Journal of Microbiological Methods 86 (2011) 82–88

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Determination of the optimal culture conditions for detecting thermophilic campylobacters in environmental water

Simon Lévesque [a], Karen St-Pierre [a], Eric Frost [a], Robert D. Arbeit [b], Sophie Michaud [a,*]

[a] Department of Microbiology and Infectious Diseases, Faculté de Médecine de l'Université de Sherbrooke, Québec, Canada
[b] Infectious Diseases Section, Tufts University School of Medicine, Boston, MA, USA

ARTICLE INFO

Article history:
Received 27 October 2010
Received in revised form 3 April 2011
Accepted 4 April 2011
Available online 12 April 2011

Keywords:
Campylobacter
Culture conditions
PCR
Environmental water

ABSTRACT

This study evaluated alternative protocols for culturing thermophilic campylobacters in environmental water. All samples were filtered through a sterile 0.45 μm pore-size membrane, which was then incubated in Preston enrichment broth. Four variables were compared: water sample volume (2000 mL vs. 500 mL), enrichment broth volume (25 mL vs. 100 mL), enrichment incubation duration (24 h vs. 48 h), and number of enrichment passages (one vs. two). In addition, DNA extracts were prepared from all final broths and analyzed using three rRNA PCR assays. River water was collected at 3 sampling sites weekly for 9 weeks. Among these 27 collections, 25 (93%) yielded *Campylobacter* spp. under at least one of the 16 culture conditions. By univariate analysis, yields were significantly better for the 2000 mL sample volume (68.5% vs. 43.0%, $p<0.0001$) and the 25 mL enrichment broth volume (64.5% vs. 47.0%, $p<0.0004$). Neither of the enrichment period had a significant effect, although there was a trend in favor of 48 h incubation (59.5% vs. 52.0%, $p=0.13$). The three PCR methods gave concordant results for 66 (33%) of the culture-negative samples and 103 (50%) of the culture-positive samples. Compared with culture results, Lubeck's 16S PCR assay had the best performance characteristics, with a sensitivity of 82% and a specificity of 94%. Of the 12 culture-negative samples positive by Lubeck's PCR assay, 11 (92%) samples were also positive by Denis' 16S PCR assay, suggesting that in these cases the culture might have been falsely negative. Based on our results, we conclude that the optimal conditions for detecting *Campylobacter* spp. in natural waters include 2000 mL sample volume and a single enrichment broth of 25 mL PB incubated for 48 h.

1. Introduction

Campylobacter jejuni is the leading reported cause of bacterial enteritis in developed countries (Altekruse et al., 1999). In 2004 in Canada, campylobacter enteritis was the leading notifiable enteric food- and waterborne disease, with 9345 reported cases (http://dsol-smed.phac-aspc.gc.ca). In Quebec province alone, nearly 3000 cases of diarrheal illness are attributed annually to *Campylobacter* enteritis, more than the combined total caused by *Salmonella* and *Shigella* species, *Escherichia coli* O157:H7 and *Yersinia enterocolitica* (Comité provincial sur les entérites à Campylobacter au Québec, 1999). Thomas et al. (2006) recently concluded that even these numbers appear to represent a substantial underestimate of the public health burden of this enteric pathogen and that for every case of campylobacter infection reported in Canada each year, there are an additional unreported 23 to 49 cases.

Raw milk, untreated surface water, and poultry have all been well-documented as sources of campylobacter outbreaks (Allerberger et al., 2003; Blaser et al., 1983; Hutchinson et al., 1985; Jones and Roworth, 1996; Miller and Mandrell, 2005; Olson et al., 2008; Pebody et al., 1997; Said et al., 2003; Skirrow et al., 1981; Stehr-Green et al., 1991; Vogt et al., 1982). Nevertheless, most clinical cases appear as isolated, sporadic infections for which the source is rarely identified (Blaser, 1997). Identifying the sources and routes of transmission of campylobacteriosis is essential for developing effective, targeted preventive measures.

There is ample opportunity for *Campylobacter* spp. to contaminate environmental water, including streams, rivers, and lakes. The members of the genus colonize a wide variety of hosts, from domestic animals to wild birds, and thus an extensive burden of organisms is excreted in animal feces (Altekruse et al., 1999; Blaser et al., 1983). Other potential sources include discharges from wastewater treatment plants.

Testing for indicator organisms (typically thermotolerant coliforms or *E. coli*) has generally been considered to reflect adequately the presence of enteric pathogens; consequently, campylobacters have not been explicitly monitored in water. However, multiple studies, albeit often limited in scope, have reported conflicting results

* Corresponding author at: Department of Microbiology and Infectious Diseases, Faculté de Médecine de l'Université de Sherbrooke, 3001, 12e Avenue Nord, Sherbrooke, Québec, Canada J1H 5N4. Tel.: +1 819 564 5321; fax: +1 819 564 5392.
E-mail address: Sophie.Michaud@USherbrooke.ca (S. Michaud).

0167-7012/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2011.04.001

regarding the correlation between the presence of *E. coli* and *Campylobacter* spp. in environmental water (Arvanitidou et al., 1995; Bolton et al., 1987; Brennhovd et al., 1992; Carter et al., 1987; Dorner et al., 2007; Eyles et al., 2003; Horman et al., 2004; Martikainen et al., 1990; Moore et al., 2001; Obiri-Danso and Jones, 1999; Savill et al., 2001; Skjerve and Brennhovd, 1992; Stelzer et al., 1989; Till et al., 2008).

The primary challenges in detecting *Campylobacter* spp. in water are (a) the small numbers of organisms present; (b) their intrinsic fastidious requirements and slow growth rate; and (c) the presence of a significant proportion of organisms that may be injured or have difficulty adapting to in vitro conditions (Jones et al., 1991; Rollins and Colwell, 1986). This study evaluated alternative protocols for the detection of *Campylobacter* spp. in environmental water, examining four key variables: water sample volume (2000 mL vs. 500 mL), enrichment broth volume (25 mL vs. 100 mL), enrichment incubation duration (24 h vs. 48 h), and number of enrichment passages (one vs. two). Culture results were also compared to three PCR methods for detecting *Campylobacter* in environmental water.

