An evaluation of methods for the isolation of *Yersinia enterocolitica* from surface waters in the Grand River watershed

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ABSTRACT

Yersinia enterocolitica is a foodborne pathogen, but the importance of water as a route of exposure for human infection is not well known. *Y. enterocolitica* isolation methods were developed primarily for food and clinical samples, and may not be effective for use with environmental samples. The objective of this study was to assess the recovery of *Y. enterocolitica* from surface water used for drinking water treatment. Four enrichment broths and an alkaline treatment protocol were compared for the isolation of *Y. enterocolitica* bioserogroup 4/O:3 spiked into surface water samples. Results showed that the methods tested were not effective for the recovery of *Y. enterocolitica*, primarily due to inadequate inhibition of interfering background microorganisms. Using one method that showed the most potential for recovery, *Yersinia* spp. were isolated from rivers in southwestern Ontario, Canada, over a 17-month period. Of 200 samples analysed, *Yersinia* spp. were isolated from 52 samples. All river isolates belonged to non-pathogenic sub-groups, including *Y. enterocolitica* biotype 1A, *Y. aldovae*, *Y. bercovieri*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii* and *Y. mollaretii*. Results of this study show that method improvements are required to more fully understand the role of water as a source of clinically important *Yersinia* strains.

Key words | Grand River watershed, isolation, occurrence, recovery, surface water, *Yersinia enterocolitica*

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INTRODUCTION

Yersinia enterocolitica is an emerging waterborne pathogen (Theron & Cloete 2002; Sharma *et al.* 2003) that has the potential to cause gastrointestinal disease and other diseases in humans (Bottone 1997). There are six *Y. enterocolitica* biogroups and more than 50 serogroups. Human infections are more commonly associated with bioserogroups 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3 and 4/O:3 (Fredriksson-Ahomaa *et al.* 2006). There are also several *Yersinia* spp. that are highly similar to *Y. enterocolitica*, including *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. aldovae*, *Y. rohdei*, *Y. bercovieri* and *Y. mollaretii* (Sulakvelidze 2000). These are often referred to as *Y. enterocolitica*-like species and are considered to be non-pathogenic (Sulakvelidze 2000; Fredriksson-Ahomaa & Korkeala 2003).

Y. enterocolitica is more commonly associated with foodborne illness, and pigs are a major reservoir of human pathogenic strains (McNally *et al.* 2004; Fredriksson-Ahomaa *et al.* 2006). *Y. enterocolitica* may also cause waterborne disease, and a study by Ostroff *et al.* (1994) found that drinking untreated water was a risk factor for *Y. enterocolitica* infection. Pathogenic *Y. enterocolitica*

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have been implicated in a few cases of waterborne illness in humans (Lassen 1972; Keet 1974; Christensen 1979; Thompson & Gravel 1986); however most cases of *Y. enterocolitica* infection are sporadic and a source is rarely identified (Bottone 1997). This has been attributed to difficulties associated with isolating the organism, in particular pathogenic strains (Fredriksson-Ahomaa & Korkeala 2003). Studies that have investigated the occurrence of *Yersinia* spp. in environmental waters have recovered mostly non-pathogenic species (Agbalika *et al.* 1985; Brennhovd *et al.* 1992; Leclerc *et al.* 2002; Fredriksson-Ahomaa & Korkeala 2003). Similarly, pathogenic strains are not often isolated from foods (Fredriksson-Ahomaa *et al.* 2006).

Several different methods are available for isolating *Y. enterocolitica*, and were developed for use with food and clinical samples. These methods have not been tested extensively with water, and may not be effective for environmental samples (Fredriksson-Ahomaa *et al.* 2007). There are three major problems encountered when attempting to isolate *Y. enterocolitica* from the environment. First, there tends to be a high concentration of organisms in environmental samples that interfere with isolation procedures, and *Y. enterocolitica* tend to grow poorly in competition with other organisms (Schiemann & Olson 1984; Calvo *et al.* 1986*b*). Second, the methods are not selective for pathogenic strains of *Y. enterocolitica* and will also isolate non-pathogenic strains of *Yersinia*, which appear to be common in the environment (Fredriksson-Ahomaa & Korkeala 2003). Third, a virulence plasmid, which imparts certain phenotypic characteristics that are used to identify pathogenic strains, is sometimes lost during culturing steps (Blais & Phillippe 1995). It is important to be aware of these challenges when evaluating and implementing *Y. enterocolitica* isolation methods.

