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Detection and Prevalence of Verotoxin-Producing *Escherichia coli* O157 and Non-O157 Serotypes in a Canadian Watershed

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Verotoxin-producing *Escherichia coli* (VTEC) strains are the cause of food-borne and waterborne illnesses around the world. Traditionally, surveillance of the human population as well as the environment has focused on the detection of *E. coli* O157:H7. Recently, increasing recognition of non-O157 VTEC strains as human pathogens and the German O104:H4 food-borne outbreak have illustrated the importance of considering the broader group of VTEC organisms from a public health perspective. This study presents the results of a comparison of three methods for the detection of VTEC in surface water, highlighting the efficacy of a direct VT immunoblotting method without broth enrichment for detection and isolation of O157 and non-O157 VTEC strains. The direct immunoblot method eliminates the need for an enrichment step or the use of immunomagnetic separation. This method was developed after 4 years of detecting low frequencies (1%) of *E. coli* O157:H7 in surface water in a Canadian watershed, situated within one of the FoodNet Canada integrated surveillance sites. By the direct immunoblot method, VTEC prevalence estimates ranged from 11 to 35% for this watershed, and *E. coli* O157:H7 prevalence increased to 4% (due to improved method sensitivity). This direct testing method provides an efficient means to enhance our understanding of the prevalence and types of VTEC in the environment. This study employed a rapid evidence assessment (REA) approach to frame the watershed findings with watershed *E. coli* O157:H7 prevalences reported in the literature since 1990 and the knowledge gap with respect to VTEC detection in surface waters.

Verotoxin-producing *Escherichia coli* (VTEC) is an important zoonotic food-borne and waterborne pathogen causing diarrhea, hemorrhagic colitis, and potentially fatal outcomes such as hemolytic uremic syndrome (HUS) in humans (1, 2). The predominant VTEC serotype associated with outbreaks and sporadic cases of serious VTEC illness is *E. coli* O157:H7 (VTEC O157) (2), and despite early recognition of non-O157 VTEC strains as human pathogens (1–3), VTEC O157 remains the major focus of clinical and food diagnostic laboratories in many jurisdictions. However, over 380 other serotypes of VTEC have been isolated from humans (2), and increasing awareness of non-O157 VTEC as causes of human illness has prompted expanded clinical diagnosis, investigation, and surveillance of these organisms (4–6). Targeted studies in Canada have indicated that non-O157 VTEC accounts for approximately 50% of VTEC infections (7, 8). Several serotypes of VTEC identified in these Canadian studies are among the four to six non-O157 serotypes most frequently causing serious human illness in the European Union (EU) and the United States (4).

Healthy cattle and other ruminants are the major animal reservoirs of many VTEC, carrying these organisms in their gastrointestinal tracts and shedding them in manure at levels ranging from 10 to $>10^5$ CFU/g (2). Human exposure occurs through numerous routes, including consumption of contaminated meats, milk, produce, fruits, juices, and water, exposure to contaminated farm environments, contact with farm animals, and person-to-person transmission (2, 9). Although contaminated ground beef has been considered the most frequent source of human exposure, recent investigations have increasingly identified numerous outbreaks and sporadic cases/clusters linked to nonmeat sources (10–12). Among these sources, rural water supplies, crop irrigation water, and contaminated municipal water supplies in agricultural areas have been implicated (13–15). In several studies, agricultural watersheds heavily impacted by ruminant livestock appear to be linked to waterborne O157 and non-O157 VTEC infections (15, 16). Consequently, effective surveillance programs designed to identify risks of human exposure to VTEC will include water as a potential source.

The Canadian integrated enteric pathogen surveillance system, FoodNet Canada (formerly C-Enternet), which was launched in 2005, relies on passive sampling of human cases and active sampling of three exposure sources (food, water, and animal manure) for a suite of enteric pathogens. Since 2005, active detection, isolation, and characterization of VTEC O157 strains and, more recently, non-O157 VTEC strains in retail foods and cattle have demonstrated their presence in these sources (17). Testing of human samples in the sentinel sites varies, with most clinical laboratories still being focused only on VTEC O157, while some are adopting methods to identify the broader suite of VTEC. Isolation-based methods are emphasized in FoodNet Canada surveillance of exposure sources, to allow enhanced characterization of VTEC strains (serotyping, phage typing, virulotyping, and pulsefield gel electrophoresis) for comparison with human VTEC strains isolated within the sentinel sites. Watersheds in these sen-

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tinel sites have also been tested for VTEC O157 and to 2009 showed low prevalences (≤1%) (17). In July 2010, an initiative was launched to include non-O157 VTEC testing for these river water samples.

Enumeration of generic *E. coli*, an indicator of recent fecal contamination, is commonly included in surface water monitoring programs; however, testing for pathogens such as VTEC O157 is not. Due to the apparent low prevalences and stressed condition of pathogens in water, most methods for detection and isolation of VTEC in water rely on filtration of a volume of water (typically 1 liter or more) and broth enrichment culture of the bacterial population captured on the filters (9, 18). For VTEC O157, immunomagnetic separation (IMS) and selective media facilitate isolation of this organism among the other bacteria in the enrichment broth. Among the many variations on these methods, those used by Jokinen et al. (19) have been applied with success in several studies (20, 21), although broth enrichment negates enumeration of the initial VTEC O157 populations. For enumeration, brief resuscitation, IMS for *E. coli* O157, and plating of the beads on a semiselective agar for VTEC O157 followed by colony lift immunoblotting with an O157-specific antibody enabled isolation and enumeration of *E. coli* O157 in animal wastewater, although half of the recovered strains were VT-negative *E. coli* O157 (22). In contrast, few methods have been used for the isolation of non-O157 VTEC in water, although several molecular methods for the detection of VT genes as evidence for the presence of any VTEC serotype have been reported (9). One method involving plating and growth of dilutions of sewage and animal waste waters on a chromogenic agar followed by colony lift hybridization with a *vt2* DNA probe enabled isolation of numerous VTEC serotypes (23).

