Exploring private water wells for fecal sources and evidence of pathogen presence in the context of current testing practices for potability in Ontario

Sophie Felleiter, Kevin McDermott, Geof Hall, Prameet Sheth and Anna Majury

ABSTRACT

Private water wells provide drinking water for an estimated 4.1 million households in Canada yet remain understudied in the context of microbial water quality or human health impacts. As there exists little systematic surveillance for enteric infections or outbreaks related to well water sources, consumers may be at risk of waterborne infectious diseases. A standard protocol in Ontario requires 200 mL of water, collected, and submitted by well owners, half of which is used to analyze for *Escherichia coli* and total coliforms (TCs). The aim of this study was to determine the efficacy of testing small water volumes and to survey for other contaminants in addition to bacterial indicators to inform pathogen prevalence and fecal source in drinking water wells. Samples were assessed for *E. coli* and TCs, by culture, and genetic markers of *Bacteroides* spp., *Campylobacter* spp., *Salmonella* spp., and Shiga toxin-producing *E. coli*, using qPCR. The source of fecal contamination varied by the geographic region and may be explained by septic tank density and underlying geology, among other factors. A small number of samples (1.9%) showed the evidence of contamination with enteric pathogens. Lastly, *E. coli* measured by qPCR, as opposed to culture, correlated more strongly to *Bacteroides* markers.

Key words | *Bacteroides*, microbial source tracking (MST), private water wells, public health, qPCR, waterborne pathogen

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INTRODUCTION

Water consumption without fear of illness is a defining characteristic of developed nations and is often taken for granted (Hrudey *et al.* 2006). Although the magnitude of drinking water-related illness in developed countries is lower than that in developing regions, waterborne disease outbreaks remain a significant cause of acute gastrointestinal illness and constitute a health risk as well as a substantial economic burden that could be better managed and prevented (Krentz *et al.* 2013). Of particular interest is waterborne disease attributable to private drinking water wells that serve a single household. Aquifers supplying these wells can become contaminated with enteric pathogens from both human and animal feces potentially causing serious illness (Charrois 2010). In Canada, an estimated 4.1 million (~12%) households rely on private supplies, most of which are rural groundwater sources (Murphy *et al.* 2016), with almost 2 million residing in Ontario (Office of the Auditor General of Ontario 2014). However, these particular water supplies are subject to little or no systematic surveillance. In Ontario, the testing

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of private wells is provided cost free by Public Health Ontario (PHO) at the discretion of the well owner. Well owners voluntarily submit 200 mL samples, of which 100 mL is used to test for the presence of indicator bacteria, namely *Escherichia coli* and total coliforms (TCs). Maier *et al.* (2014) found that, in Ontario, only 11–12% of owners who submitted water samples for testing over 5 years (2008–2012) met the historic guidelines of at least three tests per year in any given year. Furthermore, less than 1% met those guidelines every year, when followed over 5 years, further elevating risk to the consumer, given the lack of risk assessment by testing. The nature of private water supplies can vary greatly over time and one test per year, or less, is insufficient to determine the stability of the water quality. Furthermore, enteric pathogens in private wells, and their subsequent associated infections, may go largely undetected, leaving a significant proportion of the population vulnerable to waterborne infectious diseases.

Traditionally, private water supplies are tested for TCs and *E. coli* as indicators of environmental and fecal contamination. TCs include intestinal genera such as *Escherichia*, *Enterobacter*, *Klebsiella*, *Citrobacter* and *Kluyvera* (Figueras & Borrego 2010). TC testing serves best as an indicator of environmental contamination (i.e., derived from soils and plants) or of the integrity of a water distribution system (WHO 2011). *E. coli* is the most commonly employed indicator of recent fecal contamination and potential enteric pathogen presence in drinking water, given its prevalence and specificity for the gastrointestinal tract (GIT) of animals and its inability to survive and grow in aquatic environments (Saxena *et al.* 2015). These bacteria do not allow for the identification of the fecal source, given their ubiquity across multiple species and environments. The detection of *Bacteroides* 16S rRNA genes by the PCR has been through microbial source tracking (MST) methodology, as certain sequences within the 16S rRNA gene are highly host-specific (Lee *et al.* 2014). Furthermore, *Bacteroides* are relatively easy to detect in environmental waters given their high abundance in feces and, as anaerobes, do not survive outside of the GIT. Thus, detection suggests that the contamination event is recent (Lee *et al.* 2014). It should be noted that the persistence of *E. coli*, TCs, and *Bacteroides* markers differs from each other in environmental waters and that their persistence is highly dependent on factors such as dissolved oxygen concentration, temperature, sunlight, salinity, and predator presence (Okabe *et al.* 2007; Walters & Field 2009; Lee *et al.* 2010).

In southern Ontario, four discrete regions with an increased relative risk for *E. coli* contamination among private wells have been identified as seen in Figure 1 (Krolik *et al.* 2013). The vulnerability of these wells to *E. coli* contamination suggests that they are also vulnerable to pathogen contamination, particularly those spread from the feces of humans or other animals. To address this, the high-risk regions were further investigated as well as a region with high cattle density (a circled orange region in Figure 1) (Statistics Canada 2011). At its core, this study aimed to investigate the value of small water volumes obtained from convenience samples for the detection of additional markers of contamination using molecular methods, in addition to the traditional culture and quantification of TC and *E. coli* colonies for water potability determinations. Specific aims were to assess 100 mL water samples from *E. coli* and/or TC-contaminated wells for (1) the presence of molecular markers of enteric pathogens, namely *Campylobacter* spp., *Salmonella* spp. and Shiga toxin-producing *E. coli* (STEC), (2) to determine the predominant sources of fecal contamination

