2010).

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This study investigates the occurrence of Shiga toxin-encoding genes in raw and distributed water from seven small potable water supplies operated by communities using an mPCR assay and a protocol for rapid and efficient detection of STEC in water.

METHODS

Sample collection

Water samples were collected from seven small potable water systems located in the municipalities of Patillas and Guayama, in the southeast of the island of Puerto Rico (Figure 1). Because of the absence or inconsistency of treatment, for the purpose of this paper, the samples collected within the distribution systems are referred to as 'distributed' water samples. Following convention, source water samples are called 'raw'. From each site, a 1-L water sample was collected for detection and enumeration of coliforms and *E. coli*. Physicochemical parameters (pH and turbidity) were also determined. Total and free chlorine was measured from distributed water samples. Samples were kept at 4–8 °C until processing and were analyzed within 30 h of collection.

Total coliform and *E. coli*

Enumeration to log density was done by presence–absence (PA) in serial decimal dilutions for total coliforms (TC), and *E. coli* following an amendment of the Standard Methods protocol for the detection of TC. Aliquots from each sample (100, 10 and 1 mL and subsequent dilutions in 0.85% Normal Saline Solution) were added to the proper strength of selective PA broth (BD-Difco). Incubation followed *Standard Methods* (APHA 2005).

The highest and the three lowest presumptive TC aliquots were confirmed following *Standard Methods* (APHA 2005; Minnigh *et al.* 2006). Complete phase was done simultaneously in EC broth (Difco) and in 4-methylumbelliferyl-β-D-glucuronide (MUG) (Difco) to detect Thermotolerant Coliforms and *E. coli*, respectively. *E. coli* (ATCC 25922) and *E. coli* O157:H7 from the Department of Health of

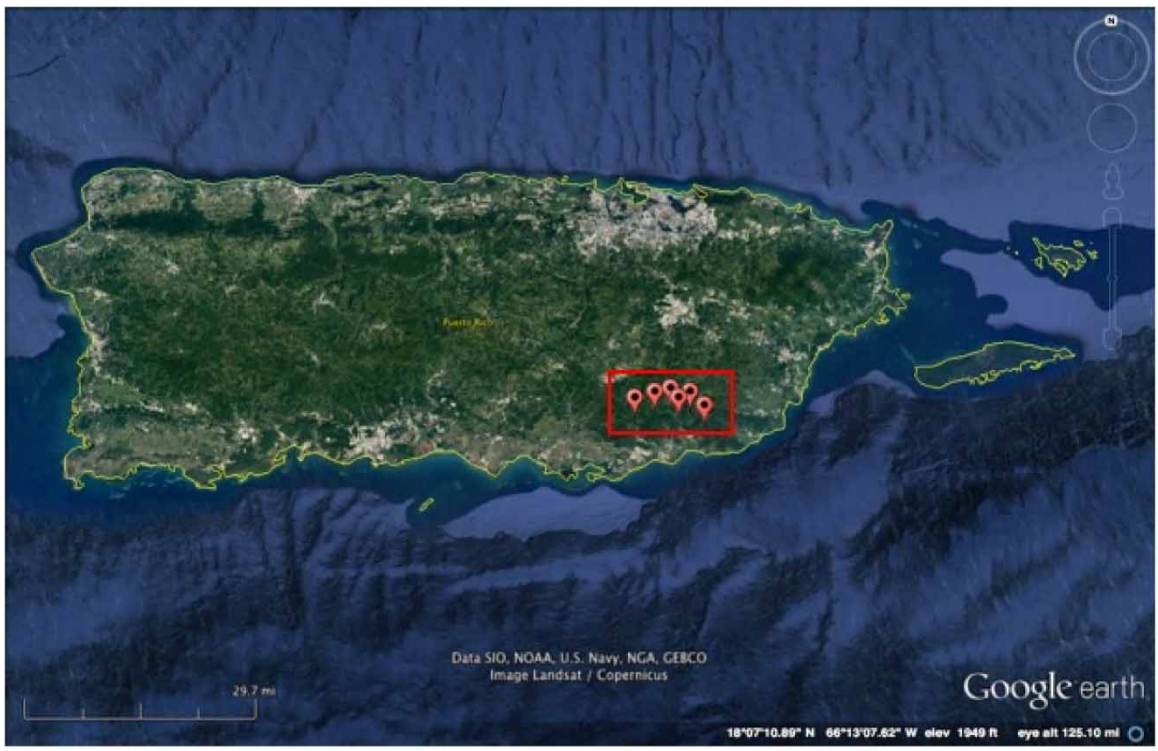

Figure 1 | Study site locations in the southeast of the island of Puerto Rico.