The objectives of this study were to implement and compare selected isolation-based methods for O157 and non-O157 VTEC strains in the testing of surface water samples in a mixed use watershed in southern Ontario (Grand River), as part of the FoodNet Canada program, and to apply the results to the examination of prevalences at the several sampling sites in the watershed. The methods chosen for VTEC O157 detection were similar to those described by Jokinen et al. (19). Methods to detect all VTEC strains (O157 and non-O157 VTEC) were derived from a VT colony immunoblotting method (VT-IB) that has been used successfully for isolation of all VTEC strains from enrichment cultures of ground beef (40), and in this study it was applied both with and without broth enrichment culture of the water filters. To contextualize the prevalence data with other studies, a rapid evidence assessment (REA) of peer-reviewed literature published between 1990 and 2013 was used to identify studies that reported the prevalence of VTEC O157 and non-O157 in surface waters of North America and Europe.

MATERIALS AND METHODS

River water sample collection. Since 2005, samples have been collected at five river sites twice per month in the FoodNet Canada sentinel site 1 within the Grand River watershed (Ontario, Canada). Three sample sites are located on the Grand River: one upstream site in the watershed (Grand River North), one site downstream of a wastewater outflow, and one site upstream of the drinking water intake. Two additional sample sites are located on tributaries of the Grand River, i.e., the Canagagigue Creek and the Conestogo River, which are both sampled prior to discharge into the Grand River. In addition, between June and September of 2011 and 2012, three local swimming sites were sampled on a biweekly basis: two beaches located on reservoirs (Laurel Creek and Shade's Mills beaches) and one site on the Grand River (the Elora Gorge). During sample collection, river water samples were collected in 1-liter sterile sampling bottles. Samples were collected from a fast-flowing portion of the river by wading into the stream or using an extendable sampling pole. The beach samples were collected at knee height, 15 cm below the surface.

Detection and isolation of VTEC O157 in water by filtration, enrichment, and IMS (2006 to 2010). From 2006 to 2010, FoodNet Canada testing was conducted by external laboratories using broth enrichment and IMS for VTEC O157. A 500-ml river water sample (2006 to 2008) and then a 1-liter river water sample (2008 to 2010) were filtered through a 0.45-μm filter membrane that was incubated in 10 ml of *E. coli* broth (EC broth) at 37°C for 4 h. Novobiocin was then added to a final concentration of 20 μg/ml, and the enriched samples were incubated at 42°C for 18 to 24 h. The broth cultures were then subjected to IMS of *E. coli* O157 using paramagnetic beads (Dynabeads *E. coli* O157; Invitrogen) following the manufacturer's instructions. After the last wash step, the beads were resuspended in 100 μl of 0.01 M phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-T) and vortexed. A 50-μl aliquot of the suspended particles was plated onto both sorbitol MacConkey agar (SMAC; Oxoid) and cefixime-tellurite-sorbitol MacConkey agar (CT-SMAC; Oxoid) and incubated at 42°C for 24 and 48 h. Suspect colonies (clear to smoky gray) were tested for the O157 antigen by latex agglutination (*E. coli* O157 Dryspot test; Oxoid). Agglutination-positive colonies were further tested by the *E. coli* MUG test (using lauryl sulfate broth with methylumbelliferyl-β-D-glucuronide), the cellobiose fermentation test, and the API 20E biotyping test. Final confirmation of isolates as VTEC O157 was conducted using the Assurance gene detection system for *E. coli* O157:H7 (Health Canada method MFLP-16). Confirmed isolates were submitted to the *E. coli* Reference Laboratory of the Public Health Agency of Canada's Laboratory for Foodborne Zoonoses (PHAC LFZ) in Guelph, Ontario, for characterization.

VTEC testing method comparison (July 2010 to December 2012). Starting in July 2010, a comparative evaluation of two approaches for the detection and isolation of VTEC O157 and all VTEC serotypes was initiated at the PHAC LFZ in Guelph, Ontario (Fig. 1). As detailed below and in Fig. 1, one approach used the common method of broth enrichment of water filters of large volumes of water, with two broth media. Each enrichment broth was tested specifically for VTEC O157 by IMS and also for detection and isolation of any VTEC strains, including VTEC O157 strains, by screening the enrichments for VTs by a VT enzyme-linked immunosorbent assay (ELISA), followed by isolation from VT-positive broths by the VT-IB method (40). In the second approach, the VT-IB method was applied to filters of smaller volumes of water directly, without broth enrichment; this was termed the direct VT-IB (method 3; Fig. 1).

(i) Detection of VTEC O157 and all VTEC with broth enrichment. Two 500-ml subaliquots of a water sample were filtered through separate 0.45-μm sterile nitrocellulose filters (Pall Corporation, Ann Arbor, MI). One filter was cultured in 50 ml of buffered peptone water (BPW; Oxoid) at 37°C for 18 to 20 h (method 1; Fig. 1), and the second filter was cultured similarly in a modified tryptic soy broth (TSB; Oxoid) containing bile salts no. 3 (1.5 g/liter), vancomycin (10 μg/ml), and cefsoludin (10 μg/ml) (mTSBVC) (method 2; Fig. 1), as described previously for the detection of VTEC in ground beef (40).

