# The detection of *Yersinia enterocolitica* in surface water by quantitative PCR amplification of the *ail* and *yadA* genes

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

## ABSTRACT

*Yersinia enterocolitica* has been detected in surface water, and drinking untreated water is a risk factor for infection. PCR-based methods have been used to detect *Y. enterocolitica* in various sample types, but quantitative studies have not been conducted in water. In this study, quantitative PCR (qPCR)-based methods targeting the *Yersinia* virulence genes *ail* and *yadA* were used to survey the Grand River watershed in southern Ontario, Canada. Initial testing of reference strains showed that *ail* and *yadA* PCR assays were specific for pathogenic biotypes of *Y. enterocolitica*; however the genes were also detected in one clinical *Yersinia intermedia* isolate. A survey of surface water from the Grand River watershed showed that both genes were detected at five sampling locations, with the *ail* and *yadA* genes detected in 38 and 21% of samples, respectively. Both genes were detected more frequently at colder water temperatures. A screening of *Yersinia* strains isolated from the watershed showed that the *ail* gene was detected in three *Y. enterocolitica* 1A/O:5 isolates. Results of this study show that *Yersinia* virulence genes were commonly detected in a watershed used as a source of drinking water, and that the occurrence of these genes was seasonal.

**Key words** | biotype 1A, PCR, surface water, virulence genes, watershed, *Yersinia*

**Bo M. Cheyne**
**Michele I. Van Dyke** (corresponding author)
**William B. Anderson**
**Peter M. Huck**
Department of Civil and Environmental
    Engineering,
University of Waterloo,
200 University Avenue West,
Waterloo Ontario,
Canada N2L 3G1
Tel.: (519) 888-4567 ext. 36193
Fax: (519) 746-7499
E-mail: *mvandyke@uwaterloo.ca*

## INTRODUCTION

*Yersinia enterocolitica* is an emerging waterborne pathogen (Sharma *et al.* 2003) and a well-known foodborne pathogen (Fredriksson-Ahomaa & Korkeala 2003). Illness caused by *Y. enterocolitica* infection can result in a wide variety of disease symptoms, including those usually associated with gastrointestinal disease such as fever, abdominal pain and diarrhoea (Bottone 1997). However, the consequences of infection can be more serious, particularly in sensitive populations (Bottone 1997; Wanger 2007). Pigs have been identified as a major reservoir of human pathogenic strains (McNally *et al.* 2004; Fredriksson-Ahomaa *et al.* 2006). While most cases of *Y. enterocolitica* infection are sporadic and a source is

rarely identified (Bottone 1997), *Y. enterocolitica* has been implicated in waterborne illness. Human cases of disease caused by *Y. enterocolitica* were linked to the consumption of water from untreated wells (Lassen 1972; Christensen 1979; Thompson & Gravel 1986) and surface water (Keet 1974). Untreated water used in a tofu manufacturing plant also caused an outbreak of yersiniosis (Tacket *et al.* 1985). Furthermore, drinking untreated water was shown to be a risk factor for *Y. enterocolitica* infections (Ostroff *et al.* 1994; Saebo *et al.* 1994; Satterthwaite *et al.* 1999). While these studies indicate that water can be route of transmission for yersiniosis, the source of most enteric disease incidents is rarely identified, indicating a need to improve

surveillance and reporting for disease occurrence and transmission routes.

Surveys for *Y. enterocolitica* in surface water, well water and other water sources have been conducted. Culture-based surveys for *Y. enterocolitica* in water have usually resulted in low occurrence rates (Shayegani *et al.* 1981; Meadows & Snudden 1982; Vajdic 1985; Massa *et al.* 1988; Gonul & Karapinar 1991; Brennhovd *et al.* 1992; Arvanitidou *et al.* 1994; Sandery *et al.* 1996; Schaffter & Parriaux 2002; Cheyne *et al.* 2009), although higher prevalence rates have been reported (Langeland 1983; Weagent & Kaysner 1983; Fukushima *et al.* 1984). In most cases, non-pathogenic *Yersinia* strains were more frequently isolated, including *Yersinia frederiksenii*, *Y. intermedia*, *Yersinia kristensenii* and non-pathogenic *Y. enterocolitica* subtypes. Pathogenic strains have been isolated from water in only a small number of studies (Fukushima *et al.* 1984; Sandery *et al.* 1996; Falcão *et al.* 2004). Investigations of pig tissues and food have found that the prevalence of pathogenic *Y. enterocolitica* was higher using PCR-based methods compared with culture-based methods (Fredriksson-Ahomaa *et al.* 2000; Johannessen *et al.* 2000; Vishnubhatla *et al.* 2000; Boyapalle *et al.* 2001; Bhaduri *et al.* 2005; Cocolin & Comi 2005; Thisted Lambertz & Danielsson-Tham 2005; Fredriksson-Ahomaa *et al.* 2006). One study that examined surface waters for pathogenic *Y. enterocolitica* showed detection rates of 1% and 10% using culture-based and PCR-based methods, respectively (Sandery *et al.* 1996). However, there have been limited studies using molecular-based approaches to evaluate the occurrence of *Y. enterocolitica* in environmental waters. Consequently, this route of transmission has not yet been thoroughly investigated.

The objective of this study was to develop and evaluate quantitative PCR (qPCR)-based methods for detecting *Y. enterocolitica* virulence genes in surface water samples. There are a number of genes involved in *Y. enterocolitica* virulence pathways (Revell & Miller 2001). The chromosomal *ail* gene plays a role in the attachment and invasion of host cells (Bottone 1997), and has been found primarily in *Y. enterocolitica* serotypes associated with disease (Miller *et al.* 1989; Revell & Miller 2001; Howard *et al.* 2006). The *yadA* gene is located on the pYV virulence plasmid and codes for a protein that promotes adherence to mucus layers, attachment to host cells and enhances serum

resistance (Bottone 1997; Cornelis *et al.* 1998), and is only associated with pathogenic subtypes of *Y. enterocolitica* (Robins-Browne *et al.* 1989; Fredriksson-Ahomaa *et al.* 2006). In this paper we describe the development and evaluation of qPCR assays that target the *ail* and *yadA* genes to assess the prevalence of pathogenic *Y. enterocolitica* in surface water from the Grand River watershed.

The Grand River watershed is located in southern Ontario, Canada. It provides part of the drinking water supply for approximately 500,000 people, and is also used for recreational activities (Dorner *et al.* 2004). This system is one of the most heavily impacted watersheds in Canada, receiving inputs from both agricultural and urban activities (Dorner *et al.* 2004), and a number of enteric pathogens typically found in surface waters have been previously detected (Dorner *et al.* 2007). This research provides information for an emerging waterborne pathogen that is not well characterized in water sources. The provision of data on pathogen occurrence in source waters is needed to better understand environmental contributions to human disease risk.

## MATERIALS AND METHODS

### DNA extraction from pure cultures

*Yersinia* strains were obtained from the Toronto Central Public Health Laboratory at the Ontario Agency for Health Protection and Promotion (Etobicoke, Ontario), the American Type Culture Collection (ATCC), or were isolated by our group from the Grand River watershed (Cheyne *et al.* 2009). *Pseudomonas aeruginosa* UG2Lr was provided by Drs H. Lee and J. T. Trevors, University of Guelph (Guelph, Ontario). *Yersinia* spp. were grown in tryptic soy broth (TSB) (BD Biosciences) at 28°C and non-*Yersinia* spp. in nutrient broth (BD Biosciences) at 37°C for 16–20 h. Genomic DNA was extracted from 1 ml of broth culture using the Qiagen DNeasy blood and tissue kit according to the manufacturer's instructions.