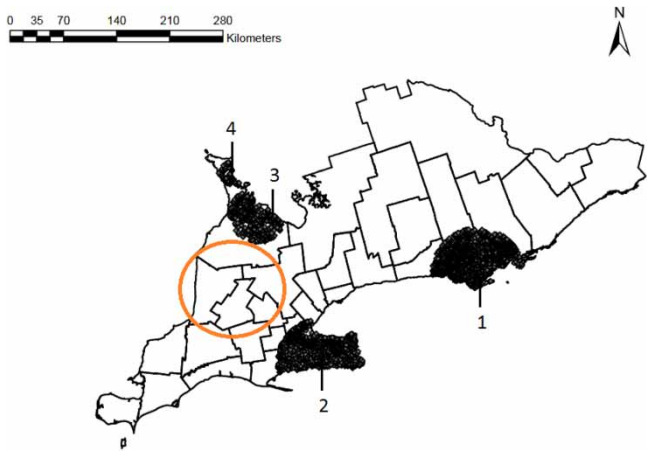

Figure 1 | The four regions, (1) Kingston/Belleville, (2) Niagara/Hamilton, and (3) and (4) Grey/Bruce, of increased relative risk of *E. coli* contamination (in black) within private drinking wells and the cattle dense region as identified by the orange circle. More specifically, regions in black represent clusters of *E. coli* contamination of private wells. Krolik *et al.* (2013) used SaTScan™ software to perform spatial analysis on multiple data sets of private well water submissions from PHO Kingston and across Ontario, visualized the spatial results, and did subsequent mapping on ArcMap version 10.0. Inner points were removed to clearly compare between clusters. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wqrj.2019.035.

as human, bovine, or other animal, and (3) to establish the nature of the relationship between the presence of these pathogens and microbial indicators, including *E. coli* and TCs (by culture), *Bacteroides fragilis*, and *E. coli* (by qPCR).

MATERIALS AND METHODS

Sampling sites and collection

Private water well convenience samples submitted for bacteriological analysis between 1 June and 31 August 2014 to three PHO laboratories located in Hamilton, Kingston and London, Ontario, were studied. Well water samples were strategically selected from the three 'hotspots' for *E. coli* contamination, as previously described by Krolik *et al.* (2013). These hotspots will be referred to as the Grey/Bruce region, the Niagara/Hamilton region, and the Kingston/Belleville region, named for their approximate geographic locations. A region of high cattle density, located in mid-southwestern Ontario, was also included, given cattle are a major reservoir of enteric pathogens (Figure 1). Well water (200 mL) was collected using PHO sample bottles (BioNuclear Diagnostics, Inc., Toronto, Canada) containing 0.8 mM sodium thiosulfate. Each water sample was transferred in coolers, to a PHO laboratory, at approximately 2–8 °C and tested within 48 h of collection. Partial vacuum filtration was used to filter each sample (100 mL) through a 0.45 μm pore size mixed cellulose esters filter (Millipore, Billerica, MA, USA). Each filter was placed on a Differential Coliform Agar (Oxoid, Nepean, Canada) containing lactose and 5-bromo-4chloro-3-indolyl-D-glucuronide (BCIG), and incubated for 18–24 h at 36°C at atmospheric conditions. After incubation, the number of presumptive *E. coli* and TC colony forming units (CFUs) were counted and reported as CFU/100 mL. Water samples positive for *E. coli* (*E. coli* (+)) as well as samples positive for TCs, but negative for *E. coli* (TC-only), were chosen for further study. All *E. coli* and TCs were declared based on the phenotype on the differential agar only and considered presumptive *E. coli* and TCs for the purpose of this paper unless stated otherwise. For the purposes of water potability testing, *E. coli* and TCs are routinely reported as presumptive positive without further biochemical or molecular confirmation.

Nucleic acid extraction

The remaining 100 mL of *E. coli* (+) samples and TC-only samples underwent a second partial vacuum filtration step. The filters were rolled and placed into 3 mL cryovials (Simport, Beloeil, Canada), lysed with NucliSENSE® easyMAG® lysis buffer (2 mL) (Biomerieux Inc., St. Laurent, Canada), and placed in a rotating drum for 1 h at 37 °C. The DNA was extracted using MagSil beads and an automated NucliSENS® easyMAG® DNA extractor (Biomerieux Inc.) to a final volume of 100 μL. Extracts were stored at −20 °C until further processing.

Quantitative PCR assays for the detection of fecal indicators, sources, and bacterial pathogens

Extracted DNA underwent qPCR using *Taq*Man® chemistry for biomarkers of the indicator organisms and bacterial pathogens previously mentioned. The primers, probes, and internal amplification control (IAC) sequences of the assays are described in Table 1. Custom assays were modified or created for *Campylobacter* spp., *Salmonella* spp., and *stx*1/*stx*2 genes to avoid difficulties in adapting and validating pre-existing assays to the ViiA™ 7 Real-time PCR system (Applied Biosystems) and the unique matrix private water well samples represent. Existing assays were used as previously described to detect *E. coli* and *Bacteroides* as these had been validated in several pre-existing studies which examined these indicators in well water (Krolik *et al.* 2014, 2016). To confirm qPCR assay efficiency and sensitivity, all sequences underwent BLAST™ analysis and were tested against available strains of ATCC organisms as indicated in the subsection for each assay. To quantitatively detect these indicator and pathogenic organisms in water samples, standard curves were created from DNA extracted from laboratory strains of the organisms in question as per the DNeasy Blood & Tissue Handbook and kit (Qiagen 2006) protocol, that is, pretreatment for Gram-negative bacteria. All assays included a set of four standards assessed in duplicate to generate a standard curve and allow quantitative analysis of samples. A no template control in which TE buffer (Invitrogen, Carlsbad, CA, USA) was added, instead of DNA, was included in each assay. All qPCR assays had amplification and detection performed in a MicroAmp® optical 96-well reaction plate sealed with optical film (Applied Biosystems, Burlington, Canada) on the ViiA™ 7