In this study we compared four different enrichment methods for the recovery of a clinically significant strain of *Y. enterocolitica* added to Grand River surface water samples. A method using enrichment in modified tryptic soy broth (mTSB) and plating on CIN agar, with and without an alkaline treatment, was selected to examine the occurrence of naturally occurring *Yersinia* in the Grand River watershed. The watershed spans an area close to 7,000 km^2 and is the largest watershed in southern Ontario. It is used as a drinking water source and for recreational activities, and is influenced by both urban and agricultural activities, including municipal wastewater discharges and livestock production (Dorner *et al.* 2004). A study of the watershed by Dorner *et al.* (2007) detected several different enteric pathogens that are common in surface waters; however *Y. enterocolitica* was not among the pathogens surveyed. The Grand River watershed is also the sentinel site for a multi-partner enteric pathogen surveillance initiative (C-EnterNet) facilitated by the Public Health Agency of Canada, whose objective is to detect trends in human enteric disease and sources of exposure in defined communities across Canada. Research on pathogen occurrence in environmental sources is important to identify water as a potential exposure route for human disease. Results of this study contribute to a better understanding of the method limitations and prevalence of *Yersinia* in water sources.

MATERIALS AND METHODS

Y. enterocolitica inoculum

Y. enterocolitica bioserogroup 4/O:3 (ATCC 700822) was maintained on tryptic soy agar (TSA) (BD) and grown in tryptic soy broth (TSB) (BD) at 28°C for 16–20 h. The culture was enumerated using a Petroff-Hausser counting chamber (Hausser Scientific) and a Zeiss Axioskop 2 microscope. Serial dilutions were prepared in phosphate-buffered water (PBW) (0.3 mM KH_2PO_4, 2 mM $MgCl_2{\cdot}H_2O$, pH 7.2), and were used to inoculate enrichment broths in the recovery experiments. Viable plate counts were also determined by plating serial dilutions on TSA, followed by incubation at 28°C for 16–20 h.

Yersinia isolation methods

Four different enrichment methods were compared: (1) Irgasan-Ticarcillin-Chlorate (ITC) (Wauters *et al.* 1988); (2) Luria-Bertani-Bile Salts-Irgasan (LB-BSI) broth (Hussein *et al.* 2001); (3) modified tryptic soy broth (mTSB) (Bhaduri *et al.* 1997); and (4) cold enrichment in phosphate-buffered saline (PBS) (Greenwood *et al.* 1975). ITC broth (Fluka) and

PBS (EMD Chemicals) were purchased from commercial suppliers. LB-BSI broth was prepared as described by Hussein *et al.* (2001). mTSB was prepared as described by Bhaduri *et al.* (1997). Ten ml aliquots of each broth were added to 20 ml glass test tubes, and then inoculated with *Y. enterocolitica* or surface water as described below. Each broth was incubated as described in Table 1. For LB-BSI and mTSB, 4 $\mu g\,ml^{-1}$ of irgasan (final concentration; Sigma-Aldrich) was added after 24 h of incubation.

Following the enrichment period, each method was conducted with and without an alkaline treatment (Johnson 1998). For the alkaline treatment, 0.5 ml of enrichment culture was added to 4.5 ml of alkaline solution (0.25% potassium hydroxide, 0.5% sodium chloride), mixed by vortexing for 2–3 s, then plated on Cefsulodin-Irgasan-Novobiocin (CIN) agar plates. CIN agar was prepared using CIN agar base containing *Yersinia* antimicrobic supplement CN (BD). CIN agar plates were incubated at 28°C for 24 h, and observed using a stereomicroscope. CIN agar is most commonly used for isolating *Y. enterocolitica*, as it can better inhibit non-*Yersinia* organisms and has a higher confirmation rate when compared with other selective agars (Head *et al.* 1982; Schiemann 1983*b*). We also found that CIN agar could inhibit and differentiate Gram-negative bacteria better than SSDC agar, and that *Y. enterocolitica* from a variety of biogroups and serogroups could grow well on CIN agar (Cheyne 2008).

Recovery experiments

The enrichment methods were compared in three separate recovery experiments (A, B and C). Experiment A compared the growth of *Y. enterocolitica* ATCC 700822 in each broth without the addition of surface water, and experiment B assessed the growth of background microorganisms from surface water. Experiment C compared the recovery of *Y. enterocolitica* ATCC 700822 added together with surface water from the Grand River. Each experiment was performed in duplicate unless indicated otherwise.