### Quantitative PCR assays

The primers and probe for the *ail* gene target (Table 1) were designed by Bhaduri *et al.* (2005) (the authors were

**Table 1** | Primers and probes for the *ail*, *yadA* and *luxB* PCR assays; oligonucleotides for the *ail* assay were designed by Bhaduri *et al.* (2005)

| Oligonucleotide | Sequence |
| --- | --- |
| *ail* forward primer | 5′-GGTCATGGTGATGTTGATTACTATTCA-3′ |
| *ail* reverse primer | 5′-CGGCCCCCAGTAATCCATA-3′ |
| *ail* probe | 5′-[FAM]-CATCTTTCCGCATCAACGAATATGTTAGC-[BHQ1]-3′ |
| *yadA* forward primer | 5′-GTATCCATTGGTCATGAAAGCCTT-3′ |
| *yadA* reverse primer | 5′-CTTTCTTTAATTGCGCGACATTCA-3′ |
| *yadA* probe | 5′-[FAM]-TTGACACATCTTGCGGCTGGCACT-[BHQ1]-3′ |
| *luxB* forward primer | 5′-GGGTACTGCCATCCAAACAATGA-3′ |
| *luxB* reverse primer | 5′-TTCTTTGCTCGTCGCATTCACA-3′ |
| *luxB* probe | 5′-[HEX]-CGCAGGACCGCCTTCAGTGAACGC-[BHQ1]-3′ |

contacted to obtain the correct sequence for the *ail* reverse primer). The *yadA* primers and probe (Table 1) were designed using Beacon Designer 2.1 software (BioRad) and gene sequence data from the National Center for Biotechnology Information (NCBI). Primers and probes were purchased from Sigma-Genosys. Probes were 5′-labelled with 6-carboxyfluorescein (FAM) and 3′-labelled with Black Hole Quencher 1 (BHQ1).

Reaction mixtures for the PCR assays contained (per 50 μl); DNA template, 300 nM forward primer, 300 nM reverse primer, 100 nM probe, 3.5 mM $MgCl_2$, 1 × PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl), 1.25 U Jumpstart Taq polymerase, and 200 μM dNTPs. All PCR reagents were purchased from Sigma-Aldrich. Cycling conditions for the *ail* assay were: one cycle at 95°C for 3 min; 50 cycles at 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 10 min. Cycling conditions for the *yadA* assay were: one cycle at 95°C for 3 min; 50 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 10 min. Reactions were performed in a 96-well plate (Bio-Rad) using a Bio-Rad iCycler iQ Real-Time PCR Detection System with version 3.1 software for data analysis. Each assay included DNA standards to generate standard curves and also negative (no template) controls.

Standard curves for the *ail* PCR assay were made using serial dilutions of genomic DNA from *Y. enterocolitica* ATCC 700822. *Y. enterocolitica* cultures used for DNA extraction were enumerated by direct microscopic cell count using SYBR Gold (Invitrogen), resulting in genomic DNA standards measured as cells per reaction. Standard curves for the *yadA* PCR assay were generated using a DNA

oligonucleotide, synthesized by Integrated DNA Technologies, designed to match the amplified region of a *yadA* gene sequence (NCBI accession number X13882) plus 30 additional base pairs at both the 5′ and 3′ end. Oligonucleotide DNA standards were measured as gene copies per reaction. Standard curve reproducibility was tested by analysing in duplicate each of two separate dilution series. The detection limit for each PCR assay was determined by testing a dilution series of DNA standards at 10 replicates per dilution.

## Surface water collection

Between March 2005 and August 2007, surface water samples were collected at five sites in the Grand River watershed (Figure 1). 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 the Canagagigue Creek, $10 \, m^3 \, s^{-1}$ in the Conestogo River, $15 \, m^3 \, s^{-1}$ at the Grand River North site and $23 \, m^3 \, s^{-1}$ in the Grand River at the drinking water intake. Samples were taken every other week and when possible following heavy rainfall and spring snow melt events, and were collected 2–3 m from the river's edge and 10–20 cm below the surface at a fast flowing area. Water was collected in sterile 1-l polypropylene, widemouth bottles (VWR) and transported on ice. Samples were stored at 4°C and analysed within 24 h of collection. Water temperature was measured in the field. Total *Escherichia coli* concentrations were determined as described by Ciebin *et al.* (1995). Turbidity was analysed using a Hach 2100P turbidity meter, and ammonia and
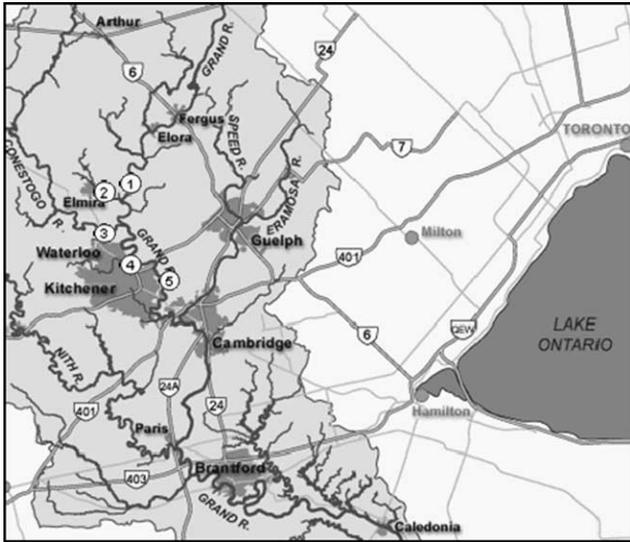
**Figure 1** | Grand River watershed sampling locations. Surface water was collected at five sites: (1) Grand River (North), (2) Canagagigue Creek, (3) Conestogo River, (4) Grand River downstream of a wastewater treatment plant effluent (WW), (5) Grand River upstream of a drinking water treatment plant intake (Intake). *Source*: Grand River Conservation Authority www.grandriver.ca

nitrate were monitored using the Hach NitraVer 5 and AmVer Test'N Tube reagent kits.

## DNA extraction and PCR amplification of surface water samples

The surface water DNA extraction protocol was based on methods by Pitcher *et al.* (1989) and Boom *et al.* (1990). For each sample, a 1.5 l volume was filtered using two 0.45 $\mu$m (47 mm) Supor membrane filters (Pall Corporation). Each filter was placed in a separate 3 ml centrifuge tube containing 1.5 ml of guanidine isothiocyanate (GITC) extraction buffer (5 M GITC, 0.1 M ethylenediaminetetraacetic acid [pH 8.0], 5 g l$^{-1}$ N-laurylsarcosine), then stored at $-20°C$ for up to 2 weeks. The tubes were then rotated vertically for at least 1 h at room temperature and centrifuged at 15,000 $\times$ *g* for 5 min. The supernatant was passed through DNeasy purification columns (Qiagen), washed and eluted in 200 $\mu$l of AE buffer according to the manufacturer's instructions. DNA preparations were stored at $-80°C$ until analysis. For each sampling event, a negative control was prepared by using the same DNA extraction and purification reagents as surface water samples.

DNA from surface water samples was analysed using the quantitative *ail* and *yadA* PCR assays described above.