Table 1 | Primers, probes and IAC sequences used in qPCR assays

Target organism	Primer/probe	Oligo sequence (5′ → 3′)	References
Campylobacter spp.	*hip*O-F	AATGCACAAATTTGCCTTATAAAAGC	Toplak *et al.* (2012)
	*hip*O-P[a,g]	ACATACTACTTCTTTATTGCTTGC	
	*hip*O-R	TICCATTAAAATTCTGACTTGCTAAATA	
	IAC-P[b,e]	TTCGAAATGTCCGTTCGGTGGC	
	IAC-Target	AATGCACAAATTTGCCTTATAAAAGCGTAT TCGAAATGTCCGTTCGGTTGGCGCTATGAA CAAGTCAGAATTTTAATCCIA	
Salmonella spp.	*fimA*-F	CACTAAATCCGCCGATCAAAC	This study
	fimA-P[a,e]	CTGGGTCAATACCGTACCGCCAGC	
	fimA-R	AGTCGTATTACCAATCGCCGTAA	
	IAC-P[b,e]	TTCGAAATGTCCGTTCGGTTGGC	
	IAC-Target	CACTAAATCCGCCGATCAAACGTATTCGAA ATGTCCGTTCGGTTGGCGCTATGAAGAGAT ACGCGGTGGAACCTGGAATTACGGCGATTG GTAATACGACT	
Shiga toxin-producing *E. coli* (STEC) – *stx*1 and *stx*2 toxin	*stx*1-F	GGATAATTTGTTTGCAGTTGATGTC	This study
	*stx*1-P[a,e]	CCGTAGATTATTAAACCGCCCTTCCTCTGGA	
	*stx*1-R	CAAATCCTGTCACATATAAATTATTTCGT	
	*stx*2-F	GGGCAGTTATTTTGCTGTGGA	
	*stx*2-P[c,e]	ATGTCTATCAGGCGCGTTTTGACCATCTT	
	*stx*2-R	GAAAGTATTTGTTGCCGTATTAACGA	
	IAC-P[d,f]	TTCGAAATGTCCGTTCGGTTGGC	
	IAC-Target	GGGCAGTTATTTTGCTGTGGAGTATTCGAA ATGTCCGTTCGGTTGGCGCTATGAAGAGAT ACGCGGTGGAACCTGGAGTCGTTAATACGG CAACAAATACTTTC	

F, forward primer; R, reverse primer; *P*, *Taq*Man® probe.
[a]6-FAM reporter.
[b]CAL Fluor Orange 560.
[c]CAL Fluor Gold 540.
[d]CAL Fluor Red 590.
[e]BHQ1 quencher.
[f]BHQ2 quencher.
[g]BHQplus quencher.

Real-time PCR System. The run method followed 50°C for 2 min, 95°C for 10 min, then 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Detection of *Campylobacter* spp.

A *Taq*Man® qPCR assay targeting the *hip*O gene of *Campylobacter* was used to analyze samples for the presence of *Campylobacter* spp. Primers and probes were adapted from Toplak *et al.* (2012) with some modifications. Specifically, the *hip*O probe was chemically synthesized with proprietary modified nucleotides or BHQplus™ technology (Biosearch Technologies) in addition to a 3′-C nucleotide to increase the probe's T_m to 71.8 °C. A further modification to the assay was the addition of an IAC, which shares the same forward and reverse primer as the *Campylobacter* assay but relies on a different probe.

Seven standards were prepared from the extracted DNA of *Campylobacter jejuni* ATCC strain 33291 for the assay as a 10-fold serial dilution in 1× TE buffer, ranging from 1.0×10^6 to 1.0×10^0 gene copies of *hip*O per reaction, and tested in duplicate. The limit of detection (LOD) was approximately one gene copy per PCR, equivalent to one *Campylobacter* cell per reaction as *hip*O is a single copy gene (Leblanc-Maridor *et al.* 2011). This assay was tested against DNA extracted from the ATCC 33291 *C. jejuni*, as well as against five clinical isolates confirmed as *C. jejuni* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (VITEK Mass

Spectrometer, Biomerieux Inc.). Furthermore, six additional bacteria tested negative by qPCR, including *Citrobacter freundii* (ATCC 8090), *Proteus mirabilis* (ATCC 12453), *E. coli* (ATCC 25922), *E. coli* O157 (ATCC 35150), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028), and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076). Briefly, the reaction mixture (25 μL) consisted of *Taq*Man® environmental master mix 2.0 at 1× (Applied Biosystems), 400 nM of each forward and reverse primer (Biosearch Technologies), 100 nM of each *hip*O and IAC probe (Biosearch Technologies), 0.1 μL of the IAC target as a final copy number of 100, 5 μL of the DNA template, and 4.5 μL nuclease-free water.

Detection of *Salmonella* spp.

A *Taq*Man® qPCR assay targeting the *fimA* gene found ubiquitously in all *Salmonella* species was custom-designed to detect the presence of *Salmonella* in samples. Primer Express Software Version 3.0.1 (Applied Biosystems) was used to design primers and probes within the *fimA* gene, using default parameters. Seven standards were prepared from DNA extracted from *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076) for the assay as a 10-fold serial dilution in 1× TE buffer, ranging from 1.0×10^6 to 1.0×10^0 gene copies of *fimA* per reaction, and tested in duplicate. The LOD was approximately one gene copy per PCR, equating to one *Salmonella* cell per reaction since *fimA* is a single copy gene (Zeiner *et al.* 2012). The *Salmonella* spp. assay was tested against *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Salmonella enterica* subsp. *enterica* serovar Enteritidis, both of which tested positive. Additionally, a number of non-*Salmonella* organisms tested negative including *C. jejuni* (ATCC 33291) and those aforementioned for the *Campylobacter* assay. The reaction mixture (30 μL) consisted of *Taq*Man® environmental master mix 2.0 at 1×, 100 μM of each forward and reverse primer, 100 μM *fimA* and IAC probe, 0.1 μL of the IAC target as a final copy number of 100, 5 μL of DNA template, and 9.54 μL nuclease-free water.