(a) Detection and isolation of VTEC O157 from enrichments by IMS. For specific isolation of VTEC O157, each filter enrichment broth was tested by IMS as described above (methods 1a and 2a; Fig. 1), and 50-μl volumes of the recovered bead suspensions were plated (undiluted and diluted 1:10 in sterile PBS) onto CT-SMAC and, in 2012, also onto CHROMagar O157 (Alere, Quebec, Canada). After incubation of the plates for 20 to 24 h at 42°C, suspect colonies were tested for the O157 antigen by slide agglutination using an O157 antiserum (Difco) and, if positive, were confirmed as VTEC by testing 3- to 6-h enrichments of each colony in 300 μl of mTSBVC for VTs by the VT ELISA (described

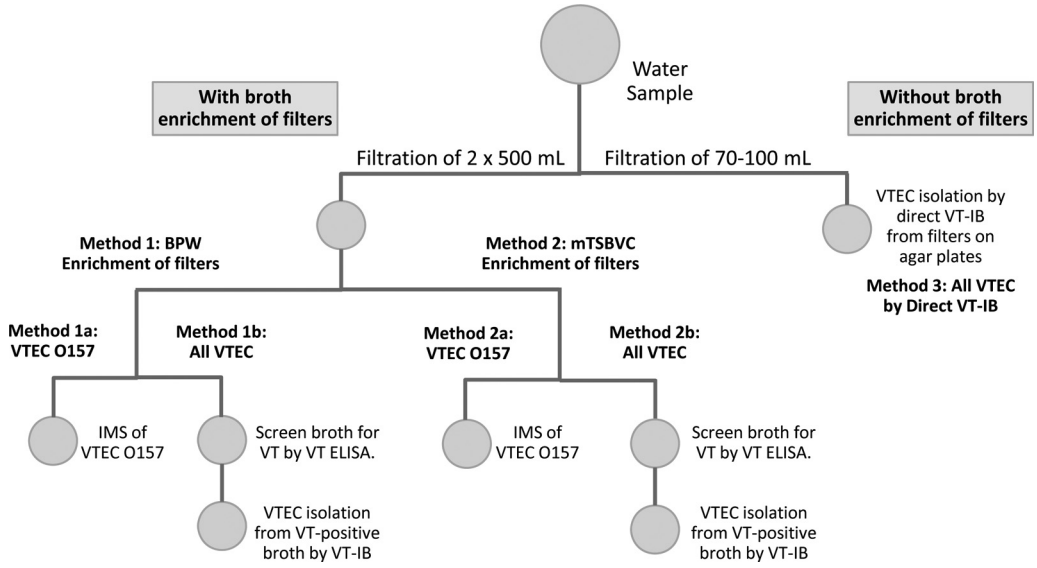

FIG 1 Schematic of comparison of VTEC isolation methods (July 2010 to December 2012). Abbreviations: VTEC, verotoxin-producing *E. coli*; VT-IB, verotoxin colony immunoblot; BPW, buffered peptone water; mTSBVC, modified tryptic soy broth containing vancomycin and cefsulodin; IMS of VTEC O157, immunomagnetic separation of *E. coli* O157 and isolation of VTEC O157 on differential agar; VT ELISA, enzyme-linked immunosorbent assay for verotoxins.

below). Up to four isolates from each sample were submitted for characterization.

(b) Detection of all VTEC serotypes in water filter enrichment broths. Enrichment broths of water filters were screened for VTs (methods 1b and 2b; Fig. 1) by a microwell VT ELISA (40), as described below. Broths scored as positive or suspicious (here jointly termed VT positive) were processed for VTEC isolation by VT-IB as described below.

(ii) VTEC detection and isolation without water filter enrichment (direct VT-IB). Approximately 70 to 100 ml of each water sample was vacuum filtered directly onto 0.45-μm hydrophobic grid membrane filters (HGMFs; Neogen), which are divided into 1,600 cells by grid lines printed with hydrophobic ink. In some cases, more than one HGMF was needed for turbid samples. The loaded HGMFs were then processed directly by the VT-IB method described below, without broth enrichment (method 3; Fig. 1).

VT ELISA. A VT ELISA developed at LFZ (40) was used to test water filter and picked colony enrichments for VTs, as evidence for the presence of viable VTEC in these cultures. Briefly, duplicate 100-μl volumes of the water enrichment broths and controls or single 100-μl volumes of enriched picked colonies were incubated in microwell plates precoated with rabbit anti-VT antibodies reactive with all known VTs. Bound VTs were detected by sequential incubation with a mixture of four monoclonal antibodies recognizing all VTs, followed by horseradish peroxidase-labeled rabbit anti-mouse IgG (Jackson ImmunoResearch) and the substrate tetramethylbenzidine (Sigma). Incubation times for samples, monoclonal antibodies, and conjugates were each 30 min. Wells were washed five times with PBS-T between steps. Color development following addition of the substrate was stopped after 10 min by the addition of 0.2 M sulfuric acid (50 μl/well). The plates were read in a microplate reader (ELx808; BioTek) at a dual wavelength of 450/630 nm, with the microplate reader set to 0 with air ("blanked to air"). Samples were scored, respectively, as suspicious or positive for VT if the mean optical densities (OD) were 1.25 to 1.5 times or >1.5 times the mean OD of the negative controls.

VTEC detection and isolation by VT-IB. The VT-IB method (40) enables effective detection and isolation of VTEC colonies in mixed cultures grown on a membrane filter over a VT capture membrane on agar plates. During colony growth, secreted VTs are bound on the capture membrane directly below VTEC colonies on the top membrane. Immunostaining the capture membrane for VTs reveals stained dots that correspond in location to individual VTEC colonies directly above on the top membrane. The procedure used in this study was as described previously (40), except that the 82-mm-diameter round top membranes were replaced by a hydrophobic grid membrane filter (HGMF). Briefly, for VT-positive water filter enrichment broths (methods 1b and 2b; Fig. 1), 100 μl of two or three 10-fold dilutions of the broths, selected based on their VT ELISA ODs, were made up to 10 ml in sterile PBS and vacuum-filtered onto HGMFs. For direct VT-IB (method 3; Fig. 1), the HGMFs were prepared as described above by filtration of 70 to 100 ml of the water samples. The HGMFs were placed over a VT capture membrane on agar plates of modified tryptic soy agar (Oxoid) containing bile salts no. 3 (1.5 g/liter), vancomycin (10 μg/ml), and cefsoludin (10 μg/ml) (mTSAVC). The capture membrane was 0.2-μm-pore-size nitrocellulose (Biotrace; Pall Life Sciences) precoated with rabbit anti-VT antibodies (LFZ Guelph) and blocked with PBS–1% gelatin. After the plates were incubated at 37°C for 16 to 18 h, the paired HGMF and capture membranes on each plate were marked by needle punctures for later reorientation, and the HGMF was transferred to a fresh agar plate. The capture membranes were probed with the same detection system that was used in the VT ELISA except that the enzyme conjugate was alkaline phosphatase-labeled rabbit anti-mouse IgG (Jackson ImmunoResearch) and the substrate was nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. The VT-IBs were scored as positive when clearly stained dark purple dots were evident on the probed membrane, as negative when there were no stained dots on the membrane, or as suspicious when faintly stained very small dots were present on the membrane. Those scored as suspicious were observed most frequently when the colony growth on the HGMFs was very dense. Up to eight individual colonies on the top HGMF corresponding in location to stained dots on the immunostained capture membrane were picked and grown for >3 h in 300 μl of mTSBVC at 37°C, and the resulting broths were tested by VT ELISA. VT-positive broths were streaked onto MacConkey agar for single colonies. Individual colonies from these plates were tested by the VT ELISA to confirm their VT status, and up to four VT-positive isolates from each sample were submitted for characterization.