A 13.3 $\mu$l volume of DNA, corresponding to 100 ml of surface water, was added to each reaction. DNA standards were analysed in duplicate to generate standard curves. PCR inhibition was tested using an external PCR assay, in which the *luxB* gene from *P. aeruginosa* UG2Lr (Fleming *et al.* 1994) was amplified together with DNA from surface water samples. An external inhibition assay was used because internal assays were found to prevent amplification of the target gene at low concentrations (data not shown). The *luxB* primers and probe (Table 1) were designed using Beacon Designer 2.1 software (Bio-Rad) and the *luxB* gene sequence (NCBI accession number E12410). The *luxB* probe was 5'-labelled with hexachloro-6-carboxyfluorescein (HEX) and 3'-labelled with BHQ1 (Sigma-Genosys). Genomic DNA containing the *luxB* gene was extracted from *P. aeruginosa* UG2Lr as described above. Each 50 $\mu$l *luxB* assay reaction was prepared as described above and contained both *luxB* DNA template (5 $\times$ 10$^2$ cells/reaction) and DNA from a Grand River sample. Control reactions containing only the *luxB* DNA template were prepared in triplicate. Cycling conditions were: one cycle at 95°C for 3 min; 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 10 min. If the *luxB* amplification signal was inhibited, as indicated by a reduced $C_T$ value or reduced signal intensity, the surface water DNA sample was repurified using the Qiagen DNeasy purification kit and tested again for inhibition.

## Recovery of *Y. enterocolitica* from surface water

*Y. enterocolitica* (ATCC 700822) inoculum was prepared in 10 ml TSB and incubated at 28°C for 24 h. A series of five dilutions ranging from 10$^2$ to 10$^5$ cells ml$^{-1}$ were prepared in phosphate-buffered water (PBW) (0.3 mM KH$_2$PO$_4$, 2 mM MgCl$_2$·H$_2$O, pH 7.2), and 1 ml of each dilution was spiked into 1 l of surface water from the Grand River. The entire sample was processed by filtration using the surface water DNA extraction protocol. DNA was also extracted from 1 l of unspiked water from the Grand River. Controls were prepared from 1 ml of each inoculum dilution using the Qiagen DNeasy kit. All treatments and controls were done in triplicate; 20 $\mu$l aliquots of each DNA sample were analysed in duplicate using the *ail* qPCR assay.

## Confirmation of PCR results

The *ail* and *yadA* PCR amplification products from selected Grand River water samples were analysed by agarose gel electrophoresis; 5 µl aliquots from each reaction were analysed on 2% agarose gels stained with ethidium bromide. Selected amplification products were also cloned using TOPO® cloning vectors and One Shot® TOP10 competent cells (Invitrogen). One or two cloned plasmids from each sample were purified using the PureLink™ Quick Plasmid miniprep kit (Invitrogen), and sequence analysis was done at Laboratory Services, University of Guelph, Guelph, Ontario.

## RESULTS

### DNA standards

DNA standards for the *ail* and *yadA* PCR assays resulted in reproducible standard curves with $R^2$ values of 0.98 or greater and slopes between 3.2 and 3.6. According to Zhang & Fang (2006), a reliable qPCR standard curve should have an $R^2$ value greater than 0.95 and a slope between 3.0 and 3.9. The detection limit was established using the criteria of Behets *et al.* (2007), and for both the *ail* and *yadA* PCR assay was between 5 and 8 cells per reaction (data not shown). When the *yadA* assay was initially used with genomic DNA standards from *Y. enterocolitica* ATCC 700822 (serotype O:3), threshold cycle ($C_T$) values were significantly higher than those of the *ail* assay. This may have been due to lower specificity of the assay for this particular strain, or possibly due to plasmid loss. *Y. enterocolitica* can lose the pYV virulence plasmid during culturing or storage (Blais & Phillippe 1995), and other researchers that had difficulties in obtaining sufficient pYV plasmid attributed the problem to plasmid loss (Robins-Browne *et al.* 1989). A synthetic oligonucleotide DNA standard derived from a different O:3 strain resulted in $C_T$ values similar to those of the *ail* PCR assay, and this standard was used for all *yadA* PCR assays.

**Table 2** | Occurrence of *ail* and *yadA* genes in *Yersinia* reference strains obtained from culture collections; all of the reference strains were isolated from clinical or porcine sources

| *Yersinia* species | Biogroup | Serogroup | Source[*] | *ail* gene | *yadA* gene |
|---|---|---|---|---|---|
| *Y. enterocolitica* | 1A | O:7,13 | OAHPP | − | − |
| *Y. enterocolitica* | 1A | O:41,42 | OAHPP | − | − |
| *Y. enterocolitica* | 1B | O:8 | ATCC 9610 | − | − |
| *Y. enterocolitica* | 1B | O:8 | OAHPP | + | + |
| *Y. enterocolitica* | 1B | O:8 | OAHPP | + | + |
| *Y. enterocolitica* | 1B | O:21 | OAHPP | + | − |
| *Y. enterocolitica* | 2 | O:5,27 | OAHPP | + | + |
| *Y. enterocolitica* | 2 | O:9 | OAHPP | + | + |
| *Y. enterocolitica* | 3 | O:1,2,3 | OAHPP | + | + |
| *Y. enterocolitica* | 4 | O:3 | OAHPP | + | + |
| *Y. enterocolitica* | 4 | O:3 | ATCC 700822 | + | +[†] |
| *Y. pseudotuberculosis* | | III | OAHPP | − | + |
| *Y. pseudotuberculosis* | | I | OAHPP | − | + |
| *Y. frederiksenii* | | | OAHPP | − | − |
| *Y. intermedia* | | | OAHPP | + | + |
| *Y. kristensenii* | | | OAHPP | − | − |
| *Y. mollaretii* | | | OAHPP | − | − |
| *Y. rohdei* | | | OAHPP | − | − |

[*]*Yersinia* strains were obtained from either the Central Public Health Laboratory at the Ontario Agency for Health Protection and Promotion (OAHPP) or the American Type Culture Collection (ATCC).
[†]Weak signal.

## Primer and probe specificity

The ail and yadA assays were tested on Yersinia strains obtained from culture collections, and included strains from clinical and porcine samples. Results for Y. enterocolitica reference strains show that the ail gene was not present in the two biotype 1A isolates tested, but the gene was present in all pathogenic biotypes (1B, 2, 3, 4) except one 1B/O:8 strain (ATCC 9610) (Table 2). The results for yadA gene detection in Y. enterocolitica were similar, except this gene was not detected in two 1B strains (serotypes O:8 and O:21) and was detected only weakly in the ATCC 700822 strain (serotype O:3). Both the ail and the yadA gene were present in the one clinical Y. intermedia isolate, and only the yadA gene was detected in Yersinia pseudotuberculosis strains. Neither gene was observed in the other Yersinia species tested (Table 2).

The detection of ail and yadA genes was also performed on Yersinia strains that were isolated from the Grand River watershed (Cheyne et al. 2009). Results showed that only the ail gene was detected in three strains of Y. enterocolitica 1A, serotype O:5 (Table 3). The yadA gene was not detected in these serotype O:5 isolates. The ail and yadA genes were not detected in any other Y. enterocolitica 1A isolates or in other Yersinia species isolated from the watershed

(Table 3). Results for ail gene detection in both reference strains and watershed isolates was confirmed using the primers designed by Thisted Lambertz et al. (2008) (data not shown).