2. Materials and methods

2.1. Water sample sites

From June 7 to August 2, 2004, samples were collected weekly from two sites on the St-François River (NAD83 latitude 45.47695462, longitude −71.9407939, and NAD83 latitude 45.4837399, longitude −71.9605028) and from one of its tributary streams (NAD83 latitude 45.3669643, longitude −71.8349593) in the Eastern Townships, Quebec. These sampling sites were selected based on their high *Campylobacter* spp. prevalence observed in a previous pilot study in 2003 (Levesque et al., 2005). There are large numbers of wild birds, mainly gulls, around these sites, and the third site is also surrounded by many dairy farms.

At each sampling, ~10 L of water was collected from near shore or from a bridge passing over the site using a horizontal alpha water sampler (Geneq inc., Montreal, QC) at a depth of 15 to 30 cm below the surface. Each sampling was made in the morning. The sample was divided into eight aliquots using four 2000-mL and four 500-mL sterile Nalgene bottles, transported on ice to the laboratory, held at 4 °C and tested within 24 h.

2.2. Comparison of culture conditions for detecting thermophilic campylobacters in water

Fig. 1 summarizes the protocol; the eight Nalgene bottles were processed in parallel and yielded 16 final cultures. The contents of each bottle were filtered through a sterile 0.45 μm pore-size membrane filter (VWR International, Mississauga, ON); the filters were transferred into the designated volume of Preston broth (PB) (Oxoid) and held in a microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2) at 37 °C for 4 h, and then at 42 °C for the remainder of the designated enrichment incubation time. Turbid samples were initially filtered with a sterile 1.5 μm pore-size membrane (VWR International) and this membrane was incubated with the corresponding 0.45 μm pore-size membrane filter. At the conclusion of the incubation period, 200 μL of the broth was streaked on Karmali agar (Oxoid) and incubated at 42 °C for 48 h under microaerobic conditions. In parallel, 1 mL was inoculated into a second enrichment broth (10 mL PB) and incubated at 42 °C in a microaerobic atmosphere for the same duration as the first. Then, 200 μL of this second enrichment broth was plated on Karmali agar. Suspect colonies (round light grey colonies with/without spreading) on the Karmali plates were subcultured on TSA and incubated at 42 °C for 24 h in a microaerobic atmosphere. In parallel, 1 mL of suspension of each enrichment broth (first or second) was processed for DNA extraction (see below).

Presumptive *Campylobacter* spp. colonies were confirmed microscopically and each plate was given a positive or negative score. One presumptive colony of *Campylobacter* spp. isolate per volume of water was identified to the species-level by routine phenotypic methods (oxidase, catalase, indoxyl acetate, sodium hippurate hydrolysis in test tube, and susceptibility to nalidixic acid and cephalotin) (Nachamkin, 2003) and by the molecular approaches described below. Bacterial isolates recovered from each condition were stored at −80 °C.

Positive control cultures were done with each new batch of PB. Aliquots of 500 mL of sterile water were spiked with 10 and 25 bacteria from a 24 h culture of *C. jejuni* ATCC 33560 and processed as above using 0.45 μm pore-size membrane filters and a single enrichment (25 mL of PB, 24 h of incubation).

2.3. DNA extraction

Genomic DNA was extracted as follows from 1 mL of enrichment broth or from 1 mL suspension of a single colony of a presumptive

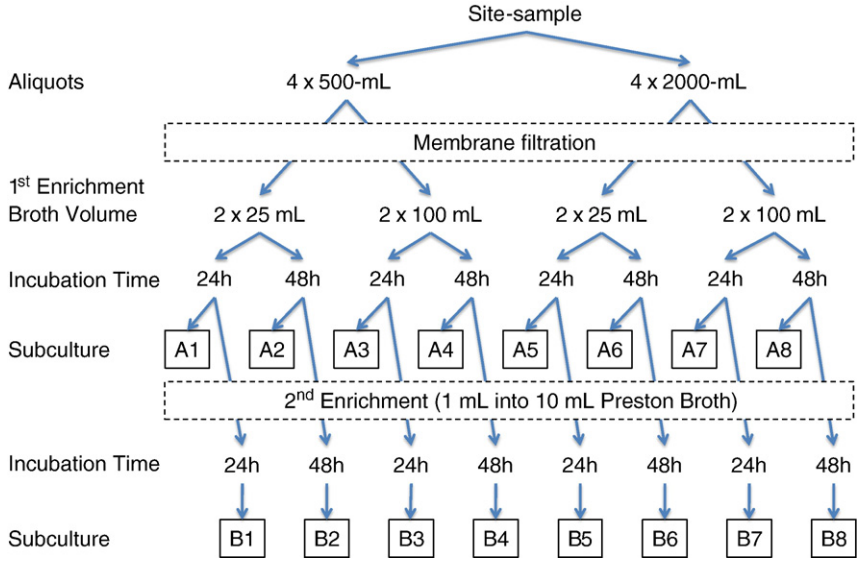

Fig. 1. Protocol for investigation of methodologic variables on the recovery of *Campylobacter* spp. from natural water, including the effect of sample volume (500 mL vs. 2000 mL), enrichment broth volume (25 mL vs. 100 mL), enrichment incubation time (24 h vs. 48 h), and one vs. two enrichments. See text for details of membrane filtration and subculture.

Campylobacter spp. isolate. 1 mL was placed in a 1.5 mL microfuge tube, centrifuged at 19,000 *g* for 10 min and the supernatant was discarded. The pellet was resuspended in 25 μL of NaOH 0.5 N, held for 5 min at room temperature, neutralized with 25 μL of Tris 1 M pH 8.0 and diluted with 100 μL (for extracts from the pellet of enrichment broth) of sterile distilled water or 450 μL (for extracts from a single colony). For the PCR assays described below, 1 μL of the final DNA extract solution was added to 50 μL of reaction mix. DNA was extracted only from the first, but not the second enrichment broth in the first week, but from both first and second enrichment broths for the 8 subsequent weeks.