Table 1 | Enrichment methods summary

Enrichment broth	Incubation temperature (°C)	Incubation period (days)	Reference
ITC	25	2	Wauters *et al.* (1988)
LB-BSI	12	3	Hussein *et al.* (2001)
mTSB	12	3	Bhaduri *et al.* (1997)
PBS	4	14	Greenwood *et al.* (1975)

For recovery experiment A, 100 μl of a 1,000 $CFU\,ml^{-1}$ inoculum of *Y. enterocolitica* ATCC 700822 (100 CFU total) was added to 10 ml of each enrichment broth, with the exception of PBS to which 100 μl of a 3,500 $CFU\,ml^{-1}$ inoculum (350 CFU total) was added. Preliminary experiments showed that, owing to limited cell growth, PBS required a higher inoculum to enable subsequent enumeration. Each broth was incubated as described above, then serially diluted in PBW and spread plated on CIN agar. Concurrently, enrichment cultures were alkaline treated (as described above), serially diluted in PBW and spread plated on CIN agar.

For recovery experiment B, each broth was inoculated with cells collected by filtering 500 ml of surface water. Surface water samples were taken from the Grand River just upstream of a drinking water treatment plant intake in Kitchener, Ontario (see Figure 1). Water was collected in 25 l plastic carboys and stored at 4°C for up to 3 days. Cells were collected from 500 ml of surface water by filtration through a 0.45 μm GN-6 Metricel® (47 mm diameter) filter (Pall Corporation) using a sterile filter unit (Nalgene) and a vacuum pump under ~500 mmHg pressure. The filter was rolled such that sample residue was on the inside, and then placed into 10 ml of each broth. Broths were incubated, treated and plated as described in experiment A. Colonies on each CIN agar plate were counted as either presumptive *Yersinia* or non-*Yersinia* (background) colonies. Presumptive *Yersinia* colonies were ~0.5–2 mm in diameter, with a red bulls-eye centre surrounded by an entire or undulated, transparent edge. All presumptive *Yersinia* colonies were restreaked on TSA plates and screened with identification tests, including growth on MacConkey agar plates (BD), oxidase reaction, urea utilization on urea agar slants (OXOID), citrate utilization on Simmons citrate agar slants (OXOID) and glucose/lactose fermentation, gas and H_2S production on Kligler iron agar slants (OXOID). Isolates were subsequently screened with the BIOLOG MicroLog Microbial Identification System, as described by the manufacturer.

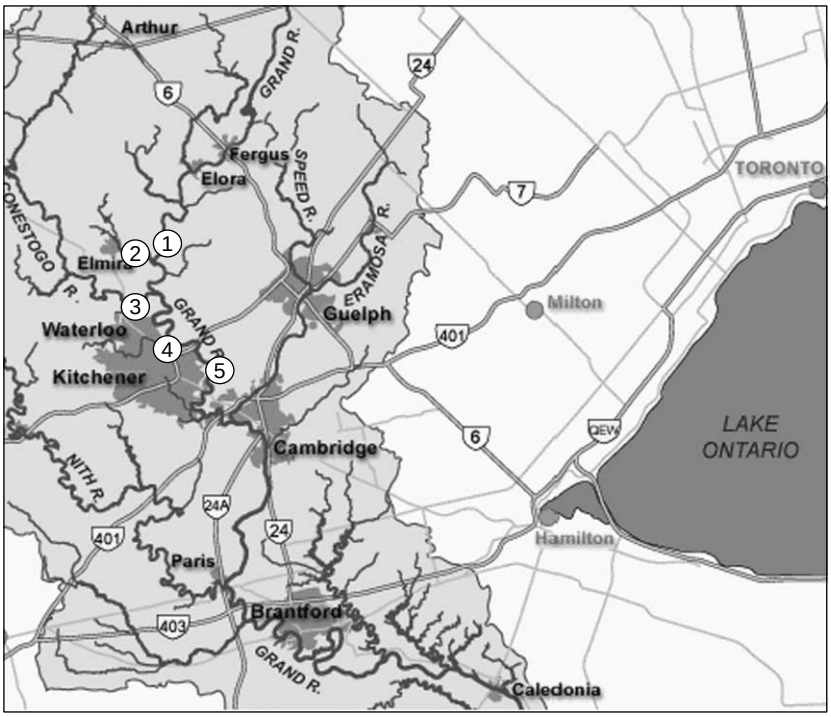

Figure 1 | Grand River watershed sampling locations. Surface water was collected from five sampling locations: (1) Grand River (North), (2) Canagagigue Creek, (3) Conestogo River, (4) Grand River (WW) downstream of a wastewater treatment plant effluent, (5) Grand River (intake) upstream of a drinking water treatment plant intake. Figure courtesy of the Grand River Conservation Authority.

In recovery experiment C, we compared the ability of each enrichment method to recover *Y. enterocolitica* ATCC 700822 added together with cells filtered from Grand River water. Only three of the four enrichment methods were compared: ITC, LB-BSI and mTSB. For each method, one tube containing 10 ml broth was inoculated with cells collected by filtering 500 ml of surface water (as described in experiment B). A second tube containing 10 ml broth was inoculated both with cells collected from 500 ml of surface water together with 100 µl of a 2,000 CFU ml^{-1} *Y. enterocolitica* ATCC 700822 inoculum (200 CFU total). All cultures were incubated, treated, plated and counted as described in experiment B.