## Recovery studies

The recovery and detection of Y. enterocolitica ATCC 700822 added to surface water samples was assessed. Y. enterocolitica was inoculated into surface water at five different concentrations that ranged from $1 \times 10^1$ to $1 \times 10^4$ cells/100 ml, and the DNA recovered from each sample was tested using the ail qPCR assay. Recovery was found to range from 24 to 37%. An ail signal could be detected in two of three water samples spiked at $1 \times 10^1$ cells/100 ml, and in all samples spiked at $5 \times 10^1$ cells/100 ml or higher. The detection of microbial pathogens in environmental samples by PCR is often affected by DNA loss during the extraction and purification steps, and incomplete removal of PCR inhibitors (Toze 1999). Surface water in the Grand River watershed has a high turbidity (1–688 NTU) and high total organic carbon content (5–10 mg l$^{-1}$) (data not shown). However, the DNA extraction and purification method used in this study

Table 3 | Occurrence of ail and yadA genes in Yersinia strains isolated from the Grand River watershed

| Yersinia species | Biogroup | Serogroup | Isolates tested | ail positive | yadA positive |
|---|---|---|---|---|---|
| Y. enterocolitica | 1A | O:5 | 5 | 3 | 0 |
| Y. enterocolitica | 1A | O:5,27 | 1 | 0 | 0 |
| Y. enterocolitica | 1A | O:7,8 | 6 | 0 | 0 |
| Y. enterocolitica | 1A | O:7,13 | 2 | 0 | 0 |
| Y. enterocolitica | 1A | O:41,43 | 1 | 0 | 0 |
| Y. enterocolitica | 1A | O:rough | 1 | 0 | 0 |
| Y. enterocolitica | 1A | O:untypeable | 1 | 0 | 0 |
| Y. aldovae | | | 6 | 0 | 0 |
| Y. bercovieri | | | 13 | 0 | 0 |
| Y. frederiksenii | | | 18 | 0 | 0 |
| Y. intermedia | 1 | | 28 | 0 | 0 |
| Y. intermedia | 4 | | 3 | 0 | 0 |
| Y. kristensenii | | | 2 | 0 | 0 |
| Y. mollaretii | | | 5 | 0 | 0 |
| Y. rohdei | | | 2 | 0 | 0 |

could effectively remove PCR inhibitors from surface water, with an average recovery of 31% for the *ail* qPCR assay.

## Grand River watershed survey

Surface water samples were collected from five sites in the Grand River watershed. These 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 (Figure 1). Surface water was collected biweekly between March 2005 and August 2007 and tested for the *ail* gene, and between January 2006 and August 2007 for the *yadA* gene. DNA extracted from surface water was tested for PCR inhibition using an external *luxB* inhibition assay. It was found that the extraction method used in this study resulted in DNA that rarely contained PCR inhibitors. Target concentrations determined by qPCR were not adjusted for recovery efficiency.

The *ail* and *yadA* genes were detected at all five sites examined in the Grand River watershed (Table 4). The *ail* gene target was detected in 121 out of 319 samples (38%), and the *yadA* gene target was detected in 44 out of 206 samples (21%). There were 31 samples (15%) that were positive for both gene targets. The frequency of detecting the *ail* target was consistently higher than the *yadA* target at all five sampling sites. The median values observed across the watershed for *ail* and *yadA* were 40 cells/100 ml and 32 gene copies/100 ml, respectively, and the maximum values

detected were 2,000 cells/100 ml and 3,276 gene copies/100 ml, respectively. Both targets were detected most frequently at the Canagagigue Creek sampling location, at a frequency of 50% and 26% for *ail* and *yadA*, respectively. Canagagigue Creek samples also showed the second highest median concentration for indicator *E. coli* (not shown). The highest median concentration for indicator *E. coli* was detected in the Grand River just downstream of a wastewater effluent discharge (data not shown), but this did not correspond with high *Y. enterocolitica* virulence gene detection (Table 4).

*Yersinia enterocolitica ail* and *yadA* genes were detected more frequently when water temperatures were colder. A seasonal trend for *ail* and *yadA* genes can be seen in Figure 2, which shows the concentration of each gene target and water temperature for two sites in the Grand River over the sampling period. The frequency for detecting the *ail* and *yadA* genes increased as the water temperature decreased (Figure 3). For samples taken at temperatures below 5°C, 67% were positive for the *ail* gene and 35% for the *yadA* gene. In contrast, at temperatures above 20°C the virulence genes were detected in less than 12% of samples. There was no relationship between *ail* or *yadA* gene occurrence and other water quality parameters, including total *E. coli*, turbidity, nitrate and ammonia (not shown).

## Confirmation of quantitative PCR results

Amplification products from selected positive *ail* and *yadA* samples were analysed by agarose gel electrophoresis, and

**Table 4** | Occurrence of *Y. enterocolitica ail* and *yadA* genes in samples from the Grand River watershed; sample locations are described in Figure 1

| Sample location | Number of samples analysed | | Frequency of positive samples (%) | | Median value[*] (cells or gene copies/100 ml) | | Maximum value (cells or gene copies/100 ml) | |
|---|---|---|---|---|---|---|---|---|
| | *ail* | *yadA* | *ail* | *yadA* | *ail* | *yadA* | *ail* | *yadA* |
| Grand River (North) | 64 | 42 | 47 | 21 | 42 | 20 | 2,000 | 53 |
| Canagagigue Creek | 64 | 42 | 50 | 26 | 39 | 25 | 576 | 76 |
| Conestogo River | 64 | 41 | 27 | 18 | 51 | 45 | 296 | 3,296 |
| Grand River (WW) | 66 | 41 | 32 | 22 | 23 | 42 | 730 | 91 |
| Grand River (Intake) | 61 | 40 | 34 | 20 | 37 | 38 | 775 | 277 |
| Overall | 319 | 206 | 38 | 21 | 40 | 32 | 2,000 | 3,276 |

[*]Median values were calculated using positive samples only.
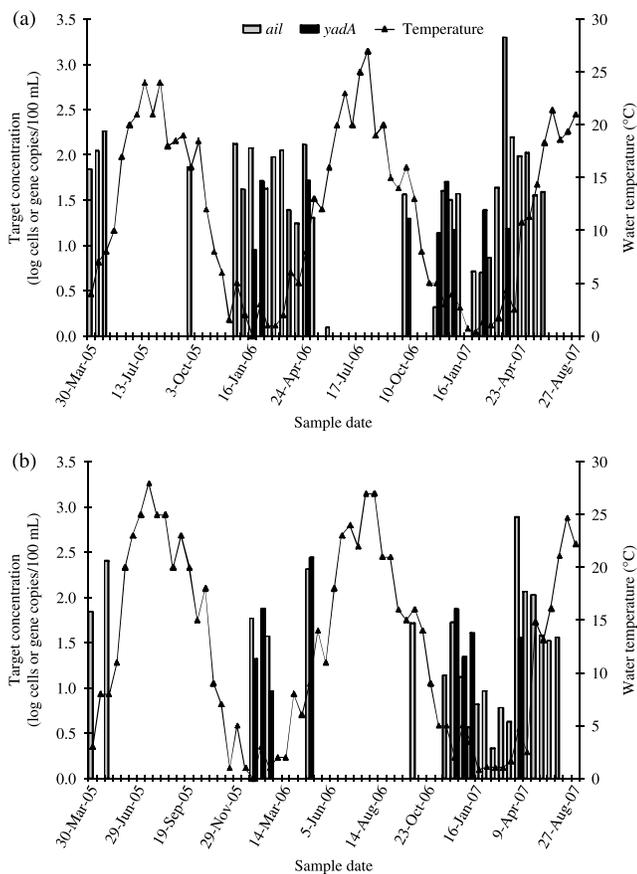
(a)

(b)

**Figure 2** | Seasonal occurrence of *Y. enterocolitica ail* and *yadA* genes in the Grand River watershed at (A) the Grand River (North) sampling site and (B) the Grand River site upstream of a drinking water treatment plant intake. Sample analysis for the *yadA* gene began in January 2006. Each tick mark along the horizontal axis represents a sample date.



**Figure 3** | Relationship between *Y. enterocolitica* gene occurrence and water temperature. The numbers above each column indicate the total number of samples (n) analysed in each temperature range.