2.4. PCR detection of Campylobacter spp. in enrichment broths

Each final enrichment broth was analyzed using three PCR assays for the detection of *Campylobacter* spp. The 23S rRNA assay detected *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*, based on amplification of a 650 bp fragment of a highly polymorphic portion of the 23S rRNA gene of *Campylobacter* spp., *Arcobacter butzleri* and *Helicobacter pylori* (Wang et al., 2002). Denis' 16S rRNA assay (Denis et al., 1999) detected *C. jejuni* and *C. coli* based on the amplification of an 857 bp fragment from the 16S rRNA gene. Lubeck's 16S rRNA assay (Lubeck et al., 2003) amplified a 287 bp fragment of the 16S rRNA gene of *C. jejuni*, *C. coli* and *C. lari*.

2.5. PCR detection of hipO gene for identification of C. jejuni isolates

DNA extracts of all presumptive *Campylobacter* spp. isolates were assayed for the presence of the *hipO* gene using the primers and PCR conditions described by Slater and Owen (1997). Isolates giving the expected 1151 bp amplicon were identified as *C. jejuni*.

2.6. PCR detection of glyA gene for identification of C. coli and C. lari isolates

DNA extracts of presumptive *Campylobacter* spp. isolates other than *C. jejuni* were assayed for the presence of the *glyA* gene using the primers and PCR conditions described by Wang et al. (2002). Isolates giving the expected 126 bp amplicon were identified as *C. coli* and isolates giving the expected 251 bp amplicon were identified as *C. lari*.

2.7. Development of an internal amplification control

For Lubeck's 16S assay, we implemented the authors' internal amplification control (IAC) based on our own chimeric DNA construct (Lubeck et al., 2003). A 420 bp fragment of the mouse Ins sequence (accession no. X00944) (Sylla et al., 1984) cloned in plasmid pB_1-20 (Piche and Bourgaux, 1987) was amplified using chimeric PCR primers Ins 16S F Short (5′-CTG CTT AAC ACA AGT TGA G**TA GG***A GCA TGG ACT GC*-3′) and Ins 16S R Long (5′-TTC CTT AGG TAC CGT **CAG AA***C GAA CCA CAC A*-3′). Bases in bold are the common bases between the *Campylobacter* spp.-specific primers and the Ins sequence, bases in italic represent the Ins sequence, and bases in normal characters represent the *Campylobacter* spp.-specific sequence. The reaction was performed in a 50 μL PCR mix containing 2 mM $MgCl_2$, 0.2 mM of a solution containing each dNTPs, 10 μg of BSA, 0.2 μM of each primer, 1 U of Taq polymerase (produced by Catherine Desrosiers, Department of Microbiology and Infectious Diseases, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, according to the Engelke et al., 1990 method) and 5 ng of pB_1-20. The amplification consisted of a denaturation step at 94 °C [3 min], 35 cycles each at 94 °C [30 s], 50 °C [30 s], and 72 °C [30 s], and a final extension step at 72 °C [4 min]. PCR products were resolved using agarose gel electrophoresis (2%) for 75 min at 125 V in TAE buffer and visualized by UV light after staining the gel with ethidium bromide (1 mg/L) for 30 min.

Next, the amplicon product from step 1 was diluted 1:100,000 in salmon sperm DNA (5 μg/μL) and used as a template in a second amplification PCR using the *Campylobacter* spp.-specific primers OT1559 and 18-1 under the conditions described (Jones et al., 1991). PCR products were visualized on 2% agarose electrophoresis gels stained with ethidium bromide as described above. The final amplicon was purified using a spin column (QIAquick PCR purification kit, Qiagen, Mississauga, Ontario, Canada) with an additional washing step with 30% guanidine hydrochloride aqueous solution as suggested by the supplier. The eluate, which contained 7 ng of DNA/μL as measured using a NanoVue spectrophotometer (GE Healthcare Life Science, Piscataway, NJ, USA), was diluted to obtain 100 copies of the DNA fragment in salmon sperm DNA (5 μg/μL) before usage. The control DNA (100 copies/reaction) was used as a positive amplification control in each reaction.

Fig. 2 demonstrates the products obtained using extracts from isolates representing different *Campylobacter* spp.

2.8. Statistical analysis

Proportions were compared with Statistix for Windows version 7.1 (Analytical Software, Tallahassee, FL), using Chi-square and Fisher's exact two-tailed tests and a significance level of 5%.

3. Results

3.1. Culture results

A total of 432 cultures were prepared during the study: 3 sampling sites were each tested at weekly intervals for 9 weeks, comparing two water volumes, two enrichment broth volumes, two incubation durations and single vs. dual enrichment passages for each of the 27 samples. Overall, 104 *C. jejuni*, 7 *C. coli*, 12 *C. lari* and 1 *Campylobacter* spp. (which died before having been identified to the species level and for which DNA extraction could not be performed) were identified, using phenotypic results, Lubeck's 16S PCR and hippurate gene PCR. Of note, 18 (17%) *C. jejuni* isolates confirmed by *hipO* gene PCR gave a negative result to sodium hippurate hydrolysis in test tubes. Control cultures of sterile water spiked with 10 and 25 *C. jejuni* cells were all positive.