Detection of STEC toxin genes, *stx1* and *stx2*

A multiplex *Taq*Man® qPCR assay targeting the *stx*1 and *stx*2 genes was custom-designed to detect the presence of STEC. Primer Express Software Version 3.0.1 was used to design primers and probes within the *stx*1 and *stx*2 genes, using default parameters. Seven standards were prepared (from DNA extracted from *E. coli* O157 ATCC 35150) as a 10-fold serial dilution in 1× TE buffer, ranging from 1×10^6 to 1×10^0 gene copies of both *stx*1 and *stx*2 per reaction. The LOD was approximately one gene copy of each *stx*1 and *stx*2 per PCR. This multiplex assay was tested against a laboratory strain of *E. coli* O157 to ensure specificity to *stx*1/*stx*2 toxin genes. Additionally, a number of non-*stx*1/*stx*2-producing organisms tested negative including a laboratory strain of non-STEC *E. coli* (*E. coli* ATCC 25922), *Enterococcus faecalis* (ATCC 29212), and those previously mentioned for the *Campylobacter* assay. It should be noted that the *stx*1 assay is unable to distinguish between the *stx*1 gene of *Shigella* and STEC; however, the contamination of well water by *Shigella* is highly unlikely, given humans are the only animal reservoir and human cases remain rare in Ontario. Accordingly, dissemination of *Shigella* into private water wells in Ontario is not anticipated, as would be expected for pathogens such as *Campylobacter* and STEC, the reservoirs of which include livestock (PHO 2018). The reaction mixture (25 μL) consisted of *Taq*Man® environmental master mix 2.0 at 1×, 100 μM each forward and reverse primer, 100 μM each *stx*1, *stx*2, and IAC probe, 0.1 μL of the IAC target as a final copy number of 100, 5 μL of the DNA template, and 1.9 μL nuclease-free water.

Detection of *E. coli* markers

A qPCR assay targeting the *E. coli* 23S rRNA gene sequence, adapted from Chern *et al.* (2011), was used to detect *E. coli* in samples. Standards were prepared using genomic DNA from ATCC strain 25922 and ranged from 1×10^6 to 1×10^0 cells per reaction. Subsequently, *E. coli* standards, to a limit of one gene copy per reaction, were assessed independently and an LOD of 2.5 gene copies per reaction or 7 cells/100 mL was repeatedly and accurately detected. The qPCR mixture and conditions are the same as detailed by Krolik *et al.* (2016).

Detection of *Bacteroides* 16S rRNA genetic markers

All samples underwent three qPCR assays for the detection of the total, human, and bovine-specific *Bacteroides* 16S rRNA genetic markers in order to reveal information about the

source of contamination. The total *Bacteroides* assay targets a conserved region for 10 host-specific species including feline, bovine, deer, canine, anserine, gull, equine, human, swine, and raccoon. These MST assays were based on earlier work by Lee *et al.* (2010) for surface water, and Krolik *et al.* (2014), for private water wells and primers, probes, and reaction conditions, have been previously described. Krolik *et al.* (2014) validated an adjusted LOD of 10 gene copies for all three singleplex assays. Standard curves for the MST assays were created as previously described using plasmids with concentrations ranging from 6.5×10^6 to 6.5×10^0 gene copies per reaction.

Statistical analyses

The distribution of all measured variables was analyzed with histograms and goodness-of-fit tests for normality, including Kolmogorov–Smirnov, Cramer–von Mises, and Anderson–Darling tests to inform the subsequent analysis. Data sets were analyzed using Pearson's chi-squared tests to determine if an association existed between a well location and the source of fecal contamination. Finally, data sets were analyzed using Spearman rank correlation tests to evaluate the relationship of microbial indicators, *E. coli* and TCs, and microbial source indicators, *Bacteroides* 16S rRNA, to each other and to pathogen detection. Spearman correlation coefficients were interpreted based on the guidelines detailed in Table 2 (Evans 1996). All statistical analyses were performed in SAS 9.3 at a level of significance of $\alpha = 0.05$.

RESULTS

Sample collection overview

In total, 557 private drinking water samples, 314 *E. coli*-positive and 243 TC-only, were obtained during the collection period. Of these, 96% (297 *E. coli* and 236 TC-only) had sufficient well location information enabling geocoding to a source well within one of the four regions. Of these, 74% (205 *E. coli* (+) and 190 TC-only) were associated with a unique source well. The remaining samples (92 *E. coli* (+) and 46 TC-only) represented 61 unique source wells (40 *E. coli* and 21 TC-only), indicating wells that were tested more than once over the study period. In terms of testing and statistical analyses, samples from wells that submitted more than once were treated the same as samples from unique source wells. Some samples could not be successfully extracted or tested for a final count of 519 samples (289 *E. coli* (+) and 230 TC-only) (Table 3).

Prevalence of enteric pathogens

The evidence of enteric pathogens was detected in 10 (1.9%) drinking water samples (Table 4). A genetic marker of *Campylobacter*, namely the *hip*O gene, was detected in five samples, and the *stx*1 gene was detected in the remaining five. Genetic markers of *Salmonella* spp. were not detected. At least one pathogen-positive sample came from each of the four regions of interest (Figure 2), suggesting no particular geographic trend for pathogen presence, although the sample size is small and no firm conclusions can be made. It should be noted that samples 9 and 10 were associated with the same source well and collected on the same day. Of the 10 samples, only two were from the *E. coli* (+) sample set, with the remaining eight being negative for culturable *E. coli* and positive for TCs. The measured variables associated with these samples, *E. coli* and TC culture results, *E. coli* qPCR, total, human, and bovine-specific *Bacteroides* 16S rRNA genetic markers do not appear to follow any particular trends. However, it

Table 2 | Interpretation of correlation coefficients for this study (Evans 1996)

Correlation coefficient	Interpretation of relationship
0.2–0.39	'Weak'
0.4–0.69	'Moderate'
0.7–0.1	'Strong'