DNA fragments of the correct size (91 and 97 bp for *ail* and *yadA*, respectively) were present (data not shown). Amplified fragments from selected PCR reactions were also sequenced. Cloned *ail* amplification products (NCBI accession #EU780661, EU780662, EU780663) from two river water samples had 100% homology, and a BLASTN search of the NCBI database showed 96–100% similarity with *Y. enterocolitica ail* gene sequences. Cloned *yadA* amplification products (EU780664, EU780665) from two river water samples had 100% homology, and 96–98% similarity with *yadA* genes from *Y. enterocolitica*, *Y. pseudotuberculosis* and *Yersinia pestis*. This was not unexpected, as the *yadA* primers and probe could detect *yadA* genes from all three species.
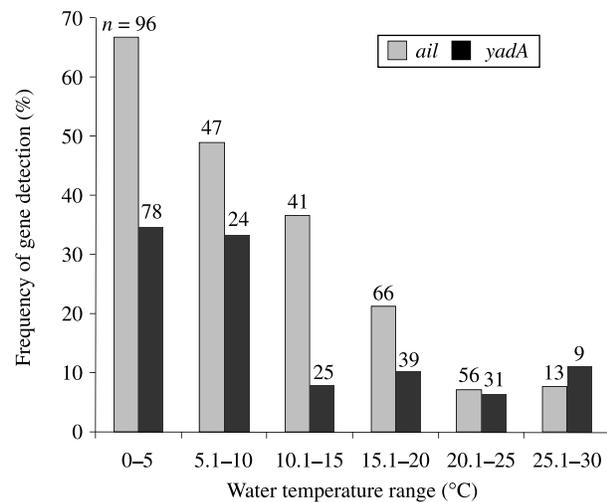
## DISCUSSION

In this study, qPCR methods for detecting *Y. enterocolitica* in surface water were evaluated that targeted two different genes, a chromosomal gene (*ail*) and a plasmid-borne gene (*yadA*). The *ail* and *yadA* genes were selected because they play critical roles in the virulence pathways of *Y. enterocolitica*. The occurrence of *ail* and *yadA* genes were tested in a number of pathogenic and non-pathogenic serotypes of *Y. enterocolitica* and also in other *Yersinia* strains. The species *Y. enterocolitica* is divided into six biogroups, 1A, 1B, and 2 through 5, and into more than 50 serogroups (Wauters *et al.* 1987). Pathogenicity has traditionally been associated with certain biogroups and serogroups, specifically 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3 and 4/O:3 (Fredriksson-Ahomaa *et al.* 2006). Other *Yersinia* species known to cause disease in humans include *Y. pseudotuberculosis* and *Y. pestis*. *Y. pseudotuberculosis* is also a zoonotic pathogen that has been identified as the causative agent in both food-borne and waterborne disease (Han *et al.* 2003; Jalava *et al.* 2006). In addition, other *Yersinia* species in the *Y. enterocolitica*-like group include *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Yersinia aldovae*, *Yersinia rohdei*, *Yersinia bercovieri* and *Yersinia mollaretii*. Although these are considered to be non-pathogenic species, the clinical significance of this group is not fully known (Sulakvelidze 2000).

We initially tested a number of reference strains isolated from clinical or porcine samples for the presence of the *ail* and *yadA* virulence genes (Table 2). Results for the *Y. enterocolitica* reference strains show that *ail* and *yadA* genes were specific for the pathogenic *Y. enterocolitica* biogroups (1B, 2, 3, 4), and they were not detected in biogroup 1A (Table 2). The *ail* and *yadA* genes were not detected in *Y. enterocolitica* ATCC 9610, which has been observed previously (Blais & Phillippe 1995; Thoerner *et al.* 2003). The *yadA* gene was also not detected in a *Y. enterocolitica* 1B/O:21 strain. The detection of chromosomal virulence genes and not pYV plasmid genes in *Y. enterocolitica* was also observed in other studies, with plasmid loss as a possible reason (Blais & Phillippe 1995; Thoerner *et al.* 2003). As expected, the *Y. pseudotuberculosis* strains examined in our study were negative for the *ail* gene and positive for the *yadA* gene (Table 2). There is an *ail* homolog in *Y. pseudotuberculosis* (Yang *et al.* 1996), but the *ail* primers used in this study were designed specifically to target *Y. enterocolitica* (Jourdan *et al.* 2000; Bhaduri *et al.* 2005). Our results also showed that both the *ail* and *yadA* virulence genes were detected in a clinical *Y. intermedia* strain (Table 2). Although *Y. intermedia* strains are considered non-pathogenic, they may have some potential for causing disease and have been isolated from symptomatic patients (Sulakvelidze 2000). In a study by Robins-Browne *et al.* (1989), a DNA probe targeting the *ail* gene hybridized with a *Y. intermedia* strain, and Kechagia *et al.* (2007) detected two plasmid-borne genes, *virF* and *yadA*, and the chromosomal gene *yst* in a *Y. intermedia* strain. The *ail* and *yadA* genes were not detected in reference strains from the other *Yersinia* species, including *Y. frederiksenii*, *Y. kristensenii*, *Y. mollaretii* and *Y. rohdei* (Table 2).

We also screened a large number of *Yersinia* isolates that were obtained from the Grand River watershed by our group from 2006–2008 (Cheyne *et al.* 2009). As shown in Table 3, only non-pathogenic bioserogroups were isolated from the watershed, which may have been due in part to the poor recovery of pathogenic serogroups by current culture-based detection methods (Cheyne *et al.* 2009). Results showed that the *ail* gene was detected in three *Y. enterocolitica* 1A/O:5 isolates. Two additional 1A/O:5 strains were negative for the *ail* gene, and all strains from

this bioserogroup were negative for the *yadA* gene. The *ail* and *yadA* genes were not detected in the other isolates tested, including 12 other isolates from biogroup 1A (serogroups O:5,27, O:7,8, O:41,43, O:rough and O:untypeable) and 77 isolates from other species including *Y. aldovae*, *Y. bercovieri*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. mollaretii* and *Y. rohdei*. Although most reports show that the *ail* gene is only present in pathogenic serogroups, some studies have shown that the *ail* gene was detected in biotype 1A strains (Thoerner *et al.* 2003; Falcão *et al.* 2006; Zheng *et al.* 2008). Serotype O:5 strains have been isolated from a number of clinical samples throughout the world (Tennant *et al.* 2003), including a nosocomial outbreak in Canada (Ratnam *et al.* 1982).

Although the clinical significance of biotype 1A strains is unclear, it is now thought that biogroup 1A may include representatives of both non-pathogenic and clinically important strains (Grant *et al.* 1998; Gulati & Virdi 2007). The virulence potential of this group of organisms is not well understood, in part because most clinical isolation methods are not suitable for biotype 1A strains, and the detection of non-pathogenic species is often not reported (Tennant *et al.* 2003). It is also thought that species other than *Y. enterocolitica* may play a role in disease (Sulakvelidze 2000); however we did not detect virulence genes in any of the *Y. enterocolitica*-like species from the Grand River. We did detect the *ail* and *yadA* genes in a *Y. intermedia* reference strain from a clinical source; however the aetiology and significance of this finding is not known. Further investigations will be necessary to better characterize these species and evaluate their potential for human pathogenicity.