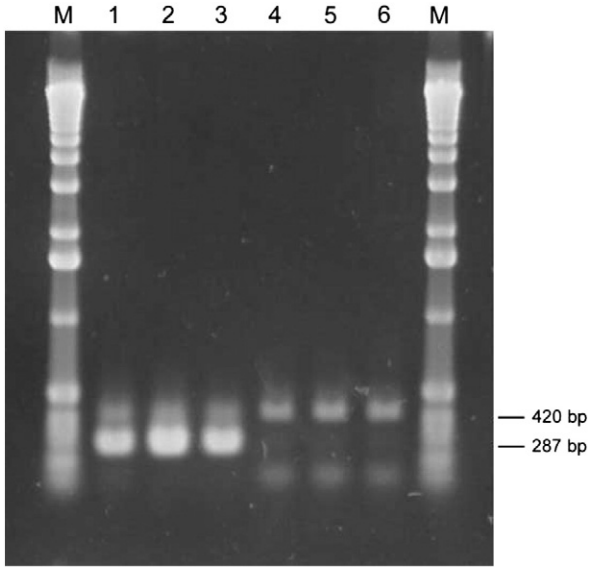

Fig. 2. Lubeck's 16S PCR with internal amplification control. Lanes: M, molecular size marker (1 kb DNA ladder); 1, *C. jejuni* ATCC 33560; 2, *C. coli* ATCC 49941; 3, *C. lari* LSPQ 3218; 4, *C. upsaliensis* ATCC 49815; 5, *C. fetus* LSPQ 2979; and 6, negative control (water).

Globally, 25 of the 27 (93%) weekly site samples were positive for *Campylobacter* spp. under at least one of the culture conditions (Table 1). The most consistent results were obtained using 2000 mL sample volumes with 25 mL enrichment broth (yield range: 72% to 84%). By univariate analysis of all cultures from these 25 samples, yields were significantly better for the 2000 mL sample volume (68.5% vs. 43.0%, $p<0.0001$) and the 25 mL enrichment broth volume (64.5% vs. 47.0%, $p<0.0004$). Neither of the enrichment period had a significant effect (57.0% vs. 54.5%, $p=0.62$), although there was a trend in favor of 48 h incubation (59.5% vs. 52.0%, $p=0.13$). Results were essentially the same by multivariate analysis (not shown). Interestingly, *C. coli* and *C. lari* isolates were mainly obtained from 2000 mL water samples and from 25 mL enrichment broths.

3.2. Comparison of the three different PCR assays with culture

The sensitivity and specificity of each PCR assay were compared to culture for 408 (94.4%) of the 432 final enrichment broths (Table 2). Lubeck's 16S PCR assay had the best performance characteristics, with a sensitivity of 82% and a specificity of 94%. Denis' 16S assay had good specificity, but modest sensitivity; the 23S assay had poor specificity. The 420 bp internal control amplicon was visualized for each negative Lubeck's 16S PCR reaction, indicating the absence of non-specific interfering substances.

All three PCR methods gave concordant results for 66 (33%) of 201 culture-negative samples and 103 (50%) of 207 culture-positive samples. Fig. 3 displays as a Venn diagram the distribution of positive PCR assays among the culture-negative samples (Panel A) and of negative PCR assays among the culture-positive samples (Panel B). Of note, among the 12 culture-negative samples that were positive by Lubeck's 16S PCR assay, 11 (92%) were also positive by Denis' 16S PCR assay. This concordance suggests that in these cases the culture might have been falsely negative.

Table 2
Sensitivity of three different PCR assays, using culture as the reference method.

Culture result	N	PCR assay results					
		23S		16S (Denis)		16S (Lubeck)	
		Positive	Negative	Positive	Negative	Positive	Negative
Positive	207	171	36	113	94	170	37
Negative	201	130	71	23	178	12	189
Sensitivity		83%		55%		82%	
Specificity		35%		89%		94%	

4. Discussion

Thermophilic campylobacters are thought to be ubiquitous in aquatic environments, but their detection can be difficult because the organisms may be injured, unable to adapt to in vitro conditions, and/or present in low numbers (Abulreesh et al., 2005). There is no standardized protocol for the detection of *Campylobacter* spp. in environmental water. This study had the advantage to compare several culture conditions using environmental samples rather than reconstructed lab samples, in order to assess organisms in their natural state, including adaptation to environmental conditions and also possibly damaged or slow replicating organisms. Our analysis of over 200 environmental water culture assays comparing four different culture conditions suggests that the highest yield of *Campylobacter* spp. is obtained by filtering 2000 mL of water and incubating the membrane filter for 48 h in 25 mL of Preston enrichment broth in a microaerobic atmosphere.

Among the three PCR assays examined, Lubeck's 16S rRNA assay had the best combination of sensitivity (82%) and specificity (94%) when compared with culture. However, the data suggest that the putative "false positive" PCR results for this assay may, in fact, represent situations where the PCR method detected organisms that failed to grow in culture.

Table 1
Results of the 25 positive sample sites for *Campylobacter* spp. using different culture conditions.

Positive site-sample number	500 mL				500 mL				2000 mL				2000 mL			
	25 mL PB				100 mL PB				25 mL PB				100 mL PB			
	1st enrich		2nd enrich		1st enrich		2nd enrich		1st enrich		2nd enrich		1st enrich		2nd enrich	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
1	pos	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos
2	pos	neg	pos	neg	pos	neg	pos	neg	pos	pos	pos	pos	pos	pos	pos	pos
3	pos	neg	pos	neg	neg	neg	neg	neg	neg	pos	pos	pos	neg	neg	neg	neg
4	neg	pos	pos	neg	neg	neg	pos	neg	pos	neg	pos	pos	neg	pos	pos	pos
5	pos	neg	pos	neg	neg	pos	pos	pos	neg	pos	pos	pos	pos	pos	neg	pos
6	neg	neg	pos	neg	neg	neg	pos	neg	neg	pos	pos	pos	neg	neg	pos	neg
7	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
8	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
9	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg
10	pos	pos	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	pos	pos	pos
11	neg	pos	neg	pos	neg	neg	neg	neg	pos	pos	pos	pos	pos	neg	pos	neg
12	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
13	neg	pos	neg	pos	neg	neg	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos
14	neg	pos	neg	pos	neg	neg	neg	neg	pos	pos	neg	pos	neg	pos	neg	pos
15	pos	pos	pos	pos	neg	neg	neg	neg	pos	pos	pos	pos	pos	pos	pos	pos
16	neg	neg	neg	neg	neg	neg	neg	pos	pos	neg	pos	neg	neg	neg	neg	neg
17	neg	neg	pos	neg	neg	neg	neg	neg	pos	pos	pos	pos	pos	pos	neg	pos
18	pos	neg	pos	pos	pos	neg	neg	neg	pos	pos	pos	pos	neg	pos	neg	pos
19	neg	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	pos	neg	pos	neg	pos
20	neg	pos	neg	pos	neg	neg	neg	neg	pos	neg	pos	neg	pos	pos	pos	pos
21	neg	neg	neg	neg	pos	pos	neg	neg	pos	pos	pos	pos	pos	pos	neg	pos
22	pos	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	pos	pos	pos	neg
23	neg	pos	pos	pos	neg	pos	neg	pos	neg	neg	pos	pos	neg	pos	neg	pos
24	neg	pos	neg	pos	neg	pos	neg	pos	neg	neg	pos	neg	neg	pos	pos	pos
25	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	pos	neg	neg	pos	neg	pos
Sensitivity (%)	40	56	60	56	32	36	28	36	76	72	84	72	48	76	48	72

