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

Bo M. Cheyne, Michele I. Van Dyke, William B. Anderson and Peter M. Huck

**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*

**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*
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 2005). 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. 1986b). 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 2005). Third, a virulence plasmid, which imparts certain phenotypic characteristics that are used to identify pathogenic strains, is sometimes lost during culturing steps (Blais & Philippe 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 km² 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) (0.5 mM KH₂PO₄, 2 mM MgCl₂·H₂O, 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 μg 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 antimicrobial 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 1983b). 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.

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 H₂S production on Kligler iron agar slants (OXOID). Isolates were subsequently screened with the BIOLOG MicroLog Microbial Identification System, as described by the manufacturer.

### Table 1 | Enrichment methods summary

<table>
<thead>
<tr>
<th>Enrichment broth</th>
<th>Incubation temperature (°C)</th>
<th>Incubation period (days)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITC</td>
<td>25</td>
<td>2</td>
<td>Wauters et al. (1988)</td>
</tr>
<tr>
<td>LB-BSI</td>
<td>12</td>
<td>3</td>
<td>Hussein et al. (2001)</td>
</tr>
<tr>
<td>mTSB</td>
<td>12</td>
<td>3</td>
<td>Bhaduri et al. (1997)</td>
</tr>
<tr>
<td>PBS</td>
<td>4</td>
<td>14</td>
<td>Greenwood et al. (1975)</td>
</tr>
</tbody>
</table>
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 Canagagique 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 m³ s⁻¹ in Canagagique Creek, 10 m³ s⁻¹ in the Conestogo River, 15 m³ s⁻¹ at Grand River North and 23 m³ 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 2005); 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
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).

### Table 2

<table>
<thead>
<tr>
<th>Media†</th>
<th>Alkaline treatment</th>
<th>Experiment A* Y. enterocolitica ATCC 700822 (log CFU ml(^{-1})) Mean (range)</th>
<th>Experiment B Background microorganisms (log CFU ml(^{-1})) Mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITC</td>
<td>–</td>
<td>8.3 (7.4–8.5)</td>
<td>8.5 (8.2–8.7)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8.2 (7.4–8.5)</td>
<td>8.5 (8.4–8.6)</td>
</tr>
<tr>
<td>mTSB</td>
<td>–</td>
<td>6.3 (5.4–6.6)</td>
<td>7.9 (7.5–8.1)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6.2 (3.8–6.6)</td>
<td>4.6 (3.9–4.9)</td>
</tr>
<tr>
<td>LB-BSI</td>
<td>–</td>
<td>4.2 (3.9–4.3)</td>
<td>8.0 (7.9–8.0)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.9 (3.0–4.1)</td>
<td>6.1 (5.8–6.4)</td>
</tr>
<tr>
<td>PBS</td>
<td>–</td>
<td>2.0 (1.8–2.1)</td>
<td>6.0 (5.8–6.2)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.0 (1.5–2.5)</td>
<td>4.0 (3.9–4.0)</td>
</tr>
</tbody>
</table>

*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.
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.

<table>
<thead>
<tr>
<th>Media†</th>
<th>Alkaline treatment</th>
<th>Environmental <em>Yersinia</em> spp. (log CFU ml−1)</th>
<th>Background microorganisms (log CFU ml−1)</th>
<th>Spiked samples‡</th>
<th>Background microorganisms (log CFU ml−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITC</td>
<td>−</td>
<td>N.D.‡ – 6§</td>
<td>9.0 (8.9–9.1)</td>
<td>N.D.</td>
<td>8.8 (8.7–8.9)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>N.D.</td>
<td>8.9 (8.7–9.0)</td>
<td>N.D.</td>
<td>8.8 (8.7–8.9)</td>
</tr>
<tr>
<td>mTSB</td>
<td>−</td>
<td>N.D. – 6§</td>
<td>7.9 (7.9–7.9)</td>
<td>N.D.</td>
<td>8.3 (8.1–8.3)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>N.D.</td>
<td>6.5 (6.2–6.7)</td>
<td>N.D.</td>
<td>6.7 (6.4–6.9)</td>
</tr>
<tr>
<td>LB-BSI</td>
<td>−</td>
<td>N.D.</td>
<td>7.7 (7.6–7.7)</td>
<td>N.D.</td>
<td>7.8 (7.8–7.9)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>N.D. – 4§</td>
<td>6.4 (6.2–6.5)</td>
<td>N.D.</td>
<td>6.0 (6.0–6.1)</td>
</tr>
</tbody>
</table>

†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.
*a*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 1983a). 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 1985b), 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. 1986b). 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. 1986a), 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 \textit{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 \textit{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 \textit{Y. enterocolitica} except in mTSB treated with an alkaline solution. With this enrichment method, \textit{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 \textit{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 \textit{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 \textit{Y. enterocolitica} from surface waters.