A focus of our study was to use the *ail* and *yadA* qPCR assays to directly assess gene occurrence in surface water samples from the Grand River watershed in southern Ontario, Canada. Samples were collected over a 2-year period from locations in the watershed upstream of a drinking water treatment plant intake (Figure 1). Results showed that the overall detection frequency was 38% for the *ail* gene and 21% for the *yadA* gene. Both gene targets were detected most frequently at the Canagagigue Creek sampling location. The Canagagigue Creek subwatershed is located in a region with the highest livestock density in the watershed (Dorner *et al.* 2007) and where estimates for daily

manure production were also highest (Dorner *et al.* 2004). Furthermore, pigs are a major reservoir for human pathogenic strains (McNally *et al.* 2004; Fredriksson-Ahomaa *et al.* 2006) and were the second most abundant livestock in the watershed (Dorner *et al.* 2004). In a study related to this current research, *Y. enterocolitica* were isolated from pig faeces on farms in the Grand River watershed in 2006, and the majority of isolates were from the pathogenic serogroup 4/O:3 (Public Health Agency of Canada 2007). In addition to agricultural runoff, it is possible that wildlife may play a role in the detection of *Yersinia* in the watershed. Although the occurrence of *Yersinia* in wildlife is not well studied, reports have shown that *Y. enterocolitica* and *Y. pseudotuberculosis*, including human pathogenic strains, have been isolated from wild birds and animals (Hacking & Sileo 1974; Shayegani *et al.* 1986; Fukushima & Gomyoda 1991; Niskanen *et al.* 2003).

A clear relationship between the occurrence of the *Y. enterocolitica* virulence genes and water temperature was demonstrated, and showed a higher frequency of detection in colder water. A relationship between water temperature and occurrence of *Yersinia* spp. has been observed previously. Massa *et al.* (1988) isolated *Y. enterocolitica* and other *Yersinia* spp. from surface waters in Italy and found most isolates during the colder months of the year when the water was between 5 and 10°C. Two other culture-based surveys for *Yersinia* in water also noted a higher rate of isolation during colder months of the year (Meadows & Snudden 1982; Fukushima *et al.* 1984). The *ail* gene has been detected in pig faeces at a higher rate during colder months of the year (Bhaduri *et al.* 2005), and *Y. enterocolitica* also seems to be isolated more frequently from humans living in countries with cooler climates (Kapperud 1991). Unlike other members of the family Enterobacteriaceae, *Y. enterocolitica* survives well at cold temperatures (Fredriksson-Ahomaa & Korkeala 2003). Moreover, Schiemann & Olson (1984) demonstrated that, while *Y. enterocolitica* grew poorly in competition with other organisms at higher temperatures (25 and 32°C), growth rates were no longer impeded by other organisms when grown at 15°C. Fukushima *et al.* (1984) also postulated that the unique growth characteristics of *Y. enterocolitica* may contribute to the observed temperature trends.

Previous surveys for *Y. enterocolitica* in surface waters have primarily used culture-based methods, and pathogenic sub-types have rarely been isolated from water. Sandery *et al.* (1996) used PCR detection of the *Y. enterocolitica ail* gene and found 11 out of 105 surface water samples (10%) were positive from creeks and reservoirs in Australia. These researchers also conducted a concurrent culture-based survey and only 1% of the isolates tested were positive for the *ail* gene. In parallel with our study, we also conducted a culture-based survey for *Y. enterocolitica* in the Grand River watershed (Cheyne *et al.* 2009). The isolation rate of *Y. enterocolitica* from Grand River samples was 4%, and the isolates were all determined to belong to the traditionally non-pathogenic biotype 1A.

In our survey of virulence genes in surface waters, it is possible that positive signals obtained for the *ail* gene target resulted from biotype 1A strains, since this gene was found in several *Y. enterocolitica* 1A/O:5 isolated from the watershed (Table 3). However, the detection of *yadA* genes in surface water samples, although at a lower frequency than that of the *ail* gene, show that our survey was likely detecting pathogenic *Yersinia* species. The *yadA* gene was not detected in any of the *Yersinia* isolates from the watershed, and in general it has been shown that the pYV plasmid is only found in pathogenic strains. Due to the high homology between the pYV plasmids of *Y. enterocolitica* and *Y. pseudotuberculosis*, it is possible that the *yadA* assay detected the presence of both species in the watershed. *Y. pseudotuberculosis* has been previously isolated from water (Fukushima 1992; Han *et al.* 2003). It is unlikely that *Y. pestis* was detected, as it is primarily contained within a sylvatic reservoir and is not transmitted by water (Stenseth *et al.* 2008). In addition, it is likely that our results are an overestimation of viable pathogenic *Y. enterocolitica* in the watershed, since PCR-based detection methods are known to also detect non-viable cells.

## CONCLUSIONS

This study demonstrates that qPCR-based methods are effective and sensitive tools for enumerating *Y. enterocolitica* in water samples. This is the first study to use qPCR methods for water samples, and results showed that *Y. enterocolitica ail* and *yadA* genes were detected in surface water samples at low levels and their occurrence was seasonal. These results

indicate that pathogenic *Y. enterocolitica* may have been present in surface waters from the Grand River watershed. Further investigation is necessary to determine whether low levels of pathogenic *Y. enterocolitica* in these surface waters pose a risk to human health, since the concentrations detected were lower than the infectious dose for *Y. enterocolitica* (Hunter 1997). Furthermore, given that gene targets were more prevalent in cold water, it seems likely that its presence would be of minimal risk to recreational users. Our findings suggest that it would be important to further investigate factors that influence *Y. enterocolitica* survival and occurrence in the environment.

## ACKNOWLEDGEMENTS

## REFERENCES

Arvanitidou, M., Stathopoulos, G. A. & Katsouyannopoulos, V. C. 1994 Isolation of *Campylobacter* and *Yersinia* spp. from drinking waters. *J. Travel Med.* **1**(3), 156–159.

Behets, J., Declerck, P., Delaedt, Y., Verelst, L. & Ollevier, F. 2007 A duplex real-time PCR assay for the quantitative detection of *Naegleria fowleri* in water samples. *Water Res.* **41**, 118–126.

Bhaduri, S., Wesley, I. V. & Bush, E. J. 2005 Prevalence of pathogenic *Yersinia enterocolitica* strains in pigs in the United States. *Appl. Environ. Microbiol.* **71**, 7117–7121.

Blais, B. W. & Phillippe, L. M. 1995 Comparative analysis of *yadA* and *ail* polymerase chain reaction methods for virulent *Yersinia enterocolitica*. *Food Control* **6**(4), 211–214.

Boom, R., Sol, C. J., Salimans, M. M., Jansen, C. L., Wertheim-van Dillen, P. M. & van der Noordaa, J. 1990 Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**, 495–503.

Bottone, E. J. 1997 *Yersinia enterocolitica*: the charisma continues. *Clin. Microbiol. Rev.* **10**(2), 257–276.

Boyapalle, S., Wesley, I. V., Hurd, H. S. & Reddy, P. G. 2001 Comparison of culture, multiplex, and 5′ nuclease polymerase chain reaction assays for the rapid detection of *Yersinia enterocolitica* in swine and pork products. *J. Food Prot.* **64**, 1352–1361.

Brennhovd, O., Kapperud, G. & Langeland, G. 1992 Survey of thermotolerant *Campylobacter* spp. and *Yersinia* spp. in three surface water sources in Norway. *Int. J. Food Microbiol.* **15**(3–4), 327–338.

Cheyne, B. M., Van Dyke, M. I., Anderson, W. B. & Huck, P. M. 2009 An evaluation of methods for the isolation of *Yersinia enterocolitica* from surface waters in the Grand River watershed. *J. Water Health* **7**(3), 392–403.

Christensen, S. G. 1979 Isolation of *Yersinia enterocolitica* 0:3 from a well suspected as the source of yersiniosis in a baby. *Acta Vet. Scand.* **20**(1), 154–156.

Ciebin, B. W., Brodsky, M. H., Eddington, R., Horsnell, G., Choney, A., Palmateer, G., Ley, A., Joshi, R. & Shears, G. 1995 Comparative evaluation of modified m-FC and m-TEC media for membrane filter enumeration of *Escherichia coli* in water. *Appl. Environ. Microbiol.* **61**, 3940–3942.