neg: negative; pos: positive; PB: Preston broth; and enrich: abbreviation for the enrichment step.

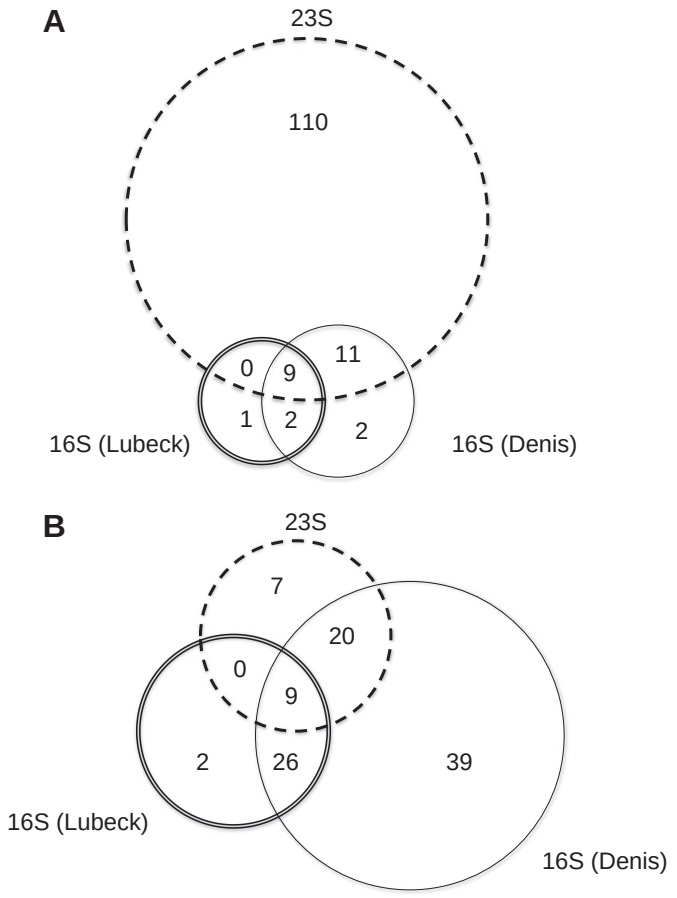

Fig. 3. Venn diagram illustrating the distribution of positive PCR assays among the culture-negative samples (Panel A) and of negative PCR assays among the culture-positive samples (Panel B).

Several studies have examined the factors that facilitate the recovery of organisms from natural waters. Direct inoculation into culture broth is typically less successful than membrane filtration, which can process up to several liters of water and thus concentrate organisms present at low density (Humphrey, 1986). Pre-enrichment at 37 °C for 2–4 h before exposure to selective agents and incubation at 42 °C also significantly increase the isolation of *Campylobacter* spp. from natural waters (Humphrey, 1986, 1989; Koenraad et al., 1994).

A recent study comparing membrane filtration with centrifugation for detecting *Campylobacter* species in 699 surface water samples from four agricultural watersheds across Canada showed that both methods found *C. jejuni* to be the most commonly detected *Campylobacter* species, and that *C. jejuni* frequency of occurrence was similar by both methods (Khan et al., 2009). However, the centrifugation method detected significantly higher frequencies of *C. coli* and other *Campylobacter* species than the membrane filtration method. It was frequently found that one method would detect *Campylobacter* in a water sample when the other method would not for a simultaneously collected, duplicate water sample. This study indicated that methods can have significantly different recovery efficiencies for *Campylobacter* species, and that caution is needed when comparing studies that report on the frequency of occurrence of waterborne *Campylobacter* at the genus level when different detection methods are used. However, as the two methods differed in the volume of water sample processed, enrichment period, the selective agar for isolation, and the genus and species-specific PCR assays used to confirm *Campylobacter* identification, the superiority of centrifugation over membrane filtration was not clearly established.

The total volume of water filtered is widely appreciated to be a critical variable, but an optimal volume for the recovery of *Campylobacter* has not been established. The International Standardisation Organisation draft proposes sample volumes of 100 to 1000 mL for detection of campylobacters in drinking water; Hanninen et al. (2003) suggested that in outbreak investigations several liters should be processed. In one outbreak associated with drinking water, *Campylobacter* spp. were not detected in a 5000-mL sample, but were detected using volumes of 8000- to 10,000-mL. It is unclear if these results represent poor recovery for technical reasons or whether the samples obtained failed to reflect the level of contamination present at the time of the outbreak. Since comparably extremely low levels of *Campylobacter* spp. have also been isolated during surveys of drinking water not implicated in outbreaks, it is difficult to see how such levels can represent a clinically or epidemiologically significant risk.

In addition, it is not logistically possible to filter such large volumes in turbid rivers because of the presence of particulate contaminants. In some cases, we pre-filtered samples using a 1.5 μm membrane to remove most of the particles, although Korhonen and Martikainen (1990) demonstrated that such filters can retain *Campylobacter* spp. cells. To avoid losses due to this effect, we incubated the pre-filtration membrane in the same enrichment broth as the subsequent 0.45 μm membrane, but we did not formally investigate this step.