Grand River survey

Based on the results of the recovery experiments, the mTSB enrichment method, with and without an alkaline treatment, was used to survey surface water from the Grand River watershed for the presence of *Yersinia* spp. Surface water samples were collected from five locations in the watershed (Figure 1) every other week from April 2006 to August 2007, and when possible following precipitation events including heavy rainfall and spring snowmelt. Sites included a point in the Grand River north of Kitchener-Waterloo, the Canagagigue Creek and Conestogo River just before each tributary meets the Grand River, and in the Grand River just downstream of a wastewater treatment plant effluent and just upstream of a drinking water treatment plant intake. Using 2006–2007 data provided by the Water Survey of Canada (www.wsc.ec.gc.ca), average flow rates were 1.4 $m^3 s^{-1}$ in Canagagigue Creek, 10 $m^3 s^{-1}$ in the Conestogo River, 15 $m^3 s^{-1}$ at Grand River North and 23 $m^3 s^{-1}$ in the Grand River at the drinking water treatment plant intake. Water samples were collected 2–3 m from the river edge and 10–20 cm below surface from a fast flowing area. Surface water was collected in sterile 1-l polypropylene, wide-mouth bottles (VWR) containing 0.5 ml of 0.1 N sodium thiosulfate, transported on ice and stored at 4°C. Samples were analysed within 24 h of collection.

Cells were collected by filtering 500 ml of surface water as described above. Filters were placed into test tubes

containing 10 ml of mTSB and incubated as described above with shaking at 100 rpm. After the enrichment period, 50 μl of non-alkaline treated enrichment broth and 100 μl of alkaline treated broth were each transferred to CIN agar plates and streaked for isolated colonies. Plates were incubated at 28°C for 16–24 h, and observed for colonies displaying typical *Yersinia* morphology. Up to eight representative colonies were selected as presumptive *Yersinia* isolates and screened with the identification tests listed in recovery experiment B. Confirmed *Yersinia* isolates were subtyped by the Ontario Ministry of Health and Long-Term Care, Toronto Regional Public Health Laboratory (Etobicoke, Ontario). The API-20E Biochemical Identification kit (bioMérieux) was used to identify the *Yersinia* species. Isolates were also biotyped based on methods developed by Wauters *et al.* (1987) and serotyped based on methods developed by Aleksić & Bockemühl (1984) and Wauters *et al.* (1991).

RESULTS AND DISCUSSION

Comparison of enrichment methods

Four different enrichment methods were compared to determine which method resulted in maximum recovery of *Y. enterocolitica* added to surface water samples. Cold enrichment (4°C) in PBS has often been used to isolate *Y. enterocolitica*, which is a psychrotrophic bacterium (Fredriksson-Ahomaa & Korkeala 2003); however this method requires a long incubation time. In contrast, ITC broth is incubated at 25°C for only 2 days, but may not provide good recovery of all *Y. enterocolitica* subtypes (Wauters *et al.* 1988). We also tested mTSB and LB-BSI, which both utilize delayed addition of the antibiotic irgasan and an incubation temperature of 12°C. Schiemann & Olson (1984) suggested that using an incubation temperature close to 15°C would enable *Y. enterocolitica* to compete better with other bacteria while reducing the incubation time required by most cold incubation methods. The laboratory strain used in the recovery experiments was *Y. enterocolitica* bioserotype 4/O:3 (ATCC 700822), which is the sub-group responsible for most human cases of yersiniosis (Bottone 1999). This strain was also chosen because it contains the pYV virulence plasmid (Cheyne 2008), which is considered crucial to imparting full virulence to *Y. enterocolitica* (Schiemann & Devenish 1982; Heesemann *et al.* 1984) and can also influence the growth of *Y. enterocolitica* in selective media (Logue *et al.* 2000, 2006).