Cocolin, L. & Comi, G. 2005 Use of a culture-independent molecular method to study the ecology of *Yersinia* spp. in food. *Int. J. Food Microbiol.* **105**, 71–82.

Cornelis, G. R., Boland, A., Boyd, A. P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M. P. & Stainier, I. 1998 The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol. Mol. Biol. Rev.* **62**, 1315–1352.

Dorner, S. M., Huck, P. M. & Slawson, R. M. 2004 Estimating potential environmental loadings of *Cryptosporidium* spp. and *Campylobacter* spp. from livestock in the Grand River Watershed, Ontario, Canada. *Environ. Sci. Technol.* **38**(12), 3370–3380.

Dorner, S. M., Anderson, W. B., Gaulin, T., Candon, H. L., Slawson, R. M., Payment, P. & Huck, P. M. 2007 Pathogen and indicator variability in a heavily impacted watershed. *J. Water Health* **5**(2), 241–257.

Falcão, J. P., Brocchi, M., Proenca-Modena, J. L., Acrani, G. O., Correa, E. F. & Falcão, D. P. 2004 Virulence characteristics and epidemiology of *Yersinia enterocolitica* and *Yersiniae*

other than *Y. pseudotuberculosis* and *Y. pestis* isolated from water and sewage. *J. Appl. Microbiol.* **96**(6), 1230–1236.

Falcão, J. P., Falcão, D. P., Pitondo-Silva, A., Malaspina, A. C. & Brocchi, M. 2006 Molecular typing and virulence markers of *Yersinia enterocolitica* strains from human, animal and food origins isolated between 1968 and 2000 in Brazil. *J. Med. Microbiol.* **55**, 1539–1548.

Flemming, C. A., Leung, K. T., Lee, H., Trevors, J. T. & Greer, C. W. 1994 Survival of *lux-lac*-marked biosurfactant-producing *Pseudomonas aeruginosa* UG2L in soil monitored by nonselective plating and PCR. *Appl. Environ. Microbiol.* **60**, 1606–1613.

Fredriksson-Ahomaa, M. & Korkeala, H. 2003 Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. *Clin. Microbiol. Rev.* **16**(2), 220–229.

Fredriksson-Ahomaa, M., Korte, T. & Korkeala, H. 2000 Contamination of carcasses, offals, and the environment with *yadA*-positive *Yersinia enterocolitica* in a pig slaughterhouse. *J. Food Prot.* **63**, 31–35.

Fredriksson-Ahomaa, M., Stolle, A. & Korkeala, H. 2006 Molecular epidemiology of *Yersinia enterocolitica* infections. *FEMS Immunol. Med. Microbiol.* **47**(3), 315–329.

Fukushima, H. 1992 Direct isolation of *Yersinia pseudotuberculosis* from fresh water in Japan. *Appl. Environ. Microbiol.* **58**, 2688–2690.

Fukushima, H. & Gomyoda, M. 1991 Intestinal carriage of *Yersinia pseudotuberculosis* by wild birds and mammals in Japan. *Appl. Environ. Microbiol.* **57**, 1152–1155.

Fukushima, H., Saito, K., Tsubokura, M. & Otsuki, K. 1984 *Yersinia* spp. in surface water in Matsue, Japan. *Zentralbl. Bakteriol. Mikrobiol. Hyg. B* **179**(3), 236–247.

Gonul, S. A. & Karapinar, M. 1991 The microbiological quality of drinking water supplies of Izmir City: the incidence of *Yersinia enterocolitica*. *Int. J. Food Microbiol.* **13**, 69–73.

Grant, T., Bennett-Wood, V. & Robins-Browne, R. M. 1998 Identification of virulence-associated characteristics in clinical isolates of *Yersinia enterocolitica* lacking classical virulence markers. *Infect. Immun.* **66**, 1113–1120.

Gulati, P. S. & Virdi, J. S. 2007 The *rrn* locus and *gyrB* genotyping confirm the existence of two clonal groups in strains of *Yersinia enterocolitica* subspecies *palearctica* biovar 1A. *Res. Microbiol.* **158**, 236–243.

Hacking, M. A. & Sileo, L. 1974 *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from wildlife in Ontario. *J. Wildlife Dis.* **10**, 452–457.

Han, T. H., Paik, I. K. & Kim, S. J. 2003 Molecular relatedness between isolates *Yersinia pseudotuberculosis* from a patient and an isolate from mountain spring water. *J. Korean Med. Sci.* **18**, 425–428.

Howard, S. L., Gaunt, M. W., Hinds, J., Witney, A. A., Stabler, R. & Wren, B. W. 2006 Application of comparative phylogenomics to study the evolution of *Yersinia enterocolitica* and to identify genetic differences relating to pathogenicity. *J. Bacteriol.* **188**, 3645–3653.

Hunter, P. R. 1997 *Waterborne Disease: Epidemiology and Ecology*. John Wiley, Chichester, UK.

Jalava, K., Hakkinen, M., Valkonen, M., Nakari, U. M., Palo, T., Hallanvuo, S., Ollgren, J., Siitonen, A. & Nuorti, J. P. 2006 An outbreak of gastrointestinal illness and erythema nodosum from grated carrots contaminated with *Yersinia pseudotuberculosis*. *J. Infect. Dis.* **194**(9), 1209–1216.

Johannessen, G. S., Kapperud, G. & Kruse, H. 2000 Occurrence of pathogenic *Yersinia enterocolitica* in Norwegian pork products determined by a PCR method and a traditional culturing method. *Int. J. Food Microbiol.* **54**, 75–80.

Jourdan, A. D., Johnson, S. C. & Wesley, I. V. 2000 Development of a fluorogenic 5′ nuclease PCR assay for detection of the *ail* gene of pathogenic *Yersinia enterocolitica*. *Appl. Environ. Microbiol.* **66**, 3750–3755.

Kapperud, G. 1991 *Yersinia enterocolitica* in food hygiene. *Int. J. Food Microbiol.* **12**, 53–65.

Kechagia, N., Nicolaou, C., Ioannidou, V., Kourti, E., Ioannidis, A., Legakis, N. J. & Chatzipanagiotou, S. 2007 Detection of chromosomal and plasmid-encoded virulence determinants in *Yersinia enterocolitica* and other *Yersinia* spp. isolated from food animals in Greece. *Int. J. Food Microbiol.* **118**, 326–331.

Keet, E. E. 1974 *Yersinia enterocolitica* septicemia. Source of infection and incubation period identified. *New York State J. Med.* **74**(12), 2226–2230.

Langeland, G. 1983 *Yersinia enterocolitica* and *Yersinia enterocolitica*-like bacteria in drinking water and sewage sludge. *Acta Pathol. Microbiol. Immunol. Scand. B* **91**, 179–185.

Lassen, J. 1972 *Yersinia enterocolitica* in drinking-water. *Scand. J. Infect. Dis.* **4**(2), 125–127.

Massa, S., Cesaroni, D., Poda, G. & Trovatelli, L. D. 1988 Isolation of *Yersinia enterocolitica* and related species from river water. *Zentralbl. Mikrobiol.* **143**(8), 575–581.

McNally, A., Cheasty, T., Fearnley, C., Dalziel, R. W., Paiba, G. A., Manning, G. & Newell, D. G. 2004 Comparison of the biotypes of *Yersinia enterocolitica* isolated from pigs, cattle and sheep at slaughter and from humans with yersiniosis in Great Britain during 1999–2000. *Lett. Appl. Microbiol.* **39**(1), 103–108.

Meadows, C. A. & Snudden, B. H. 1982 Prevalence of *Yersinia enterocolitica* in waters of the lower Chippewa River Basin, Wisconsin. *Appl. Environ. Microbiol.* **43**(4), 953–954.