Table 3 | Total samples organized by the 'hotspot' location

Hotspot	*E. coli* (+) samples	TC-only samples
Grey/Bruce	132	45
Niagara/Hamilton	68	77
High Cattle	62	88
Kingston/Belleville	27	20
Total	289	230

Table 4 | Full profile of private water well samples that demonstrated molecular evidence of targets of interest

Sample	Target of interest	TC (CFU/100 mL)	*E. coli* (CFU/100 mL)	*E. coli* gene copies and cell equivalents/100 mL qPCR	Human *Bacteroides* 16S rRNA markers	Bovine *Bacteroides* 16S rRNA markers	Total *Bacteroides* 16S rRNA markers	Hotspot
1	*hip*O	5	0	0/0	0	0	0	High cattle
2	*hip*O	64	0	22.4/3.2	21	0	50	K/B
3	*hip*O	3	0	0/0	0	0	15	High cattle
4	*hip*O	8	0	0/0	0	0	0	G/B
5	*hip*O	81	45	140/20	8,498	44	37,515	G/B
6	*stx*1	81	2	9.1/1.3	0	0	0	High cattle
7	*stx*1	1	0	0/0	0	0	1,333	N/H
8	*stx*1	81	0	29.4/4.2	1,740	0	11,890	G/B
9	*stx*1	22	0	79.8/11.4	0	0	0	N/H
10	*stx*1	20	0	130.2/18.6	0	0	17	N/H

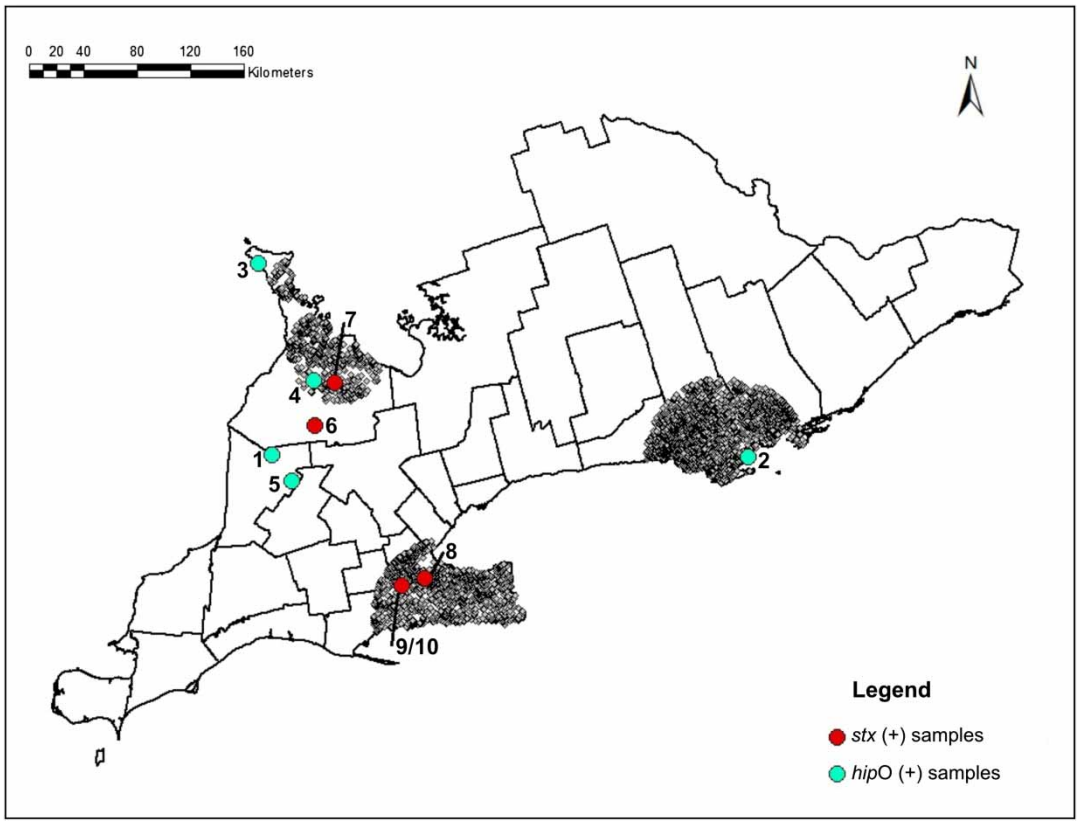

Figure 2 | Location of private water wells that were associated with a water sample positive for a target of interest in each of the 'hotspot' regions (shaded in gray).

should be noted that eight of the 10 samples were positive for at least one indicator of fecal contamination, either in the form of culturable *E. coli* and/or *E. coli* or *Bacteroides* detected by qPCR.

Microbial source tracking using *B. fragilis*

Samples were tested via three MST qPCR assays in order to determine the pre-eminent source of fecal contamination.

The following MST statuses were assigned to each sample based on the established gene copy thresholds: negative for all *Bacteroides* (none), positive for total *Bacteroides* only (not human or bovine), positive for human *Bacteroides*, positive for bovine *Bacteroides*, and positive for both human and bovine *Bacteroides*. Among the *E. coli* (+) samples, a higher proportion tested positive for human-specific contamination (41%) compared with the TC-only samples (22%) (Figure 3). The TC-only group had a higher proportion of samples negative for all *Bacteroides* genetic markers (50%) compared with the *E. coli* (+) sample set (23%).

Prior work by Krolik *et al.* (2016) found that the same *E. coli* (+) samples assessed in this study were highly contaminated with human-specific contamination, especially within the Kingston/Belleville (2012 data) and Grey/Bruce regions (56% and 53%, respectively). A Chi-square test and post-hoc comparisons revealed that these two regions had significantly more wells positive for human-sourced contamination compared with the Niagara/Hamilton and High Cattle regions. The latter two regions appeared to have a higher number of wells with only total *Bacteroides* genetic markers detected (38% and 37%, respectively).