Although overall 2000 mL gave higher yields, in some instances the 500 mL volume was positive while the concurrent 2000 mL volume was negative under the same culture conditions. Several factors might contribute to such apparent discrepancies. As noted above, with larger volumes, the final cultures might include greater numbers of competitive bacteria that deplete critical nutrients, grow faster than *Campylobacter* spp. organisms, or even actively kill them. Further, the concentration of *Campylobacter* spp. cells in water samples could be very low. In another study, we showed that 82.2% of water samples had a concentration of *Campylobacter* spp. cells ranging from <0.04 MPN/L to 4.0 MPN/L (St-Pierre et al., 2009). Also, the organisms present may be distributed less evenly than in control samples spiked with in vitro grown organisms. These factors increase the probability of finding bacteria in a random 500 mL sample, but not in a concurrent 2000 mL volume.

Following filtration, membranes are typically incubated in an enrichment broth, however few studies have examined the impact of the enrichment procedures (broth volume, incubation duration or serial passage) on the recovery of *Campylobacter* spp. Abulreesh et al. (2005) suggested that for large turbid water samples, a larger volume of enrichment broth (up to 1000 mL) would prevent the interference of background bacteria and allow *Campylobacter* spp. to grow to detectable levels. In our study, for 18 (72%) of the 25 positive site-samples, aliquots processed using 25 mL of PB were more likely to yield *Campylobacter* spp. than those processed using 100 mL of PB. We believe that the larger volume results in lower concentrations of *Campylobacter* organisms in the broth and the lower probability of transferring organisms to the selective agar.

Ribeiro and Price (1984) examined the use of PB for the isolation of thermophilic campylobacters from water and found that the maximum yield was obtained with enrichment for 48 h in a microaerobic atmosphere. Although the yield at 48 h was numerically better than at 24 h, this did not reach statistical significance. However, the yield at 48 h was significantly better than at 72 h and it was felt that the optimal time of enrichment was 48 h.

One study suggested that extending the enrichment time from 24 h to 48 h did not improve the isolation of *Campylobacter* spp. and did increase the growth of contaminants (Korhonen and Martikainen, 1990), which may interfere with the recovery of smaller *Campylobacter* spp. colonies. *Campylobacter* spp. generally reach stationary phase by 24 h and, at this time, nutrients have been consumed (He et al., 2008). In general we observed little difference

between 24 h and 48 h enrichment incubations. However, for 3 (12%) of the 25 positive site-samples (see Table 1, #13, 14, and 23), aliquots incubated for 48 h were almost uniformly positive compared to poor yields from aliquots incubated for 24 h. It is unclear if the *Campylobacter* spp. in these site-samples represent particular genotypes that had longer generation times or required additional time to acclimate to in vitro conditions.

The relative benefits of two enrichment passages are not clear in the literature. Several authors include this procedure (Devane et al., 2005; Korhonen and Martikainen, 1990; Savill et al., 2001; Till et al., 2008), but a significant increase of *Campylobacter* spp. recovery has never been documented. We observed no difference between aliquots processed using one vs. two enrichment broths.

PCR represents a culture-independent approach to detecting *Campylobacter* spp. in the environment and offers several possible advantages, including increased sensitivity with the detection of injured and even non-culturable organisms, greater throughput permitting evaluation of larger numbers of samples, and more rapid results. Several PCR methods for detecting campylobacters in water samples have been described (Hernandez et al., 1995; Jackson et al., 1996; Kirk and Rowe, 1994; Moreno et al., 2003; Oyofo and Rollins, 1993; Sails et al., 2002; Waage et al., 1999), although only a few have been applied to the detection of campylobacters in naturally contaminated waters (Jackson et al., 1996; Moreno et al., 2003; Sails et al., 2002; Waage et al., 1999). Direct PCR amplification from environmental water samples has proved to be difficult due to the extremely low concentrations of *Campylobacter* spp. present (Giesendorf et al., 1993). In addition, the Taq polymerase used in PCR is readily inhibited by factors frequently found in surface waters, such as humic acids, metal ions and high concentrations of non-target DNA (On and Jordan, 2003). Therefore, filtration and enrichment are required prior to extracting bacterial DNA and applying PCR (Hernandez et al., 1995; Waage et al., 1999).

We calculated the sensitivity and specificity of each PCR reaction using culture results as the “gold standard”. However, putative “false positive” results could indicate the presence of viable, potentially infectious organisms that were not recovered under the conditions used. This may be the case for several samples that were positive by the two 16S PCR assays, both of which had reasonable specificity. The apparent “false negative” PCR assays could represent inhibitory substances or technical errors; however, these seem unlikely given that the internal control was visualized in each negative reaction obtained with Lubeck's 16S assay. Stochastic effects may have contributed to the negative PCR assays. A full 200 μL of enrichment broth was cultured, but, because of the need for centrifugation, resuspension and considerable dilution, the PCR assays were seeded with DNA solution that potentially represented fewer organisms.

Based on this extensive investigation we conclude that the optimal conditions for detecting *Campylobacter* spp. in natural waters include a 2000 mL sample volume and a single enrichment broth of 25 mL PB incubated for 48 h. We employed this protocol in the CAMPYLOGIS project, integrating clinical observations, environmental microbiologic data, and a Geographic Information System in an effort to better understand the risk factors involved in sporadic campylobacter infections (St-Pierre et al., 2009).

Acknowledgments

This study was funded by the Centre de Recherche Clinique Etienne Le-Bel du Centre Hospitalier Universitaire de Sherbrooke. We thank the Corporation de Gestion CHARMES for providing water samples. We thank Sylvie Deslandes for assistance in the IAC development and Catherine Desrosiers for providing Taq polymerase. We also thank the Canadian Infectious Disease Society (CIDS) for the Safe Drinking Water/CFID Studentship Award (S.L.).