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Puerto Rico were used as controls for the culture stages. Data presented here include only the results for TC and for *E. coli*.

Processing *E. coli* positive samples and enriched samples

The entire volume of *E. coli* positive tubes was transferred to 15 mL sterile conical centrifuge tubes (Tornado) using sterile 10 mL pipettes (Fisherbrand). The tubes were then centrifuged for 15 min at 1008 rcf × g in a Hermle© z230 to pellet the cells and the supernatant was discarded. The cells were then washed twice with DNA-grade 1× phosphate-buffered saline (PBS) (Ramirez-Toro 1992) to remove the remaining media. The resulting pellets were stored at −20 °C until processing.

E. coli O157:H7 strains do not fluoresce in MUG media (Krishnan *et al.* 1987; Thompson *et al.* 1990); to ensure their detection and to extend the range of the method, the remaining 780–900 mL of the original water sample was enriched by adding 50 mL of 3× selective PA broth and incubated at 35 °C (±0.5 °C) for 24 h (±2 h). These samples will be referred to as 'enriched' in the Results and Discussion sections. Enriched samples were transferred to sterile Nalgene© centrifuge bottles and centrifuged for 7 min at 3360 × g in a Hermle© Z513 K. The supernatant was discarded, and the pellet was transferred using a sterile 10 mL serological pipette to a 50 mL sterile centrifuge tube. The pellet was then washed twice using DNA-grade 1× PBS and stored at −20 °C until processing. Pellets were split into 2 mL portions to ease examination.

DNA extraction

DNA from all samples (*E. coli* positive and enriched samples) was extracted using a PureLink™ Genomic DNA Mini Kit (Invitrogen™) following manufacturer's protocols. Initial digestion was done for 2 h at 55 °C in a dry bath. DNA from all samples was stored at −20 °C.

Multiplex PCR

All mPCR reactions were performed in an Eppendorf unit Mastercycler ep Realplex 4S in a total reaction volume of 20 μL. The mPCR protocol for the mPCR kit (QIAGEN©) was used for all PCR reactions. The genes targeted by our mPCR assay included *stx1* and *stx2* genes which encode the two main forms of the toxin; the *lt* gene which encoded the heat-labile toxin produced by the enterotoxigenic *E. coli* (ETEC); the *eaeA* gene which encodes for the adhesion protein intimin; and an *E. coli* marker gene *mdh*.

Each reaction included 10 μL of 2× QIAGEN Multiplex PCR Master Mix, 2 μL of the primer mix (0.1 μM of *mdh* and *lt* primers forward (F) and reverse (R), 0.3 μM of *eaeA* and *stx1* primers F and R and 0.5 μM of *stx 2* F and R), 2 μL of sample DNA and 4 μL of water. The cycle consisted of an initial activation step at 95 °C, followed by 35 cycles which consisted of a denaturing step at 94 °C for 45 s, annealing at 55 °C for 45 s, extension at 68 °C for 2 min and finally elongation at 72 °C for 5 min (Omar & Barnard 2014). PCR amplifications were analyzed in a 2.5% (w/v) agarose gel stained with 10 μL of SYBR® Safe DNA Gel Stain for 30 min at 100 V and visualized under UV light.

Statistical analyses

Statistical analyses were run in SPSS version 23 and SYSTAT 13. Analyses were non-parametric correlation and Chi-squared tests including McNemar's for an appropriate sample.