Characterization of isolates. All putative VT-positive isolates from O157 IMS and from VT-IB with or without enrichment were characterized by serotyping and confirmation as VTEC by PCR at the PHAC LFZ *E. coli* Reference Laboratory in Guelph, Ontario, Canada.

Systematic literature review on the state of knowledge of *E. coli* O157:H7 and VTEC detection in surface waters in the peer-reviewed literature. The REA (24) was conducted to capture the current state of the science on culture-based detection methods and reported VTEC prevalences in surface water studies in selected countries between 1990 and January 2013. Studies completed in one of the following countries were included: Canada, United States, United Kingdom, Australia, New Zealand, and countries within the European Union. Articles that were based on molecular methods for VTEC detection (e.g., PCR and quantative PCR [qPCR]), sampled marine water, wastewater, or groundwater exclusively, reported on outbreaks or human illness, and/or quantified only generic *E. coli* were excluded. Articles were identified by searching four electronic databases (PubMed [1950 to 2013], CAB Direct [1900 to 2013], Web of Science [1864 to 2013], and ProQuest [1900 to 2013]) and scanning reference lists of articles. Search terms included *E. coli* O157, *E. coli* O157:H7, detection, culture, surface water, watershed, VTEC, STEC, verotoxigenic, and Shiga-toxin producing *E. coli*. The search strategy incorporated single-term searches as well as iterative combinations of the terms using the Boolean expressions "OR" and "AND." Eligibility assessments for articles were performed independently by two reviewers. Any disagreements between reviewers were resolved by consensus.

Article abstracts were preliminarily assessed based on the above predefined eligibility criteria. Upon receipt of the full-length papers, remaining articles were further examined in more detail to confirm their eligibility. In addition, the reference sections of all selected articles/publications were hand searched to identify any further relevant studies (19). Information was extracted from each included article on (i) culture methods used to investigate the presence of VTEC O157 and other VTEC strains and (ii) prevalence estimates obtained by the authors for VTEC O157 and other VTEC strains in water samples. Variables of interest collected for the culture methods included volume of sample analyzed, medium type, incubation temperature, enumeration capacity, filter use, enrichment steps, immunocapture methods, sensitivity, and specificity. Variables of interest collected for the prevalence estimates included type of water, geographic location, time of collection, collection bottle type, number of samples taken, and prevalence of *E. coli* O157:H7 and other VTEC types. The last search was run on 14 January 2013.

Statistical analyses. All data cleaning and coding were performed in MS Excel 2010. All statistical analyses (including Pearson's chi-squared tests and Cohen's kappa coefficient) were performed in SAS version 9.3 (SAS Institute Inc., Cary, NC).

RESULTS

Detection method comparison. The use of filtration, enrichment, and IMS for the entire surveillance period (from 2006 to 2012) resulted in relatively low prevalence estimates for VTEC O157 in the study watershed (Table 1), with an average of 1.4% (9/657). No consistent trend was observed among sample sites, as sites positive for VTEC O157 varied from year to year (Table 2). Because prevalence was low and sample volume and IMS methods changed from year to year, statistical comparisons of method prevalences by year or site were not performed.

The subsequent comparison of VTEC detection methods was performed on 236 surface water samples collected between July 2010 and December 2012. After testing of the first 94 samples, enrichment in BPW (method 1; Fig. 1) was discontinued because no samples were VT positive in the VT ELISA or yielded VTEC O157 by IMS, whereas six of the same samples enriched in mTSBVC were VT positive in the VT ELISA, another yielded VTEC O157 by IMS after mTSBVC enrichment, and VTEC had been isolated from 33% (31/94) of these samples by direct VT-IB without enrichment.

By the remaining methods, VTEC strains of any serotype, including VTEC O157, were isolated from 32% (75/236) of samples, 30% (72/236) by direct VT-IB without enrichment (method 3) and 3% (7/236) after mTSBVC enrichment plus VT ELISA and VT-IB (method 2b) (Fig. 2). Among these seven samples, two were positive for non-O157 VTEC only by this method, and five also yielded isolates by direct VT-IB, two of which were VTEC O157 positive by all three methods. VTEC O157 was isolated from 4% (10/236) of samples (Fig. 2), six by direct VT-IB only, one by mTSBVC enrichment plus IMS only, one by direct VT-IB and mTSBVC enrichment plus IMS, and two by all three methods.

Several samples were VTEC positive by more than one method (Fig. 2). In some cases, we were not able to isolate VTEC from samples scored as positive or suspicious after initial testing. VTEC strains were isolated from 50% (7/14) of samples scored as positive or suspicious by the VT ELISA after enrichment and from 95% (72/75) of samples after positive or suspicious direct VT-IB.