\section*{Grand River survey}

Samples from the Grand River watershed (Figure 1) were analysed for the presence of \textit{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. \textit{Yersinia} spp. were detected in 52 samples (26\%) (Table 4) and \textit{Y. enterocolitica} isolates were detected in eight samples (4\%). In previous studies that surveyed surface water, \textit{Yersinia} were isolated more frequently during colder months of the year (Meadows \& Snudden 1982; Fukushima \textit{et al.} 1984; Massa \textit{et al.} 1988); however, no seasonal trends were observed in \textit{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 \textit{Yersinia}, a total of 97 \textit{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 \textit{Y. enterocolitica}-like species, including \textit{Y. aldovae} (11\%), \textit{Y. bercovieri} (9\%), \textit{Y. frederiksenii} (16\%), \textit{Y. intermedia} (42\%), \textit{Y. kristensenii} (2\%) and \textit{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 \textit{Yersinia} spp. isolated from the Grand River are typically considered non-pathogenic species. \textit{Y. enterocolitica} strains accounted for 11\% of the \textit{Yersinia} isolates. All of the \textit{Y. enterocolitica} isolates belonged to biogroup 1A, which is also considered to be a non-pathogenic biotype. \textit{Y. frederiksenii}, \textit{Y. intermedia} and \textit{Y. kristensenii} have all been isolated from water previously (Shayegani \textit{et al.} 1981; Meadows \& Snudden 1982; Fukushima \textit{et al.} 1984; Massa \textit{et al.} 1988;). The occurrence of \textit{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.

<table>
<thead>
<tr>
<th>Sampling location</th>
<th>Samples analysed</th>
<th>Samples positive for Yersinia</th>
<th>Yersinia isolates(^\ast)</th>
<th>Y. enterocolitica isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grand River (North)</td>
<td>40</td>
<td>15</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>Canagagigue Creek</td>
<td>40</td>
<td>11</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>Conestogo River</td>
<td>40</td>
<td>10</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Grand River (WW)</td>
<td>40</td>
<td>9</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Grand River (intake)</td>
<td>40</td>
<td>7</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>All locations</td>
<td>200</td>
<td>52</td>
<td>97</td>
<td>11</td>
</tr>
</tbody>
</table>

\(^\ast\)Yersinia isolate counts include \textit{Y. enterocolitica} isolates.
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. 2005) 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. (2005) 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.

ACKNOWLEDGEMENTS

Special thanks to Vanessa Morton and Nicole McLellan for technical assistance, and to Cynthia Hallé for water quality data (University of Waterloo). The authors would like to acknowledge the Public Health Agency of Canada (C-EnterNet) and the Ontario Ministry of Health and Long-Term Care, Toronto Regional Public Health Laboratory for subtyping analysis of the Yersinia isolates. Our funding partners were: the Canadian Water Network, the Public Health Agency of Canada, the Natural Sciences and Engineering Research Council of Canada (NSERC) and Partners of the NSERC Chair in Water Treatment (http://www.civil.uwaterloo.ca/watertreatment/). The current Chair partners include: American Water Canada Corp., Associated Engineering Group Ltd, the cities of Brantford, Guelph, Hamilton, Ottawa and Toronto, Conestoga-Rovers & Associates Limited, EPCOR Water Services, GE Water & Process Technologies (Zenon), Lake Huron and Elgin Area Water Supply Systems, the Ontario Clean Water Agency (OCWA), RAL Engineering Ltd, the Region of Durham, the Regional Municipalities of Niagara and Waterloo, and Stantec Consulting Ltd.

REFERENCES


Schiemann, D. A. 1983b Comparison of enrichment and plating media for recovery of virulent strains of Yersinia enterocolitica from inoculated beef stew. J. Food Protect. 46(11), 957–983.


Characteristics of *Yersinia enterocolitica* and related species
isolated from human, animal, and environmental sources.

Sulakvelidze, A. 2000 *Yersiniae* other than *Y. enterocolitica*,
*Y. pseudotuberculosis*, and *Y. pestis*: the ignored species.

Tennant, S. M., Grant, T. H. & Robins-Browne, R. M. 2003
Pathogenicity of *Yersinia enterocolitica* biotype 1A.

Theron, J. & Cloete, T. E. 2002 Emerging waterborne infections:
contributing factors, agents, and detection tools.

Thompson, J. S. & Gravel, M. J. 1986 Family outbreak of
gastroenteritis due to *Yersinia enterocolitica* serotype 0:3 from

Wauters, G., Kandolo, K. & Janssens, M. 1987 Revised biogrouping

Wauters, G., Goossens, V., Janssens, M. & Vandepitte, J. 1988 New
enrichment method for isolation of pathogenic *Yersinia enterocolitica*

and flagellar antigens of *Yersinia enterocolitica* and related

First received 18 July 2008; accepted in revised form 11 October 2008. Available online May 2009