References

Abulreesh, H.H., Paget, T.A., Goulder, R., 2005. Recovery of thermophilic campylobacters from pond water and sediment and the problem of interference by background bacteria in enrichment culture. Water Res. 39, 2877–2882.
Allerberger, F., Al-Jazrawi, N., Kreidl, P., Dierich, M.P., Feierl, G., Hein, I., Wagner, M., 2003. Barbecued chicken causing a multi-state outbreak of *Campylobacter jejuni* enteritis. Infection 31, 19–23.
Altekruse, S.F., Stern, N.J., Fields, P.I., Swerdlow, D.L., 1999. *Campylobacter jejuni*—an emerging foodborne pathogen. Emerg. Infect. Dis. 5, 28–35.
Arvanitidou, M., Stathopoulos, G.A., Constantinidis, T.C., Katsouyannopoulos, V., 1995. The occurrence of *Salmonella*, *Campylobacter* and *Yersinia* spp. in river and lake waters. Microbiol. Res. 150, 153–158.
Blaser, M.J., 1997. Epidemiologic and clinical features of *Campylobacter jejuni* infections. J. Infect. Dis. 176 (Suppl. 2), S103–S105.
Blaser, M.J., Taylor, D.N., Feldman, R.A., 1983. Epidemiology of *Campylobacter jejuni* infections. Epidemiol. Rev. 5, 157–176.
Bolton, F.J., Coates, D., Hutchinson, D.N., Godfree, A.F., 1987. A study of thermophilic campylobacters in a river system. J. Appl. Bacteriol. 62, 167–176.
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, 327–338.
Carter, J.M., Pacha, R.E., Clark, G.W., Williams, E.A., 1987. Seasonal occurrence of *Campylobacter* spp. in surface waters and their correlation with standard indicator bacteria. Appl. Environ. Microbiol. 53, 523–526.
Comité provincial sur l'entérite à Campylobacter au Québec, 1999. L'entérite à *Campylobacter* au Québec: état de situation et propositions d'action. Comité provincial sur l'entérite à Campylobacter au Québec.
Denis, M., Soumet, C., Rivoal, K., Ermel, G., Blivet, D., Salvat, G., Colin, P., 1999. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. Lett. Appl. Microbiol. 29, 406–410.
Devane, M.L., Nicol, C., Ball, A., Klena, J.D., Scholes, P., Hudson, J.A., Baker, M.G., Gilpin, B.J., Garrett, N., Savill, M.G., 2005. The occurrence of *Campylobacter* subtypes in environmental reservoirs and potential transmission routes. J. Appl. Microbiol. 98, 980–990.
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, 241–257.
Engelke, D.R., Krikos, A., Bruck, M.E., Ginsburg, D., 1990. Purification of *Thermus aquaticus* DNA polymerase expressed in *Escherichia coli*. Anal. Biochem. 191, 396–400.
Eyles, R., Niyogi, D., Townsend, C., Benwell, G., Weinstein, P., 2003. Spatial and temporal patterns of *Campylobacter* contamination underlying public health risk in the Taieri River, New Zealand. J. Environ. Qual. 32, 1820–1828.
Giesendorf, B.A., van Belkum, A., Koeken, A., Stegeman, H., Henkens, M.H., van der Plas, J., Goossens, H., Niesters, H.G., Quint, W.G., 1993. Development of species-specific DNA probes for *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* by polymerase chain reaction fingerprinting. J. Clin. Microbiol. 31, 1541–1546.
Hanninen, M.L., Haajanen, H., Pummi, T., Wermundsen, K., Katila, M.L., Sarkkinen, H., Miettinen, I., Rautelin, H., 2003. Detection and typing of *Campylobacter jejuni* and *Campylobacter coli* and analysis of indicator organisms in three waterborne outbreaks in Finland. Appl. Environ. Microbiol. 69, 1391–1396.
He, Y., Frye, J.G., Strobaugh, T.P., Chen, C.Y., 2008. Analysis of AI-2/LuxS-dependent transcription in *Campylobacter jejuni* strain 81-176. Foodborne Pathog. Dis. 5, 399–415.
Hernandez, J., Alonso, J.L., Fayos, A., Amoros, I., Owen, R.J., 1995. Development of a PCR assay combined with a short enrichment culture for detection of *Campylobacter jejuni* in estuarine surface waters. FEMS Microbiol. Lett. 127, 201–206.
Horman, A., Rimhanen-Finne, R., Maunula, L., von Bonsdorff, C.H., Torvela, N., Heikinheimo, A., Hanninen, M.L., 2004. *Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., noroviruses, and indicator organisms in surface water in southwestern Finland, 2000–2001. Appl. Environ. Microbiol. 70, 87–95.
Humphrey, T.J., 1986. Techniques for the optimum recovery of cold injured *Campylobacter jejuni* from milk or water. J. Appl. Bacteriol. 61, 125–132.
Humphrey, T.J., 1989. An appraisal of the efficacy of pre-enrichment for the isolation of *Campylobacter jejuni* from water and food. J. Appl. Bacteriol. 66, 119–126.
Hutchinson, D.N., Bolton, F.J., Hinchliffe, P.M., Dawkins, H.C., Horsley, S.D., Jessop, E.G., Robertshaw, P.A., Counter, D.E., 1985. Evidence of udder excretion of *Campylobacter jejuni* as the cause of milk-borne campylobacter outbreak. J. Hyg. (Lond.) 94, 205–215.
Jackson, C.J., Fox, A.J., Jones, D.M., 1996. A novel polymerase chain reaction assay for the detection and speciation of thermophilic *Campylobacter* spp. J. Appl. Bacteriol. 81, 467–473.
Jones, I.G., Roworth, M., 1996. An outbreak of *Escherichia coli* O157 and campylobacteriosis associated with contamination of a drinking water supply. Public Health 110, 277–282.
Jones, D.M., Sutcliffe, E.M., Curry, A., 1991. Recovery of viable but non-culturable *Campylobacter jejuni*. J. Gen. Microbiol. 137 (Pt 10), 2477–2482.
Khan, I.U., Gannon, V., Loughborough, A., Jokinen, C., Kent, R., Koning, W., Lapen, D.R., Medeiros, D., Miller, J., Neumann, N., Phillips, R., Robertson, W., Schreier, H., Topp, E., van Bochove, E., Edge, T.A., 2009. A methods comparison for the isolation and detection of thermophilic *Campylobacter* in agricultural watersheds. J. Microbiol. Methods 79, 307–313.
Kirk, R., Rowe, M.T., 1994. A PCR assay for the detection of *Campylobacter jejuni* and *Campylobacter coli* in water. Lett. Appl. Microbiol. 19, 301–303.
Koenraad, P., Hazeleger, W.C., Van der Laan, T., Beumer, R.R., Rombouts, F.M., 1994. Survey of *Campylobacter* spp. in sewage plants in the Netherlands. Food Microbiol. 11, 65–73.