The direct VT-IB method without broth enrichment, though more effective than other methods of isolation, often resulted in dense growth on the HGMFs. When growth was very dense, immunostained dots were often smaller and less numerous than in samples with less dense growth. Typically, direct VT-IB of 70 to 100 ml of water resulted in fewer than 10 stained dots on each membrane, with recovery of confirmed VTEC isolates from up to 5 of the 8 colonies picked from cells of the corresponding HGMFs. In some cases, initially picked colonies from cells of the HGMF were mixed cultures, requiring a second round of culture and testing of single colonies to obtain pure isolates.

The high frequency of VTEC isolation by direct VT-IB (72/236 samples) compared to the other methods (7/236 samples by method 2b) is significant. In comparison of the results of method 2b (mTSBVC enrichment plus VT ELISA and VT-IB) to the direct VT-IB (method 3) for detection of VTEC, the Cohen's kappa of 0.267 (standard error [SE], 0.031) indicates poor to fair comparability. A comparison of the direct VT-IB (method 3) with mTSBVC enrichment and IMS (method 2a) is similar (Cohen's kappa = 0.275; SE, 0.031). Our results illustrate that the direct immunoblot method is more sensitive than the VT ELISA method, and from a laboratory perspective, it is also a more efficient approach for the detection of VTEC from river water.

Serotypes detected. VTEC isolates identified between 2010 and 2012 belonged to a diverse range of 53 serotypes, and several samples yielded isolates of two or three serotypes. In all, 28 samples (12% of all samples and 37% of 75 VTEC-positive samples) contained one or more VTEC strains belonging to six of the top seven serogroups of human health significance: O26, O103, O111, O121, O145, and O157 (Table 3).

VTEC sample site and season prevalence of VTEC (following the direct immunoblotting method). Between July 2010 and December 2012, VTEC prevalences varied significantly ($P < 0.01$) by sampling site. VTEC detection was more frequent at the sampling location downstream of a wastewater effluent outflow than at other sampling sites, with an overall prevalence of 85% (Table 2). Statistical comparisons did not include the three beach sampling locations due to small sample sizes. However, it is important to note that the beach sites showed the lowest occurrence for VTEC overall. Similar VTEC prevalence was observed among seasons (no significant difference; $P = 0.33$).

Rapid evidence assessment (REA) search and extraction results. To capture the reported prevalences observed by others for surface water VTEC studies, a predefined search strategy conducted in PubMed, CAB Direct, Web of Science, and ProQuest

TABLE 1 VTEC O157 prevalence over the study period, 2006 to 2012

Yr	Laboratory[b]	Sample vol	Test method	No. of samples		VTEC O157 prevalence (%)	Sampling location(s) where VTEC O157 was isolated[a]
				Positive	Tested		
2006	External	500 ml	Filter enrichment in EC broth + IMS O157	2	175	1.1	Conestogo River, Grand River North
2007	External	500 ml	Filter enrichment in EC broth + IMS O157	3	176	1.7	Canagagigue Creek, Grand River (near wastewater outflow)
2008	External	1 liter	Filter enrichment in EC broth + IMS O157	1	100	1.0	Conestogo River
2009	External	1 liter	Filter enrichment in EC broth + IMS O157	0	112	0.0	NA
2010	External	1 liter	Filter enrichment in EC broth + IMS O157	3	94	3.2	Conestogo River, Grand River North, Canagagigue Creek
2010	PHAC LFZ	500 ml	Filter enrichment in mTSBVC + IMS O157 and VT ELISA + VT-IB for all VTEC	2	19	10.5	Canagagigue Creek, Grand River (near wastewater outflow)
		70 ml	Direct VT-IB without broth enrichment				
2011	PHAC LFZ	500 ml	Filter enrichment in mTSBVC + IMS O157 and VT ELISA + VT-IB for all VTEC	2	99	2.0	Grand River (near intake), Grand River North
		70 ml	Direct VT-IB without broth enrichment				
2012	PHAC LFZ	500 ml	Filter enrichment in mTSBVC + IMS O157 and VT ELISA + VT-IB for all VTEC	6	118	5.1	Conestogo River, Grand River North, Grand River (near wastewater outflow)
		70 ml	Direct VT-IB without broth enrichment				
Total, all years				19	893	2.1	
Total, 2010–2012 at PHAC LFZ				10	236	4.2	
Total, external labs				9	657	1.4	

[a] NA, not applicable.
[b] The numbers of positive samples at the PHAC LFZ Laboratory are the combined results of testing the two sample volumes by the corresponding test methods shown in the fourth column from the left.

databases yielded a total of 979 citations. After duplicates were removed, 627 studies remained. Of these, 587 studies were excluded, as they did not meet the eligibility criteria of our study. Two additional studies were excluded because the full-length papers were not available in English.

Upon review of the full-length papers of the remaining 38 studies, another 14 papers were removed, as they did not meet the predefined eligibility criteria. An additional nine papers were removed, as they were summary/consensus reports. A total of 15 studies were identified for final inclusion in the REA. The predetermined data variables of interest were found in 11 of the 15 papers and synthesized. The remaining four studies that reported results using inoculated water samples thus were excluded.

Previously reported prevalence of *E. coli* O157:H7 in surface water in Canadian studies. Six Canadian studies, published after 1990, reported a variety of culture-based VTEC O157 prevalence estimates from several different watersheds across Canada. The reported prevalence estimates from the six studies ranged from 0% to 6.7% in river watershed samples, with a crude combined prevalence of 1.2% (55/4,922) (19–21, 25–27). All of these studies reported use of a combination of filtration, enrichment of filters, and IMS (Table 4).