In addition to the *E. coli* (+) samples, the TC-only samples, from the same four regions, were investigated for sources of fecal contamination (Figure 4). Fewer samples were positive for human contamination than for *E. coli* (+) samples, and a chi-squared test determined there was no statistically significant association between the region and human-specific contamination ($\chi^2 = 0.62$, $p = 0.89$). The Grey/Bruce and High Cattle regions had a higher proportion of samples negative for all *Bacteroides* genetic markers (63% and 57%, respectively). In contrast, the Kingston/Belleville and Niagara/Hamilton regions had a lower proportion of samples with no *Bacteroides* genetic markers detected (30% and 39%, respectively) and a higher proportion of samples with only total *Bacteroides* (40% and 31%, respectively). There was a statistically significant association between regions sampled and the proportion of samples with no *Bacteroides* genetic markers detected ($\chi^2 = 12.0$, $p < 0.01$).

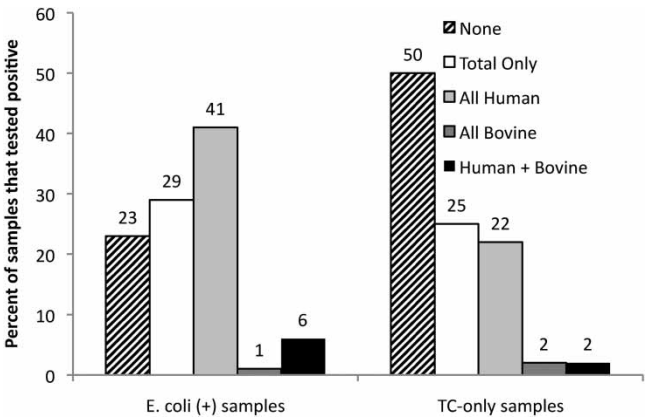

Figure 3 | Distribution of microbial sources arranged for *E. coli* (+) and TC-only samples.

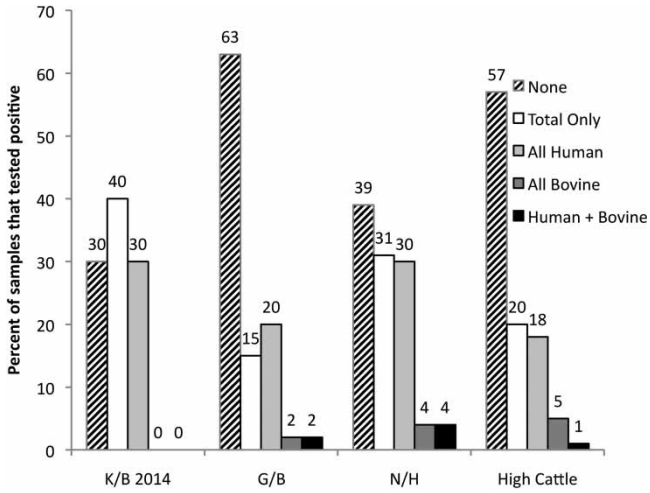

Figure 4 | Distribution of microbial sources in TC-only samples stratified by the region. K/B, Kingston/Belleville; G/B, Grey/Bruce; N/H, Niagara/Hamilton.

All regions had a low percentage of samples contaminated by a bovine fecal source. The High Cattle region had the lowest proportion of *E. coli* culture (+) samples with bovine-specific *Bacteroides* genetic marker contamination (2%), and only 5% of TC-only samples in the High Cattle region were positive for bovine-specific contamination. Due to the low amount of observed bovine-specific contamination, not enough data were available to statistically test association with the region, nor its relationship with a number of other measured variables.

Correlation between alternative and traditional indicators

Total *Bacteroides* versus *E. coli*

All measured variables, including *E. coli* CFU/100 mL, TC CFU/100 mL, *E. coli* cells as detected via qPCR, and total, human, and bovine-specific *Bacteroides* 16S rRNA genetic

markers, showed non-normal skewed distributions. Therefore, Spearman rank correlations were used to analyze the relationship between variables. When data from all wells were aggregated, there was a weak but significant correlation ($\rho = 0.20$, $p < 0.01$) between total *Bacteroides* genetic markers and culturable *E. coli* (CFU/100 mL). There was also a weak but significant correlation ($\rho = 0.34$, $p < 0.001$) between total *Bacteroides* genetic markers and *E. coli* cells/100 mL as detected by qPCR.

Human-specific *Bacteroides* versus *E. coli* and TCs

No significant correlation was found between human-specific *Bacteroides* genetic markers and culturable *E. coli* (CFU/100 mL) ($\rho = 0.16, p = 0.07$). However, there was a significant correlation overall ($\rho = 0.3$, $p < 0.05$) when investigating the relationship between human-specific *Bacteroides* genetic markers and *E. coli* cells/100 mL as detected by qPCR.

TCs and *Bacteroides*

There was no significant correlation between total or human-specific *Bacteroides* genetic markers and TC CFU/100 mL in the TC-only sample set (data not shown).