In experiment A, each enrichment method was evaluated for the ability to support the growth of a laboratory *Y. enterocolitica* strain. Results show that *Y. enterocolitica* grew in most enrichment methods tested (Table 2). *Y. enterocolitica* concentrations at the end of the incubation period (without an alkaline treatment) were the highest in ITC (8.3 log CFU ml^{-1}), followed by mTSB (6.3 log CFU ml^{-1}), LB-BSI (4.2 log CFU ml^{-1}), and were lowest in PBS (2.0 log CFU ml^{-1}). Since the starting concentration in PBS was 1.5 log CFU ml^{-1}, there was a 0.5 log increase following incubation at 4°C. Given that PBS does not contain nutrients, growth may be attributed to the addition of exponential phase cells or the carry over of nutrients from the diluted TSB culture used as inoculum. Another study found that *Y. enterocolitica* could grow by over 1.0 log after 72 hours in sterile distilled water at 4°C, and viable cell concentrations were maintained for an additional 6 days (Highsmith *et al.* 1977). Results in Table 2 also show that *Y. enterocolitica* remained at high levels following a post-enrichment alkaline treatment. Almost no difference was observed between alkaline treated and non-treated cultures. Overall, results from experiment A show that *Y. enterocolitica* grows best in ITC broth probably because this broth was incubated at a higher temperature (25°C) compared with mTSB and LB-BSI (12°C). However, an ideal enrichment method must also effectively inhibit the growth of non-*Yersinia* bacteria.

In experiment B, each enrichment method was evaluated for the ability to inhibit the growth of non-*Yersinia* (background) microorganisms from the Grand River. Similar to other heavily impacted surface waters, the Grand River contains a high concentration of microorganisms. In water samples taken at this location over the sampling period ($n = 37$) the median heterotrophic plate count and total direct cell count were 5.7 log CFU ml^{-1} and 6.2 log cells ml^{-1}, respectively. Results from experiment B (Table 2) show that background microorganisms were not inhibited following enrichment and plating on CIN agar. At the end of the incubation period, the concentration of background

Table 2 | Results from recovery experiments A and B, which separately tested the recovery using each enrichment method of either *Y. enterocolitica* ATCC 700822 (experiment A) or background microorganisms from surface water (experiment B). Results show the target cell concentration (log CFU ml^{-1}) in each broth at the end of the incubation period

		Experiment A*	Experiment B
Media†	Alkaline treatment	*Y. enterocolitica* ATCC 700822 (log CFU ml^{-1}) Mean (range)	Background microorganisms (log CFU ml^{-1}) Mean (range)
ITC	−	8.3 (7.4–8.5)	8.5 (8.2–8.7)
	+	8.2 (7.4–8.5)	8.5 (8.4–8.6)
mTSB	−	6.3 (5.4–6.6)	7.9 (7.3–8.1)
	+	6.2 (3.8–6.6)	4.6 (3.9–4.9)
LB-BSI	−	4.2 (3.9–4.3)	8.0 (7.9–8.0)
	+	3.9 (3.0–4.1)	6.1 (3.8–6.4)
PBS	−	2.0 (1.8–2.1)	6.0 (5.8–6.2)
	+	2.0 (1.5–2.5)‡	4.0 (3.9–4.0)

*The starting concentration of *Y. enterocolitica* ATCC 700822 was 1.0 log CFU ml^{-1}, except for PBS which was 1.5 log CFU ml^{-1}.
†For each medium $n = 2$, except for mTSB $n = 3$.
‡Approximate values based on low CFU counts.

microorganisms that grew on CIN agar (without an alkaline treatment) was highest in ITC (8.5 log CFU ml^{-1}), followed by mTSB (7.9 log CFU ml^{-1}), LB-BSI (8.0 log CFU ml^{-1}), and was lowest in PBS (6.0 log CFU ml^{-1}). Although an alkaline treatment did not reduce the concentration of background microorganisms in ITC, it was effective in reducing counts by 3.3 log in mTSB, 1.9 log in LB-BSI and 2.0 log in PBS. Environmental (non-spiked) *Yersinia* strains were not detected in this experiment using any of the enrichment methods. The results showed that background microorganisms from the Grand River were at lower concentrations in mTSB and LB-BSI compared with ITC. Again, this is probably due to the lower incubation temperature (12°C) used in the mTSB and LB-BSI methods compared with the ITC method (25°C). High background concentrations in PBS after cold incubation confirm that other bacteria in the Grand River can also survive for an extended period at cold temperatures. This challenge was noted previously by Fredriksson- Ahomaa & Korkeala (2003). Also, the filtered river water would have resulted in the addition of nutrients to the PBS, and this may have allowed microorganisms to grow. Based on these results, it was concluded that cold enrichment in PBS was not an appropriate method for the recovery of *Yersinia* in surface water samples. Consequently, PBS was not tested in recovery experiment C.