Previously reported prevalence of *E. coli* O157:H7and VTEC in surface water in U.S. studies. Four reports of studies in the United States, published after 1990, reported a variety of prevalence estimates from three different watersheds in the United

TABLE 2 VTEC O157 and non-O157 prevalence by season and sampling site, July 2010–December 2012

Season or sampling site	No. of samples		
	Collected (2010–2012)	VTEC positive	VTEC prevalence (%)
Seasons			
Spring (March, April, May)	57	22	29
Summer (June, July, August)	84	22	26
Fall (September, October, November)	51	16	31
Winter (December, January, February)	44	15	34
Sampling sites			
Canagagigue Creek	35	10	31
Conestogo River	35	7	20
Grand River (Elora Gorge, summer sampling)	15	4	27
Grand River North	31	4	13
Grand River (near intake)	50	13	26
Grand River (near wastewater outflow)	40	34	85
Laurel Creek Beach (summer sampling)	15	2	13
Shade's Mills Beach (summer sampling)	15	1	7

States. These four studies reported prevalence estimates for VTEC O157 between 0.0% and 54.0% in samples from river watersheds and ponds within agricultural watersheds, between 2002 and 2008 (28–31). In total, these four studies comprise 1,903 water samples, 716 of which were classified as positive for VTEC O157, yielding a prevalence of 37.6% (716/1,903). However, in two studies (28,

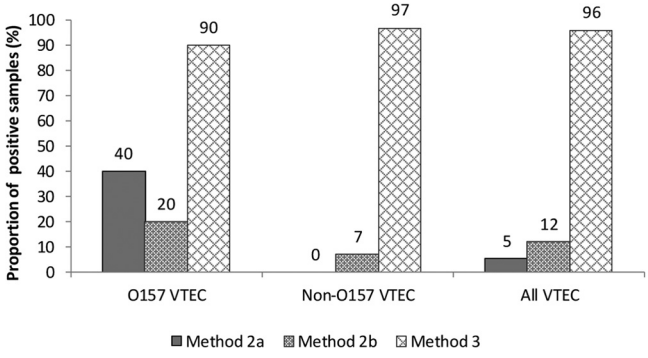

FIG 2 Proportion of water samples positive for O157 VTEC ($n = 10$), non-O157 VTEC ($n = 70$), and all VTEC serotypes ($n = 75$) by three compared isolation methods: method 2a, mTSBVC enrichment plus IMS for O157 VTEC; method 2b, mTSBVC enrichment plus VT ELISA and VT-IB of VT-positive broths; and method 3, direct VT-IB without broth enrichment. Of the 10 VTEC O157-positive samples, one was positive only by method 2a, and nine were positive by method 3, including six by method 3 only, one by methods 2a and 3, and two by methods 2a, 2b, and 3. Of the 70 non-O157 VTEC-positive samples, two were positive only by method 2b, 63 were positive only by method 3, and five were positive by methods 2b and 3. Of the 72 samples positive for all VTEC types by method 3, four contained only VTEC O157, five contained O157 and non-O157 VTEC, and 63 contained only non-O157 VTEC. Of these 72 samples, 15 each yielded VTEC strains of two different serotypes, two each yielded three different serotypes, and the remaining 55 samples each yielded one serotype.

TABLE 3 Prevalence and serotype distribution of O157 and non-O157 VTEC strains, July 2010 to December 2012[a]

Yr	No. of samples		VTEC prevalence (%) (no. positive/no. tested)			VTEC serotypes isolated[b] (no. of samples yielding the serotype)
	Positive for VTEC by isolation	Tested	VTEC O157	Top 7 VTEC serogroups[c]	VTEC (any serotype)	
2010	3	19	11 (2/19)	11 (2/19)	16 (3/19)	O109:NM (1), **O111:H8** (1), **O157:H7** (2)
2011	33	99	2 (2/99)	7 (7/99)	33 (33/99)	O1:H20 (2), **O26:H11** (1), O39:H38 (1), O41:NM (2), O46:H11 (1), O52:H45 (1), O55,128ab:NM (1), O76:H21 (1), O88:NM (1), O91:H14 (2), O96:H19 (1), **O103:H2** (1), O105:H18 (2), O106:NM (1), **O111:H8** (1), **O111:NM** (1), O113:H21 (1), O113:H4 (1), O116:NM (1), **O121:H10** (1), O140:H32 (1), O153:H32 (1), **O157:H7** (2), O168:H? (1), O171:H2 (1), O174:H21 (1), O174:H28 (1), OR:H18 (1), OR:H2 (1), OR:H8 (1) OR:NM (1), O?:H21 (1), O?:NM (1)
2012	39	118	5 (6/118)	16 (19/118)	33 (39/118)	O1:H20 (1), O2:H29 (1), O22:H? (1), **O26:H11** (4), **O26:NM** (2), O36:H42 (2), O39:H38 (1), O55,128ab:NM (1), O79:H7 (1), O91:NM (1), O103:H2 (8), O103:H25 (1), O103:NM (1), O104:H7 (1), O105:H18 (1), O107:H7 (1), O113:H21 (2), O116:NM (1), O121:H19 (1), O126:H27 (1), O128:NM (1), O130:H11 (1), O139:H19 (1), O145:NM (1), O153:NM (1), O157:H7 (6), O158:H23 (1), O168:H8 (1), O174:H21 (1), O174:H8 (1), O179:H8 (1), OR:NM (2)
Total	75	236	4 (10/236)	12 (28/236)	32 (75/236)	

[a] Serotypes were isolated from 72/75 samples by direct immunoblotting without enrichment, from 2/75 samples by only postenrichment immunoblotting, and from 1/75 samples by only postenrichment VTEC O157 immunomagnetic separation. For method details, see the text.

[b] Several positive samples had more than one serotype: 55 samples had one serotype, 15 had two serotypes, and two had three serotypes. VTEC strains isolated from two samples in 2012 were not serotyped. Serotypes in bold belong to the top 7 VTEC serotypes. The total no. of serotypes isolated was 53.

[c] O26, O45, O103, O111, O121, O145, and O157.