RESULTS

In this study, two distinct sampling programs were performed; one in 2015 where 52 water samples were collected and the second one in 2016 where 46 samples were collected, for a total of 98 samples. For the purpose of this research, a positive *stx* sample means that the samples were positive to either *stx*1, *stx*2 or to both. Fifty-two per cent of the samples were *stx* positive (Table 1). A complete dataset with all individual samples can be found in Supplementary Material, Table S1. Most of the distributed samples (58%, 18 out of 31 samples) were negative for TC and *E. coli*. Eight (8 of 31) samples were positive for TC with densities between 10^0 and 10^2 per 100 mL, and *E. coli* between 0 and 10^2. Three samples (3 of 31) were in the 10^3 log for TC and in the 10^2 log for *E. coli*. Additionally,

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Table 1 | Total number of positive samples for *stx1*, *stx2* or both per sampling site per sampling campaign

System	Type	Year	Number of samples	Number of *stx* positive samples[a]	% positive	mPCR results (number of positive samples)		
						stx1	*stx2*	*stx1* and *stx2*
1	D	2015	7	1	14	0	0	1
		2016	4	3	60	2	1	0
	R	2015	8	5	63	0	0	5
		2016	9	4	44	0	4	0
2	R	2015	8	3	38	0	0	3
		2016	5	3	60	0	3	0
3	D	2015	4	2	50	0	0	2
		2016	10	3	30	0	3	0
	R	2015	5	2	40	0	0	2
		2016	13	7	54	0	7	0
4	R	2015	0	0	0	0	0	0
		2016	1	1	100	0	0	1
5	R	2015	9	6	67	0	0	6
		2016	1	1	100	0	1	0
6	R	2015	8	4	50	0	3	1
		2016	1	1	100	0	1	0
7	D	2015	4	3	75	0	2	1
		2016	2	2	100	0	2	0
Total			**98**	**51**	**52**	**2**	**27**	**22**

[a]*stx* positive implies that the sample was positive to *stx1*, *stx2* or to both.

two distributed samples (2 of 31) were in the 10^5 and 10^4 log for TC and were in the 10^3 and the 10^0 log, respectively, for *E. coli* (Table S1). The two distributed samples with the highest TC log had no chlorine and the turbidity for the sample with higher log counts was 4.6 NTUs (Table S1). Raw water samples had TC log ranging from 10 to 10^5 and 0 to 10^4 *E. coli* per 100 mL (Table S1).

Even though all systems under this study showed *stx* positive samples in either or both raw and distributed water (Table 1), when comparing the results, a higher per cent of raw water samples was positive for *stx*; 44 out of 67 raw water samples (66%), while 14 out of 31 distributed samples (45%) were positive for *stx*; a difference that was not statistically significant (Chi-square = 0.270, df = 1, $p = 0.603$). The occurrence of *stx2*, which encodes for the form of the toxin that is usually associated with more acute illness (Orth *et al.* 2007), is higher than the *stx1* form; abundance being significantly different (McNemar's = 4.315, df = 1, exact $p < 0.000$). This finding is consistent with previous reports that bacteriophages containing *stx2* were more prevalent than *stx*1 phages (Grau-Leal *et al.* 2015). Two samples were positive to *stx1* only, while 27 samples were positive to *stx*2 only. Twenty-two samples had the combination of both *stx1* and *stx*2 (Table 1).

Only one system showed a sample positive to the gene encoding the Lt toxin (data not shown). The mPCR method used included an internal control, the *mdh* gene. However, the amplification of this gene was very difficult to screen in the 2.5% agarose gel used; the bands were small and faint and their visualization in the gel was not constant, thus mPCR results for this gene were not considered accurate and were not included in the analysis (data not shown).

The gene *eae*A was detected in 63 of the 98 samples (Table 2). The two samples that contained only *stx1* were also positive for *eaeA*. A total of 21 of the 27 samples that were positive to just *stx2* were also positive to *eaeA* (78%). A total of 19 of the 22 samples that had a combination of *stx1* and *stx2* were also positive to *eaeA* (86%) (Table 2). The only sample that was positive to the gene encoding

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Table 2 | Detection of *eae*A gene in raw and distributed water samples per sampling site

System	Type	*stx*1 + *eaeA*	*stx*2 + *eaeA*	*stx* 1 + *stx* 2 + *eaeA*	*eaeA* only	Total
1	D	2	1	1	1	**5**
1	R	0	2	4	4	**10**
2	R	0	0	4	6	**10**
3	D	0	3	1	0	**4**
3	R	0	5	1	4	**10**
4	R	0	0	1	0	**1**
5	R	0	1	6	2	**9**
6	R	0	5	0	3	**8**
7	D	0	4	1	1	**6**
Total		**2**	**21**	**19**	**22**	**63**

Data from both sampling campaigns compiled.

the Lt toxin was also *eaeA* positive (data not shown). Twenty-one samples had only *eaeA*, with no *stx1* or *stx2* associated (Table 2).