In experiment C, we tested the recovery of *Y. enterocolitica* ATCC 700822 added together with microorganisms from the Grand River. Results showed that *Y. enterocolitica* ATCC 700822 was not recovered using any of the enrichment methods tested (Table 3). Two *Yersinia* colonies were detected on plates from spiked samples of LB-BSI combined with an alkaline treatment; however it was determined that these isolates were not *Y. enterocolitica* ATCC 700822, but an environmental *Yersinia* strain from the river sample. Environmental *Yersinia* strains were detected in non-spiked samples that were inoculated only with microorganisms from river water, showing that *Yersinia* are present in the Grand River. It is important to note that in Table 3, *Yersinia* concentrations are based on very low CFU counts, meaning that only one or two confirmed *Yersinia* colonies were detected on one plate in a dilution series. Normally, less dilute samples are plated to obtain higher and more reliable CFU counts. However, in this case, agar plates from lower dilutions were completely overgrown with background microorganisms from the Grand River. For this reason, the *Y. enterocolitica* ATCC 700822 strain that was spiked into samples may have been present following enrichment and plating, but at concentrations below that of the background microorganisms and consequently could not be detected.

The concentrations of background microorganisms were relatively similar in non-spiked and spiked broths (Table 3), and these results were similar to those in recovery experiment B (Table 2). Background colony

Table 3 | Results of recovery experiment C, which tested each enrichment method for recovery of *Y. enterocolitica* ATCC 700822 added together with cells from surface water (spiked samples). Controls (non-spiked samples) contained cells from surface water only, without *Y. enterocolitica* inoculum. Results show cell concentrations (log CFU ml^{-1}) in each broth at the end of the incubation period

		Non-spiked samples		Spiked samples*	
Media†	Alkaline treatment	Environmental *Yersinia* spp. (log CFU ml^{-1})	Background microorganisms (log CFU ml^{-1})	*Y. enterocolitica* (ATCC 700822) (log CFU ml^{-1})	Background microorganisms (log CFU ml^{-1})
ITC	−	N.D.‡–6§	9.0 (8.9–9.1)	N.D.	8.8 (8.7–8.9)
	+	N.D.	8.9 (8.7–9.0)	N.D.	8.8 (8.7–8.9)
mTSB	−	N.D.–6§	7.9 (7.9–7.9)	N.D.	8.3 (8.1–8.3)
	+	N.D.	6.5 (6.2–6.7)	N.D.	6.7 (6.4–6.9)
LB-BSI	−	N.D.	7.7 (7.6–7.7)	N.D.	7.8 (7.8–7.9)
	+	N.D.–4§	6.4 (6.2–6.5)	N.D.	6.0 (6.0–6.1)

*The starting concentration of *Y. enterocolitica* ATCC 700822 in spiked samples was 1.3 log CFU ml^{-1}
†For each medium $n = 2$.
‡N.D. = Not Detected.
§Approximate values based on low CFU counts.

concentrations in non-alkaline treated samples were highest in ITC (8.8–9.0 log CFU ml^{-1}), followed by mTSB (7.9–8.3 log CFU ml^{-1}) and LB-BSI (7.7–7.8 log CFU ml^{-1}). As in experiment B, the alkaline treatment was effective at reducing the background counts in mTSB and LB-BSI, but not in ITC (Table 3). For the mTSB cultures, the alkaline treatment was less effective in experiment C compared with experiment B. This is probably a result of difficulties in reproducing the alkaline treatment consistently each time, primarily due to the short alkaline exposure time of only 2–3 seconds. Inconsistent results using a post-enrichment alkaline treatment have also been noted in previous studies (Schiemann 1983*a*). Also, it has previously been demonstrated that resistance to alkaline conditions varies between *Y. enterocolitica* strains (Doyle & Hugdahl 1983). Nonetheless, results suggest that the use of an alkaline treatment can inhibit the growth of background microorganisms from surface water samples. Plating both alkaline treated and non-treated enrichment cultures on *Yersinia* isolation agar is recommended in the USDA/FSIS Microbiology Laboratory Guidebook, which outlines methods for isolation and identification of pathogenic *Yersinia enterocolitica* from meat and poultry products (Johnson 1998).

The objective of this experiment was to enumerate levels of *Yersinia* and non-*Yersinia* organisms in the enriched samples. However, the high concentration of non-*Yersinia* (background) microorganisms from the Grand River that grew in the enrichment broths and on CIN agar made it difficult to detect and enumerate low levels of *Yersinia*. Furthermore, many of the background colonies on CIN agar looked very similar to *Yersinia* colonies, and consequently many presumptive isolates were not confirmed as *Yersinia* after identification tests were conducted. Although CIN agar has been identified as the preferred selective agar for isolating *Y. enterocolitica* (Head *et al.* 1982; Schiemann 1983*b*), it could not sufficiently select for and differentiate *Y. enterocolitica* from other bacteria in surface waters tested in this study.