TABLE 4 Rapid evidence assessment of reported VTEC O157 and other VTEC prevalence estimates from published surface water studies since 1990

Authors and reference	Water type[a]	Study location	Time period	No. of samples	Vol analyzed	Enumeration capacity	Filtration of water sample	VTEC targets	Isolation technique[b]	Prevalence
Edge et al. (27)	River watersheds	Canada: watersheds of Sumas River, British Columbia; Oldman River, Alberta; South Nation, Ontario; Bras d'Henri and Fourchette Rivers, Quebec	Biweekly sampling over three field seasons, 2005–2007	902	500 ml	No	Yes	VTEC O157	Enrichment + IMS-O157; confirmation of VTEC O157 isolates by PCR, serotyping	VTEC O157: At agriculture sites, 3%; at reference sites, 1%
Heijnen and Medema (32)	Surface water (lakes and wastewater treatment plant effluent and influent)	Netherlands	April-July, 2004	27 surface water; 4 wastewater	100 ml, surface water samples; 10 ml, wastewater samples	Yes	Yes	VTEC O157	Enrichment + IMS-O157; confirmation of VTEC O157 isolates by PCR	VTEC O157 isolation by IMS-O157, surface water, 0/27 (0%) samples; wastewater, 2/4 (50%) samples
Jenkins et al. (30)	Pond in agricultural watershed (pond inflow, within pond, pond outflow)	United States: Bishop Pond, southern Piedmont of northeast Georgia	2006–2007	10 pond inflow; 10 within pond; 10 pond outflow	20 liters	Yes	Yes	VTEC O157	Modified MPN with concn, culture, and isolation; confirmation of putative VTEC O157 by O157 agglutination and PCR for Eae gene	VTEC O157 isolated on 6/10 (60%) sampling times; pond inflow, 4/10 (40%) samples; within pond, 4/10 (40%) samples; pond outflow, 1/10 (10%) samples
Jenkins et al. (31)	Ponds in agricultural watersheds (pond inflow, within pond, pond outflow)	United States: one pond located in the Piedmont and two ponds in the coastal plain of Georgia	February, 2006-August, 2008	17 pond inflow; 17 within pond; 17 pond outflow	20 liters	Yes	Yes	VTEC O157	Modified MPN with concn, culture and isolation. Confirmation of putative VTEC O157 by O157 agglutination and PCR for Eae gene	VTEC O157; pond inflow, 9/17 (53%) samples; within pond: 8/17 (47.1%) samples; pond outflow: 6/17 (35.3%) samples
Johnson et al. (25)	River watershed	Canada: Oldman River watershed, Alberta	1999–2000	1,483	90 ml	No	No	VTEC O157	Enrichment + IMS-O157; confirmation of VTEC O157 isolates by PCR, serotyping	VTEC O157: 13/1,483 (0.9%) samples
Jokinen et al. (19)	River watershed	Canada: Salmon River, British Columbia	September–November, 2004–2006	Site 1, 30 Site 2, 71 Site 3, 31 Site 4, 54	500 ml	No	Yes	VTEC O157	Enrichment + IMS-O157; confirmation of VTEC O157 isolates by PCR, serotyping	VTEC O157; site 1, 2/30 (6.7%) samples 2 (2.8%) samples 1 (3.1%) samples 0 (0.0%) samples
Jokinen et al. (26)	River watershed	Canada: Oldman River watershed, Alberta	July 2005-November 2007	342	500 ml	No	Yes	VTEC O157	Enrichment + IMS-O157; confirmation of VTEC O157 isolates by PCR, serotyping	VTEC O157, 8/342 (2.3%) samples
Shelton et al. (28)	Watershed	United States: Gwynns Falls, Baltimore, MD	July-September, 2002	72	500 ml	No	Yes	*E. coli* O157 serogroup	Enrichment; IM-ECL-O157	33/72 (45.8%) samples positive for *E. coli* O157 by IM-ECL; not confirmed as VTEC O157

Shelton et al. (29)	Watershed	United States: Baltimore metropolitan area watersheds	Weekly sampling, March 2002–August 2004	1,303	100 ml	No	Yes	*E. coli* O157 serogroup; Stx and Eae genes	Enrichment + separate testing of broth by IM-ECL-O157 and by PCR for Stx and Eae genes	*E. coli* O157 by IM-ECL-O157: 50% (652/1,303) samples; *stx* by PCR: 26% (336/1,293) samples; none of 17 *E. coli* O157 isolates had *stx* genes (i.e., non-VTEC)
Wilkes et al. (20)	Watershed	Canada: South Nation River basin	April-November in 2004–2006	823	500 ml	Yes	Yes	VTEC O157	Enrichment + IMS-O157; confirmation of VTEC O157 isolates by PCR, serotyping	VTEC O157: 5/823 (0.6%) samples
Wilkes et al. (21)	Watershed	Canada: South Nation River basin	2004–2008	1,186	500 ml	Yes	Yes	VTEC O157	Enrichment + IMS-O157; confirmation of VTEC O157 isolates by PCR, serotyping	VTEC O157: 15/1,186 (1.3%) samples

[a] Categorization based on classifications made by original paper authors.
[b] IMS-O157, immunomagnetic separation of *E. coli* O157; IM-ECL-O157, immunomagnetic electrochemiluminescent detection of *E. coli* O157.

29), none of the *E. coli* O157 isolates were confirmed as VTEC O157 by further molecular testing for toxin genes.

Previously reported prevalence of VTEC O157 in surface water in EU studies. Only one study from the European Union, published after 1990, was included in this study. Surface water samples, collected in 2004, from lakes and a wastewater treatment plant (influent and effluent) in the Netherlands were assessed for the presence of VTEC O157 using a combination of culture-based detection and PCR confirmation of isolates. The prevalence was 7.4% (2/27) in surface water samples and 50.0% (2/4) in wastewater samples (32).