In an effort to detect serotypes that could be missed by the standard MUG assay, such as *E. coli* O157:H7, we enriched the remaining portion of the sample (i.e., the remainder of the initial sample after aliquots for dilutions) with PA media. Five enriched samples that were negative to TC and to *E. coli* were *stx* positive by mPCR. These might suggest that the results from the mPCR analysis is above the limit of detection of our culture-based assay and/or might indicate either the presence of the genes in the sample matrix, as cell-free DNA or the presence of other bacteria carrying the *stx* gene. The latter two scenarios can be attributed to the activity of Stx-converting bacteriophages, which can lyse their STEC host and transfer the *stx* genes to other bacteria (Schmidt 2001) or can be found as free Stx-converting phages (Grau-Leal *et al.* 2015).

In any event, this technique increased the sensitivity of our analyses. Of the 98 samples analyzed for TC and *E. coli*, 71 were enriched; 37 of these 71 (52%) were *stx* positive. All enriched samples were >700 mL or a sensitivity of ≥0.0014 CFU per mL, measuring the occurrence by minimal organism density. To further test the efficacy of enrichment, we tested *stx* occurrence in each of the aliquots from the discrete samples; there were 338 portions for the 98 discrete samples. Enriched portions were significantly more likely to elucidate *stx* (47 of 136 portions) than portions not enriched (40 of 202 portions) (Chi-square 9.260, df = 1, $p = 0.002$).

Of the 19 distributed samples with chlorine measurements (Table S1), *stx* genes were not found in waters with total chlorine >0.5 mg/L (Fishers exact test $P = 0.004$) and not found after enrichment in samples with total chlorine >0.05 mg/L. Turbidity was not significantly related to *stx* occurrence in either raw or distributed samples.

As to the efficacy of tested indicators to predict *stx* in the study; only *stx2* presence was significantly associated with TC (Chi-square = 23.529, df = 5, $p = <0.000$) and with *E. coli* (Chi-square 20.412, df = 4, $p = <0.000$).

DISCUSSION

Small (serving <10,000 people) and very small (serving < 3,300) public water supply systems represent the largest numbers of systems around the world. Even though present in large numbers in all countries, these are usually not included in comprehensive sampling campaigns to evaluate the prevalence and occurrence of emerging pathogens and other contaminants. This is driven by the perception that they represent a smaller risk because they serve a lesser population at any given time.

Small systems are found in all geographical settings and typically serve remote underserved populations that are most at risk from waterborne illness due to lack of technical, economical and managerial capacity that results in improper treatment and disinfection and can represent a risk to their users and the rest of the population (Minnigh & Ramirez-Toro 2004; US EPA 2006; Hunter *et al.* 2010). For example, in the USA, in 2016, the US EPA reported that small systems violations comprised the vast majority of the most serious of health-based standards (US EPA 2016). Health-based standards include monitoring for *E. coli,* turbidity and treatment techniques, such as disinfection.

Moreover, surveillance for waterborne illness might not provide information about the contribution of small systems to waterborne illness. Hunter *et al.* (2010) indicate that 'One of the weaknesses with the current reporting of waterborne outbreaks in the US is that all community systems are classed together' and that '… it is often not possible to distinguish between the risk of an outbreak in large or small systems',

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while Nichols (2002) concluded that the approach can result in undetected waterborne outbreaks in small systems.

In the USA and its territories, there are over 148,000 small and very small potable water systems; and at least 351 of them are in Puerto Rico. Of those, about 240 are operated by local communities, and treatment in these systems, when present, is limited to chlorine disinfection.