Results from experiment C did not reveal that any one enrichment method was better at recovering *Y. enterocolitica* spiked into surface water. The problems encountered with *Y. enterocolitica* recovery can probably be attributed to the survival and growth of non-target background microorganisms from the Grand River in the enrichment broths and on CIN agar. In addition, *Y. enterocolitica* may have been inhibited by other microorganisms from the river water growing in the enrichment broths. *Y. enterocolitica* tend to grow poorly in competition with other organisms (Schiemann & Olson 1984; Calvo *et al.* 1986*b*). One study found that *Y. enterocolitica* growth may be impeded by bacteriocin-like agents produced by *Y. frederiksenii*, *Y. kristensenii* and *Y. intermedia* (Calvo *et al.* 1986*a*), organisms that have been detected in surface waters (Fredriksson-Ahomaa & Korkeala 2003) and were found

to be present in the Grand River, as discussed below. Results indicate that the culture-based methods tested may not be effective for recovering low levels of *Y. enterocolitica* from the Grand River, and would probably not be effective with other surface water matrices. It is possible that these methods may be useful in samples containing higher *Y. enterocolitica* concentrations or lower levels of background microorganisms. However the purpose of this study was to examine the recovery of low pathogen concentrations that are typically found in surface water samples.

Conclusions can be drawn by comparing the results of experiments A and B. Table 2 shows that the concentrations of background bacteria were usually higher than that of *Y. enterocolitica* except in mTSB treated with an alkaline solution. With this enrichment method, *Y. enterocolitica* grew to concentrations that were higher (just over 1.5 log) than background bacteria. In ITC, LB-BSI and PBS, background concentrations (without an alkaline treatment) were about 0.2, 3.8 and 4.0 log higher, respectively, than *Y. enterocolitica* concentrations. Even after an alkaline treatment, background concentrations were 0.3 log (ITC), 2.2 log (LB-BSI) and 1.7 log (PBS) higher than *Y. enterocolitica*. These results suggest that the mTSB enrichment method in combination with an alkaline treatment may perform better than the other methods tested in isolating *Y. enterocolitica* from surface waters.

Grand River survey

Samples from the Grand River watershed (Figure 1) were analysed for the presence of *Yersinia* using enrichment in mTSB followed by plating on CIN agar. Between April 2006 and August 2007, 200 surface water samples were collected and analysed. *Yersinia* spp. were detected in 52 samples (26%) (Table 4) and *Y. enterocolitica* isolates were detected in eight samples (4%). In previous studies that surveyed surface water, *Yersinia* were isolated more frequently during colder months of the year (Meadows & Snudden 1982; Fukushima *et al.* 1984; Massa *et al.* 1988); however, no seasonal trends were observed in *Yersinia* isolation rates from the Grand River, nor were there trends associated with precipitation events (data not shown).

From the 52 water samples that were positive for *Yersinia*, a total of 97 *Yersinia* isolates were collected (Table 5). The distribution of these isolates among sampling locations is outlined in Table 4. It should be noted that multiple isolates of the same subtype were sometimes isolated from one sample, and it is possible these isolates originated from a single strain in the river. The majority of isolates (89%) were *Y. enterocolitica*-like species, including *Y. aldovae* (11%), *Y. bercovieri* (9%), *Y. frederiksenii* (16%), *Y. intermedia* (42%), *Y. kristensenii* (2%) and *Y. mollaretii* (7%). These identifications are based on results from testing isolates with the API-20E Biochemical Identification kit. Isolates were initially tested using the BIOLOG system, and species identity showed a 61% match with results obtained using the API-20E system.

The *Yersinia* spp. isolated from the Grand River are typically considered non-pathogenic species. *Y. enterocolitica* strains accounted for 11% of the *Yersinia* isolates. All of the *Y. enterocolitica* isolates belonged to biogroup 1A, which is also considered to be a non-pathogenic biotype. *Y. frederiksenii*, *Y. intermedia* and *Y. kristensenii* have all been isolated from water previously (Shayegani *et al.* 1981;

Table 4 | The occurrence of *Yersinia* spp. in samples taken from the Grand River watershed. Samples were collected from April 2006 to August 2007 and sample locations are shown and described in Figure 1

Sampling location	Samples analysed	Samples positive for *Yersinia*	*Yersinia* isolates*	*Y. enterocolitica* isolates
Grand River (North)	40	15	32	4
Canagagigue Creek	40	11	17	4
Conestogo River	40	10	14	1
Grand River (WW)	40	9	16	0
Grand River (intake)	40	7	18	2
All locations	200	52	97	11

*_Yersinia_ isolate counts include _Y. enterocolitica_ isolates.