Miller, V. L., Farmer, J. J., III, Hill, W. E. & Falkow, S. 1989 The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. *Infect. Immun.* **57**, 121–131.

Niskanen, T., Waldenström, J., Fredriksson-Ahomaa, M., Olsen, B. & Korkeala, H. 2003 *virF*-positive *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* found in migratory birds in Sweden. *Appl. Environ. Microbiol.* **69**(8), 4670–4675.

Ostroff, S. M., Kapperud, G., Hutwagner, L. C., Nesbakken, T., Bean, N. H., Lassen, J. & Tauxe, R. V. 1994 Sources of sporadic *Yersinia enterocolitica* infections in Norway: a

prospective case-control study. *Epidemiol. Infect.* **112**(1), 133–141.

Pitcher, D. G., Saunders, N. A. & Owen, R. J. 1989 Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* **8**, 151–156.

Public Health Agency of Canada 2007 *Canadian National Enteric Pathogen Surveillance System (C-EnterNet) 2006 Annual Report*. Government of Canada, Guelph, Ontario. Available at www.phac-aspc.gc.ca/c-enternet/publications-eng.php, (accessed 19 October 2009).

Ratnam, S., Mercer, E., Picco, B., Parsons, S. & Butler, R. 1982 A nosocomial outbreak of diarrheal disease due to *Yersinia enterocolitica* serotype O:5, biotype 1. *J. Infect. Dis.* **145**, 242–247.

Revell, P. A. & Miller, V. L. 2001 *Yersinia* virulence: more than a plasmid. *FEMS Microbiol. Lett.* **205**, 159–164.

Robins-Browne, R. M., Miliotis, M. D., Cianciosi, S., Miller, V. L., Falkow, S. & Morris, J. G., Jr 1989 Evaluation of DNA colony hybridization and other techniques for detection of virulence in *Yersinia* species. *J. Clin. Microbiol.* **27**, 644–650.

Saebo, A., Kapperud, G., Lassen, J. & Waage, J. 1994 Prevalence of antibodies to *Yersinia enterocolitica* O:3 among Norwegian military recruits: association with risk factors and clinical manifestations. *Eur. J. Epidemiol.* **10**, 749–755.

Sandery, M., Stinear, T. & Kaucner, C. 1996 Detection of pathogenic *Yersinia enterocolitica* in environmental waters by PCR. *J. Appl. Bacteriol.* **80**, 327–332.

Satterthwaite, P., Pritchard, K., Floyd, D. & Law, B. 1999 A case-control study of *Yersinia enterocolitica* infections in Auckland. *Aust. N.Z. J. Public Health* **23**, 482–485.

Schaffter, N. & Parriaux, A. 2002 Pathogenic-bacterial water contamination in mountainous catchments. *Water Res.* **36**(1), 131–139.

Schiemann, D. A. & Olson, S. A. 1984 Antagonism by Gram-negative bacteria to growth of *Yersinia enterocolitica* in mixed cultures. *Appl. Environ. Microbiol.* **48**(3), 539–544.

Sharma, S., Sachdeva, P. & Virdi, J. S. 2003 Emerging water-borne pathogens. *Appl. Microbiol. Biotechnol.* **61**(5–6), 424–428.

Shayegani, M., DeForge, I., McGlynn, D. M. & Root, T. 1981 Characteristics of *Yersinia enterocolitica* and related species isolated from human, animal, and environmental sources. *J. Clin. Microbiol.* **14**(3), 304–312.

Shayegani, M., Stone, W. B., DeForge, I., Root, T., Parsons, L. M. & Maupin, P. 1986 *Yersinia enterocolitica* and related species isolated from wildlife in New York State. *Appl. Environ. Microbiol.* **52**(3), 420–424.

Stenseth, N. C., Atshabar, B. B., Begon, M., Belmain, S. R., Bertherat, E., Carniel, E., Gage, K. L., Leirs, H. & Rahalison, L. 2008 Plague: past, present, and future. *PLoS Med.* **5**(1), e3 (doi:10.1371/journal.pmed.0050003).

Sulakvelidze, A. 2000 *Yersiniae* other than *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*: the ignored species. *Microbes Infect.* **2**(5), 497–513.

Tacket, C. O., Ballard, J., Harris, N., Allard, J., Nolan, C., Quan, T. & Cohen, M. L. 1985 An outbreak of *Yersinia enterocolitica* infections caused by contaminated tofu (soybean curd). *Am. J. Epidemiol.* **121**, 705–711.

Tennant, S. M., Grant, T. H. & Robins-Browne, R. M. 2003 Pathogenicity of *Yersinia enterocolitica* biotype 1A. *FEMS Immunol. Med. Microbiol.* **38**(2), 127–137.

Thisted Lambertz, S. & Danielsson-Tham, M. L. 2005 Identification and characterization of pathogenic *Yersinia enterocolitica* isolates by PCR and pulsed-field gel electrophoresis. *Appl. Environ. Microbiol.* **71**, 3674–3681.

Thisted Lambertz, S., Nilsson, C., Hallanvuo, S. & Lindlblad, M. 2008 Real-time PCR methods for detection of pathogenic *Yersinia enterocolitica* in food. *Appl. Environ. Microbiol.* **74**(19), 6060–6067.

Thoerner, P., Bin Kingombe, C. I., Bogli-Stuber, K., Bissig-Choisat, B., Wassenaar, T. M., Frey, J. & Jemmi, T. 2003 PCR detection of virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and investigation of virulence gene distribution. *Appl. Environ. Microbiol.* **69**, 1810–1816.

Thompson, J. S. & Gravel, M. J. 1986 Family outbreak of gastroenteritis due to *Yersinia enterocolitica* serotype 0:3 from well water. *Can. J. Microbiol.* **32**(8), 700–701.

Toze, S. 1999 PCR and the detection of microbial pathogens in water and wastewater. *Water Res.* **33**, 3545–3556.

Vajdic, A. 1985 *Yersinia in Municipal Ground Water Supplies in Southern Ontario*. Ontario Ministry of the Environment, Rexdale, Ontario.

Vishnubhatla, A., Fung, D. Y., Oberst, R. D., Hays, M. P., Nagaraja, T. G. & Flood, S. J. 2000 Rapid 5′ nuclease (TaqMan) assay for detection of virulent strains of *Yersinia enterocolitica*. *Appl. Environ. Microbiol.* **66**, 4131–4135.

Wanger, A. 2007 *Yersinia*. In *Manual of Clinical Microbiology* (ed. P. R. Murray & E. J. Baron), pp. 688–697. ASM Press, Washington, DC.

Wauters, G., Kandolo, K. & Janssens, M. 1987 Revised biogrouping scheme of *Yersinia enterocolitica*. *Contrib. Microbiol. Immunol.* **9**, 14–21.

Weagant, S. D. & Kaysner, C. A. 1983 Modified enrichment broth for isolation of *Yersinia enterocolitica* from nonfood sources. *Appl. Environ. Microbiol.* **45**, 468–471.

Yang, Y., Merriam, J. J., Mueller, J. P. & Isberg, R. R. 1996 The *psa* locus is responsible for thermoinducible binding of *Yersinia pseudotuberculosis* to cultured cells. *Infect. Immun.* **64**, 2483–2489.

Zhang, T. & Fang, H. H. 2006 Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. *Appl. Microbiol. Biotechnol.* **70**, 281–289.

Zheng, H., Sun, Y., Mao, Z. & Jiang, B. 2008 Investigation of virulence genes in clinical isolates of *Yersinia enterocolitica*. *FEMS Immunol. Med. Microbiol.* **53**(3), 368–374.