DISCUSSION

Enteric pathogens in private drinking water wells

It was hypothesized that water from private drinking wells in previously identified high-risk regions would be vulnerable to contamination by enteric pathogens. This study explored the value of 100 mL convenience samples as a potential resource for on-going water surveillance purposes and in the context of previously established indicators of drinking water potability, namely *E. coli* and TCs. In total, 10 (1.9%) samples were positive for targets of interest by qPCR, including five for the *hip*O gene specific to *Campylobacter* spp. and five for the *stx*1 gene that encodes the Stx1 toxin produced by STEC and *Shigella* spp. Although low, these detections are significant, given this study was limited by a small volume of water available for testing. Moreover, for *E. coli* (+) samples, it was determined, via qPCR, that the human-specific *Bacteroides* genetic marker was detected more frequently than non-human-specific *Bacteroides* markers, indicating that human feces and/or sewage was the primary contaminating source (Figure 3). Although humans are capable of becoming ill with *Campylobacter*, *Salmonella*, and *E. coli* O157, these diseases remain relatively rare occurrences (PHO 2018), and humans are not considered natural reservoirs of these pathogens unlike food-producing animals (e.g. cattle and chickens). As such, it is not surprising that these zoonotic enteric pathogens for which this study surveyed were rarely detected. Furthermore, *Campylobacter* spp. and *Salmonella* spp. have a wide range of animal hosts (i.e., wild life, livestock, and domestic pets), and some them are outside of that which the *Bacteroides* assay can detect. In particular, birds are a notable source of both pathogens, and although the total *Bacteroides* assay can detect gull and anserine contamination, there is a gap in poultry sources. Additionally, only a very small subset of samples were positive for bovine-specific contamination (6%), and as cattle are the primary reservoir of STEC, this finding may, in part, explain the low number of *stx*1/*stx*2 detections.

Although there is vast literature indicating that surface water, wastewater, and sewage effluent are often contaminated with pathogens, there is relatively less evidence for private groundwater supplies (Hörman *et al.* 2004; Walters *et al.* 2007; Viau *et al.* 2011). Won *et al.* (2013) showed that in private wells proximal to bovine operations in northeastern Ohio, *Campylobacter* spp. DNA was not detected by qPCR in any samples (2 L filtered) and *E. coli* O157:H7 by qPCR was detected in just 4% of samples. Similarly, Schets *et al.* (2005) tested drinking water from private water supplies in the Netherlands and found that *E. coli* O157:H7 was isolated from just 2.7% of samples. Investigation of the microbial quality of private groundwater wells generally requires large volume samples (e.g., greater than 1 L) relative to less protected sources, such as surface or wastewater. Hänninen *et al.* (2003) studied waterborne outbreaks in Finland caused by *C. jejuni* in private groundwater supplies and found that the use of large sample volumes of several liters, versus the $\leq$1 L proposed by the International Standardization Organization (2002) for the detection of *Campylobacter* from drinking water, increased the chance of pathogen detection. Schets *et al.* (2005) discovered that *E. coli* O157, as detected by

qPCR, was isolated more frequently from 1 L versus 100 mL sample volumes of private groundwater drinking wells.

Statistical analyses were not performed to assess the predictive value of fecal indicators as it relates to pathogen presence as only a few samples tested positive for targets of interest. Overall, eight of 10 samples in which pathogen presence was detected showed the evidence of fecal contamination, either *E. coli* (by culture or qPCR) or *Bacteroides* 16S rRNA genetic markers. However, only two were positive for culturable *E. coli*, with the remaining positive for culturable TC-only, suggesting that TCs remain a relevant and useful indicator of water quality. Furthermore, only four samples with the molecular evidence of pathogens were positive for *E. coli* by qPCR when applying the LOD of seven *E. coli* cells/100 mL. Numerous studies provide evidence that *E. coli* does not always accurately predict the presence of pathogens within aquatic environments. Won *et al.* (2013) found that in the samples positive for *E. coli* O157 by qPCR, only one out of seven was also positive for *E. coli* by a commercial MPN test. Wu *et al.* (2011) assessed 540 cases representing independent indicator–pathogen correlations from 1970 to 2009 and found when *E. coli* was used as the indicator organism, only 25% of cases were correlated to pathogen presence.

Microbial fecal sources of contamination in Ontario *E. coli* hotspot well waters

Relative to TC-only samples, a higher percentage of *E. coli* (+) samples (by culture) were also contaminated with *Bacteroides* 16S rRNA genetic markers (77% versus 50%). Interestingly, 23% of these *E. coli* (+) samples were negative for *Bacteroides* genetic markers. This apparent discrepancy may be consequent to the fact that: (1) *E. coli* detection is via a membrane filtration method followed by culture on differential agar, whereby *E. coli* are identified based on phenotype alone (color change), and not subject to further confirmatory assays. This aligns with the current testing practices for the presumptive identification of *E. coli* in drinking water in Ontario, (2) these samples may have fecal contamination in the form of *Bacteroides* that is attributable to hosts other than the 10 sources detected by the total *Bacteroides* assay, and (3) the *Bacteroides* qPCR assays may have been less sensitive for small volume water samples especially in instances where *E. coli* CFU/100 mL counts were low. Similarly, Krolik *et al.* (2014) showed that for those private drinking water samples that were positive for culturable *E. coli*, 23.6% were negative for all three *Bacteroides* genetic markers. Although the TC-only samples had less total *Bacteroides* contamination than *E. coli* (+) samples, 50% were still positive for *Bacteroides*. Many of these samples, although negative for culturable *E. coli*, were positive for *E. coli* by qPCR, suggesting that the samples were fecally contaminated at one time with organisms that are either nonviable or viable but non-culturable (VBNC) (Oliver 2010).

TC-only sample analysis revealed that all regions, with the exception of Kingston/Belleville, had a high proportion of TC-only samples that were negative for the presence of *Bacteroides* genetic markers. The Kingston/Belleville region included a small data set and was excluded for this particular analysis. The Grey/Bruce and High Cattle regions have a significantly higher percentage of samples with no *Bacteroides* genetic markers detected compared with the Niagara/Hamilton region. This may suggest that wells with TC-only contamination in these two regions are less susceptible to future or past fecal contamination compared with TC-only wells from the Niagara/Hamilton region. This may be due to well integrity and structure, frequent testing and treatment, and appropriate distance from septic tanks as well as different characteristics of the hydrogeological system (Tallon *et al.* 2005).