Table 5 | Characterization of *Yersinia* strains isolated from the Grand River watershed

Species	Biotype	Serotype	Number of isolates
Y. enterocolitica	1A	O:5	2
Y. enterocolitica	1A	O:5,27	1
Y. enterocolitica	1A	O:7,8	4
Y. enterocolitica	1A	O:7,13	1
Y. enterocolitica	1A	O:41,43	1
Y. enterocolitica	1A	O:rough	1
Y. enterocolitica	1A	O:Untypeable	1
Y. aldovae			11
Y. bercovieri			9
Y. frederiksenii			16
Y. intermedia	1		37
Y. intermedia	4		4
Y. kristensenii			2
Y. mollaretii			7
All species			97

Fukushima *et al.* 1984; Agbalika *et al.* 1985; Aleksić & Bockemühl 1988; Massa *et al.* 1988; Brennhovd *et al.* 1992; Arvanitidou *et al.* 1994; Schaffter & Parriaux 2002; Falcão *et al.* 2004). However, *Y. bercovieri* and *Y. mollaretii* have never previously been isolated from water. *Y. enterocolitica* 1/O:5, 1/O:5,27 and 1/O:7,8 have also previously been isolated from water (Fukushima *et al.* 1984; Aleksić & Bockemühl 1988; Massa *et al.* 1988; Arvanitidou *et al.* 1994), but these studies did not distinguish between biotype 1A and 1B. A more recent study by Falcão *et al.* (2004) characterized several *Y. enterocolitica* isolates from water, and detected bioserotype 1A/O:5, which was among the bioserotypes isolated from the Grand River.

Although the *Yersinia* strains isolated from the Grand River watershed are typically considered non-pathogenic, subtyping schemes may not accurately predict human pathogenicity. *Y. enterocolitica* 1A (Tennant *et al.* 2003) and all *Y. enterocolitica*-like species except *Y. aldovae* (Sulakvelidze 2000) have been isolated from patients displaying gastrointestinal disease symptoms. Virulence-associated genes found in pathogenic *Y. enterocolitica* subtypes have been detected in *Y. enterocolitica* biotype 1A strains (Falcão *et al.* 2006; Bhagat & Virdi 2007). Tennant *et al.* (2003) lists *Y. enterocolitica* 1A clinical isolates that have been associated with enteric disease, and this includes 1A strains of serotype O:5 and O:7,8, both of which were isolated in our survey of the Grand River (Table 5). In a study related to our project, *Y. enterocolitica* 1A O:6,30, 1A O:rough and *Y. intermedia* were obtained from separate patients in the Region of Waterloo in 2007 (Public Health Agency of Canada 2008), although it is unclear whether this finding is incidental and perhaps not the aetiological agent in these patients. All other *Yersinia* strains isolated from clinical samples were *Y. enterocolitica* bioserotype 4/O:3. Although *Y. enterocolitica* 1A O:6,30 was not isolated from river water in our study, biotype 1A O:rough and *Y. intermedia* were isolated from water.

Pigs are a major reservoir for *Y. enterocolitica* strains of clinical importance to humans (Fredriksson-Ahomaa *et al.* 2006). In the same study related to our current research, *Y. enterocolitica* were isolated from pig faeces from farms in the Grand River watershed in 2005 to 2007. *Y. enterocolitica* were isolated from 6% of samples (Public Health Agency of Canada 2006, 2007, 2008). While the majority of the strains isolated were *Y. enterocolitica* 4/O:3, *Y. enterocolitica* biotype 1A strains were also isolated, including one *Y. enterocolitica* 1A/O:5 strain. *Y. enterocolitica* 1A/O:5 strains were also isolated from Grand River surface water (Table 5). Since close to 80% of the land in the watershed is farmed and pigs are the second most prevalent form of livestock found in the watershed (Dorner *et al.* 2004), it seems possible that *Yersinia* may be entering surface water through agricultural run-off. However, information on occurrence in other animals, including wildlife, is still needed to evaluate all possible sources of *Yersinia* in the watershed.

CONCLUSIONS

This study demonstrates that current culture-based methods are not sufficiently optimized for isolating pathogenic *Y. enterocolitica* from surface water samples. Culture-based methods need to be improved to isolate low levels of *Y. enterocolitica* from a surface water matrix containing diverse bacterial species. The poor sensitivity of current methods suggests that previously published culture-based surveys have probably underestimated the prevalence of

Y. enterocolitica in water. Despite the limitations of these methods, *Y. enterocolitica* 1A and other *Yersinia* spp. were successfully isolated in surface water from the Grand River. While the isolated strains are generally considered non-pathogenic, reports have implicated these strains in human gastrointestinal cases. Further studies using the *Yersinia* strains isolated from the Grand River are needed to assess their risk to human health. Prior to this study, occurrence data for *Yersinia* was not available for this watershed, which is used both as a drinking water source and for recreational activities.

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