This study took place in seven of these community-operated systems in the southeast of the island. Currently, there is no available information in the Department of Health of Puerto Rico or in the literature about the incidence of STEC in Puerto Rico, and the Center for Disease Control and Prevention's (CDC) *Escherichia coli* (STEC) Surveillance program does not include data from Puerto Rico in their annual reports (CDC 2012). However, in a previous study, we were able to determine that the rate of diarrhea in these systems was 3.5% in a 4-week period and lowered to 2.5% after an educational intervention (Hunter *et al.* 2010). In a follow-up study, we were able to confirm the presence, for the first time, of *Cryptosporidium* in some of the systems under this study, and urged the additional investigation of waterborne agents presenting health risks for these communities (Robinson *et al.* 2015).

Following that objective, this pilot study attempted to adapt methods currently used in the monitoring of *E. coli* in potable water to develop a protocol for the detection of Shiga toxin-encoding genes in drinking water focusing on small systems serving less than 3,300 persons. In an effort to verify the presence of STEC in these systems, we adapted previously published methods (Omar & Barnard 2014) to examine raw and distributed water samples. A total of 52% of the samples analyzed were positive to the genes encoding either one or both of the forms of Shiga toxin (*stx*: *stx*1 and *stx*2) indicating that the method we used is effective in the detection of the gene encoding the toxin in water.

The STEC that also carries the enterocyte effacement locus (LEE), a moderately conserved pathogenicity island, uses an adhesion protein called intimin, which is encoded by the *eaeA* gene. Although the intimin protein is not exclusively produced by STEC (Donnenberg *et al.* 1993), the strains that can synthesize this protein (*eae* positive strains) are more likely to cause disease than intimin-negative strains (Jerse *et al.* 1990), because the toxins are more likely to attach to the receptor in the target cell membrane (Jerse *et al.* 1990). Since *eaeA* encodes for an attachment protein, which can be associated with other virulence factors (Donnenberg *et al.* 1993), the presence of this gene alone, without the presence of one or more toxin-encoding genes might suggest the presence of other virulence factors beyond the scope of this assay, which might represent an additional risk for the population.

Our study has the same limitation of surveillance systems that rely on the use of *E. coli*, in the sense that *E. coli* O157:H7, a serotype highly associated with outbreaks related to Shiga toxin, is not detected by the conventional *E. coli* detection method using MUG (Thompson *et al.* 1990). To account for this limitation, we also analyzed the remaining portion of the sample (780–900 mL) after TC and *E. coli* assays with enrichment media and molecular methods as described in the Methods section of this paper.

If there was *E. coli* O157:H7 present in our samples, we expect their detection in the enrichment samples. Of the 71 samples that had enrichments, 44 were *stx* positive and 30 of those were positive only in the large-volume enrichment portion and negative in their respective *E. coli* MUG test (McNemar's $= 12.291$, $df = 1$, $p = 0.0004$), suggesting the possibility that *E. coli* O157:H7, other Stx producing bacteria, or free Stx-converting phages were present in our samples. When comparing the results of each of the 338 portions of the 98 discrete samples, we determined, again, that enrichment was more likely to elucidate *stx* than using only conventional TC/*E. coli*-positive sample, also suggesting that larger sample volumes may be useful in elucidating *stx*. Future work will involve the sequencing of *stx* positive in order to discern which Stx producing bacteria were present in our enriched samples.

Potable water regulations use TC and *E. coli* as bacterial indicators of fecal contamination. In this study, we investigated if TC and *E. coli* could be used as indicators for *stx*. Results indicated that TC and *E. coli* positive samples were significantly associated with the presence of *stx2*, but not with the presence of *stx*1 suggesting that a sample containing only *stx*1 might be missed by conventional methods.

CONCLUSION

This pilot study confirms the detectable presence of *stx1* and *stx2* in surface and distributed waters used by small potable

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water systems and demonstrated the use of an easy method to detect STEC in samples taken for compliance monitoring.

This study also shows the need to include small systems in surveillance studies and efforts to gather information to assess the risk to the total population. The presence of the genes encoding Shiga toxin represents a health risk for the people drinking water from these systems, especially since most of these systems provide little or no disinfection. The presence of *eaeA* gene without Shiga toxin-encoding genes might represent an additional health risk, suggesting that other virulent factors might be present at these sites. Additional studies are needed for the development of methods to detect *E. coli* serotype O157:H7 as it represents a health risk and its presence is easily missed by conventional methods.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could construct a potential conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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