DISCUSSION

The comparison of methods for detection and isolation of VTEC in this study was prompted by the apparent low prevalence of VTEC O157 found in this watershed between 2006 and 2010 using traditional methods (Table 1) and a desire to address the increasing importance of non-O157 VTEC strains as human pathogens. It quickly became evident that direct filtration and VT-IB of 70 to 100 ml of surface water without filter enrichment was more sensitive for detection and isolation of VTEC O157 than the more common methods based on enrichment of filters from larger volumes and IMS. Nine of the 10 VTEC O157-positive samples were detected by direct filtration without enrichment, compared to four samples after enrichment and IMS. The direct immunoblot method without enrichment was also much more effective than enrichment methods for the isolation of the broader group of VTEC (30% versus 3%). Rather than increasing the sensitivity of detection and isolation of VTEC in surface waters, broth enrichment of water filters appears to reduce recovery rates. The reasons for such differences with and without broth enrichment were not investigated but deserve further study. Contributing factors might include a lack of sensitivity of the VT ELISA and/or that VTEC populations are reduced during broth enrichment. The VT ELISA has proven very sensitive in testing cultures of meats, produce, and animal and human feces. In optimizing our assay, polymyxin B extraction disproportionately increased background and negative-control signals, resulting in false-negative results (R. P. Johnson, unpublished data), and hence was not adopted. Mitomycin C, which induces VT production, may improve the assay sensitivity, though it acts primarily on VT2- and not VT1-producing strains (33). Perhaps more likely is that the VTEC populations are reduced during enrichment by predatory microorganisms such as bacteriophages and protozoa (34). Verotoxin-encoding bacteriophages are abundant in water, sewage, and cattle feces (43), as are virulent VTEC O157 phages in cattle environments (35). Replicative growth of VTEC during broth enrichment is more likely to favor phage predation of VTEC than growth on filters on agar plates, as is used for direct immunoblotting. The presence of bacteriophages may also contribute to the failure to isolate VTEC from VT-IB of 7/14 broth enrichments and 3/75 direct VT-IBs. They may be picked along with a VTEC colony, resulting in lysis during the broth incubation step for confirmation of VT status of the colony. Additionally, abundant VT-encoding bacteriophages may unstably lysogenize *E. coli* in water, leading to prompt loss of the VT-encoding bacteriophage on colony subculture (36). Nevertheless, VTEC isolation from direct VT-IB was unsuccessful in only 3/75 samples, and it appears that the direct immunoblotting method would help to improve our understanding of the occurrence and potential sources of O157 and non-O157 VTEC in the environment.

What this study also demonstrated through the use of a rapid

evidence assessment is that many studies that have attempted to understand the potential risks of VTEC presence in surface water systems (i) used some type of IMS method and (ii) focused only on VTEC O157. A systematic review, or rapid evidence assessment, can be used to inform study design, conduct meta-analyses, or contextualize study findings (41). In this case, the REA was used to identify the current gap in detection methodology related to identifying VTEC in surface water systems in North America and the European Union. In this study, the formal nature of the REA allowed the critical appraisal of the search terms, and reproducibility in future years, to measure how much the science has advanced and to quantify any potential shift in our understanding of enteric disease transmission and exposures of public health significance.

When the literature was examined using the structured review process, it became evident that few studies have attempted to estimate, with isolation-based methods, the prevalence of VTEC O157 in surface water, which ranged from 1% (13/1,483) to 50% (1/2), and few reported the detection of non-O157 VTEC serotypes (Table 4). Of the methods reported in the studies that were captured in the final review, most were based on a combination of filtration and IMS or capture. Various approaches to IMS were taken, and many used an enrichment step to further improve method sensitivity, with varying success. Large variations in sample size were reported, though the most extensive sampling effort, by Johnson et al. (25), resulted in a VTEC O157 prevalence of 1% in a surface water system in Alberta, Canada, using IMS.

The study watershed, the Grand River, is located in southwestern Ontario, Canada, and is impacted by both point and nonpoint sources of fecal material, including human, wildlife, and agriculture (39), suggesting that VTEC could be present at least some of the time in the river. By improving the sensitivity of the detection method employed, prevalence estimates for VTEC O157 in our study increased. Overall, the prevalence of VTEC was approximately 32%, yet sampling site influenced this value. The samples collected in the river downstream of a wastewater treatment plant outflow were positive for VTEC 85% of the time. This finding does not represent a new threat to water treatment in the watershed.

Seasonal trends in surface water prevalence of a number of enteric pathogens, including *Campylobacter*, *Salmonella*, and *Cryptosporidium*, in Canadian river systems have previously been reported (19, 21, 25, 38). While the trend was not statistically significant, samples were more frequently positive for VTEC in the winter and spring months than in the fall and summer months. This study was not designed to elucidate predictors for seasonality of this pathogen, but others suggest that some common land use management practices, as well as the seasonal dynamics of human infections, which contribute to wastewater loading levels, could contribute to seasonal trends (39), although the seasonality in water appears to be different from the seasonality in human infections, which shows a summer peak (42). Knowledge of seasonal trends at the watershed level could help inform interventions that will minimize impacts on the watercourse through the control of land use activities such as manure runoff to surface waters and septic tank leakage, as well as highlighting the need for additional treatment of wastewater. The use of a more sensitive detection method will help to inform future efforts to understand the effects of seasonality and land use practices on the prevalence of VTEC in surface water systems.

This study illustrates the utility of an integrated surveillance system framework like FoodNet Canada to support research hypothesis formulation and develop new detection methods while informing the broad goal of understanding public health risks from exposures to food, water, animals, and the environment. The results illustrate how a change in a detection method can help enhance our understanding of the presence of human-pathogenic strains of *E. coli* in surface waters and inform efforts in watershed surveillance, with a farm-to-fork and source-to-tap perspective.

These data, collected over a 7-year period, demonstrate consistent trends from year to year. Samples were positive at all sampling locations in the watershed, illustrating that both urban and agricultural streams, the larger river body, and inland freshwater beaches are being impacted by fecal wastes that carry VTEC. The study also indicates that testing by direct VT-IB without broth enrichment is an efficient approach that can significantly improve test sensitivity and provide a more comprehensive understanding of potential environmental sources and serotypes of VTEC present in surface water.

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