Relationship of an alternative to traditional fecal indicators

Given the low prevalence of *E. coli* (by culture and qPCR) in samples positive for genetic markers of enteric pathogens, the relationship between *E. coli* and the alternative indicator, *Bacteroides*, was investigated. A weak but significant correlation between total *Bacteroides* and culturable *E. coli* was observed, which is supported by similar investigations. Lee *et al.* (2010) determined that total *Bacteroides* exhibited a weak correlation to *E. coli* ($r^2 = 0.22$) in Ontario surface water samples. However, the relationship between total *Bacteroides* genetic markers and *E. coli* (qPCR) was stronger than to culturable *E. coli*, a trend that has been previously documented for other water sources, but rarely for private drinking well waters. Sauer *et al.* (2011) saw a stronger correlation of *E. coli* as detected by qPCR to total

Bacteroides ($\rho = 0.464$) than to culturable *E. coli* ($\rho = 0.336$) in Wisconsin stormwater samples.

It is known that the persistence of *E. coli* differs from that of *Bacteroides* markers in environmental waters and is highly dependent on various factors. Okabe & Shimazu (2007) demonstrated that decay rates for the *Bacteroides* genes were greater at high temperatures and lower between 4 and 12 °C, whereas TC and fecal coliform (FC) decay rates, measured by culture, were lower at temperatures above 10 °C and higher at 4 °C. Further differences may be explained by the different methods of detection, that is, culture for *E. coli* and qPCR for *Bacteroides* genetic markers. Culture will only detect viable cells, whereas qPCR detects viable cells, VBNC and DNA from dead cells. Furthermore, the culture method used in this study is based upon the phenotypic identification of *E. coli* colonies. Although the detection of β-D-glucuronidase activity is generally a good indicator of *E. coli*, this enzyme has been reported in other organisms, including *Salmonella*, *Shigella*, *Bacteroides*, and *Clostridium* (Cabral 2010). Therefore, culture tests may be accounting for more or less *E. coli* than is actually present, affecting the correlation with total *Bacteroides* genetic markers.

Human-specific *Bacteroides* genetic markers and *E. coli* as detected by qPCR, but not by culture, were significantly correlated. Sauer *et al.* (2011) showed that although total *Bacteroides* genetic markers were correlated with *E. coli* (culture and qPCR), human-specific markers exhibited non-significant correlation with culturable *E. coli* and Enterococci and weak correlation with *E. coli* by qPCR ($\rho = 0.16$). Okabe *et al.* (2007) demonstrated that in river water samples, total *Bacteroides* genetic markers showed a moderate level of correlation ($r^2 = 0.49$) with FC, but human-specific *Bacteroides* gene markers showed no significant correlation with FC ($r^2 = 0.12$) which was thought to be a consequence of multiple sources of fecal contaminants other than human.

The weak correlation of *E. coli* to human-specific *Bacteroides* genetic markers is partially due to the inherent differences between traditional culture methods and qPCR and the differences in survival of *E. coli* to *Bacteroides* as mentioned above. However, it is also thought that the strength of correlation may be weak because *E. coli* is not indicative of human contamination specifically. Strains of *E. coli* are prominent within the GIT of the majority of warm-blooded animals. Therefore, a number of sources besides humans, including pets, domestic livestock, and various wildlife could be responsible for *E. coli* contamination in private water wells.

Total and human-specific *Bacteroides* 16S rRNA genetic marker analyses in the context of correlation to TCs showed non-significant correlation. This was to be expected given TCs are not strictly a fecal indicator but also an indicator of environmental contamination and demonstrates well vulnerability.

CONCLUSIONS

This study aimed to investigate the feasibility of using private water well samples, submitted to the provincial laboratory for bacterial testing (convenience samples) for the purpose of pathogen surveillance, and to examine the findings in the context of various markers of fecal contamination for select enteric pathogens. Accordingly, the relationship between well location, fecal indicators, and pathogen presence was assessed. This study did not explore other drivers of well water quality, such as well screen depth, well age, depth to water, land use, septic system density, geology, or others. Current studies in our laboratory are examining these potential drivers of contamination for private water wells in Ontario. The detection of genes of various enteric pathogens was low and may, in part, be explained by small water volumes, but our findings, and those of others, might further be explained by the fact that human, not bovine, fecal contamination is responsible for the majority of private water well contamination in the regions studied. Future studies should consider including a similar assessment, but expanding the pathogens investigated to those most frequently associated with human fecal sources (e.g. norovirus) and with a comparative analysis to larger volumes in order to explore the limit of detection in the context of water volume as a future aim. If the results are comparable, this would further validate the use of small volume waters as adequate for pathogen monitoring.

Given that private drinking water samples are frequently impacted by fecal contamination, especially from humans, attention must be paid to improving barriers for fecal transport between wells, septic, or household waste management

systems, and the environment. The evidence of both *Campylobacter* and STEC contamination was found, albeit in few samples, and is typically associated with cattle or livestock, demonstrating that multiple sources need to be considered and addressed, even though infrequently. The presence of target DNA for fecal markers and pathogens also suggests recent or past contamination, confirming the vulnerability of the well to contamination by these organisms.

In developed nations, clean and safe drinking water is the expected standard, and the presence of enteric pathogens is considered unacceptable (Safe Water Drinking Act 2002; WHO 2011). Many enteric pathogens have a very low infectious dose and if ingested may cause infection, especially in immunocompromised individuals or those persons with first exposure (naïve) to the drinking water source; for example, visitors.

This study further demonstrates that fecal indicators, in addition to *E. coli*, and detection methods, beyond culture, could be considered both at the laboratory and the regulatory level, for private water well testing in Ontario. In this investigation, *E. coli* did not accurately predict the presence of enteric bacterial pathogens within those samples tested, and the majority of samples with enteric pathogen presence had TC contamination only. Furthermore, *E. coli*, as detected by standard culture methodology, did not strongly correlate with other indicators of fecal contamination, notably species-specific *Bacteroides*. However, a larger scale study looking at additional regions of interest, with larger sample size, against which these findings could be compared could be considered in future as a validation step.

This study and future-related studies serve to inform water testing and maintenance practices for both the private water well owners and source drinking water stewards, as well as further enhance the understanding of well water quality implications and impacts on humans, animals, and the environment, from both the public health and policy perspectives.

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