Korhonen, L.K., Martikainen, P.J., 1990. Comparison of some enrichment broths and growth media for the isolation of thermophilic campylobacters from surface water samples. J. Appl. Bacteriol. 68, 593–599.
Levesque, S., Martel, J., Frost, E., Michaud, S., 2005. Prevalence of *Campylobacter* in river and tributary water in the Eastern Townships, Québec, Canada. Presented to the 13th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms. Gold Coast Queensland, Australia, September 5, 2005.
Lubeck, P.S., Wolffs, P., On, S.L., Ahrens, P., Radstrom, P., Hoorfar, J., 2003. Toward an international standard for PCR-based detection of food-borne thermotolerant campylobacters: assay development and analytical validation. Appl. Environ. Microbiol. 69, 5664–5669.
Martikainen, P.J., Korhonen, L., Kosunen, T.U., 1990. Occurrence of thermophilic campylobacters in rural and urban surface waters in central Finland. Water Res. 24, 91–96.
Miller, W.G., Mandrell, R.E., 2005. Prevalence of *Campylobacter* in the food and water supply: incidence, outbreaks and detection. In: Ketley, J.A., Konkel, M.E. (Eds.), *Campylobacter*: Molecular and Cellular Biology. Horizon Bioscience, Wymondham, Norfolk, U.K., pp. 101–163.
Moore, J.E., Caldwell, P.S., Millar, B.C., Murphy, P.G., 2001. Occurrence of *Campylobacter* spp. in water in Northern Ireland: implications for public health. Ulster Med. J. 70, 102–107.
Moreno, Y., Botella, S., Alonso, J.L., Ferrus, M.A., Hernandez, M., Hernandez, J., 2003. Specific detection of *Arcobacter* and *Campylobacter* strains in water and sewage by PCR and fluorescent in situ hybridization. Appl. Environ. Microbiol. 69, 1181–1186.
Nachamkin, I., 2003. *Campylobacter* and *Arcobacter*. In: Murray, P.R., Baron, E.J., Pfaller, M.A., Jorgensen, J.H., Yolken, R.H. (Eds.), Manual of Clinical Microbiology, 8th ed. American Society for Microbiology, Washington, D.C., pp. 902–914.
Obiri-Danso, K., Jones, K., 1999. Distribution and seasonality of microbial indicators and thermophilic campylobacters in two freshwater bathing sites on the River Lune in northwest England. J. Appl. Microbiol. 87, 822–832.
Olson, C.K., Ethelberg, S., Van Pelt, W., Tauxe, R.V., 2008. Epidemiology of *Campylobacter jejuni* infections in industrialized nations. In: Nachamkin, I., Szymanski, C.M., Blaser, M.J. (Eds.), *Campylobacter*. ASM Press, Washington, D.C., U.S.A., pp. 163–189.
On, S.L., Jordan, P.J., 2003. Evaluation of 11 PCR assays for species-level identification of *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. 41, 330–336.
Oyofo, B.A., Rollins, D.M., 1993. Efficacy of filter types for detecting *Campylobacter jejuni* and *Campylobacter coli* in environmental water samples by polymerase chain reaction. Appl. Environ. Microbiol. 59, 4090–4095.
Pebody, R.G., Ryan, M.J., Wall, P.G., 1997. Outbreaks of campylobacter infection: rare events for a common pathogen. Commun. Dis. Rep. CDR Rev. 7, R33–R37.
Piche, A., Bourgaux, P., 1987. Resolution of a polyomavirus-mouse hybrid replicon: release of genomic viral DNA. J. Virol. 61, 840–844.
Ribeiro, C.D., Price, T.H., 1984. The use of Preston enrichment broth for the isolation of 'thermophilic' campylobacters from water. J. Hyg. (Lond.) 92, 45–51.
Rollins, D.M., Colwell, R.R., 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. 52, 531–538.
Said, B., Wright, F., Nichols, G.L., Reacher, M., Rutter, M., 2003. Outbreaks of infectious disease associated with private drinking water supplies in England and Wales 1970–2000. Epidemiol. Infect. 130, 469–479.
Sails, A.D., Bolton, F.J., Fox, A.J., Wareing, D.R., Greenway, D.L., 2002. Detection of *Campylobacter jejuni* and *Campylobacter coli* in environmental waters by PCR enzyme-linked immunosorbent assay. Appl. Environ. Microbiol. 68, 1319–1324.
Savill, M.G., Hudson, J.A., Ball, A., Klena, J.D., Scholes, P., Whyte, R.J., McCormick, R.E., Jankovic, D., 2001. Enumeration of *Campylobacter* in New Zealand recreational and drinking waters. J. Appl. Microbiol. 91, 38–46.
Skirrow, M.B., Fidoe, R.G., Jones, D.M., 1981. An outbreak of presumptive food-borne campylobacter enteritis. J. Infect. 3, 234–236.
Skjerve, E., Brennhovd, O., 1992. A multiple logistic model for predicting the occurrence of *Campylobacter jejuni* and *Campylobacter coli* in water. J. Appl. Bacteriol. 73, 94–98.
Slater, E.R., Owen, R.J., 1997. Restriction fragment length polymorphism analysis shows that the hippuricase gene of *Campylobacter jejuni* is highly conserved. Lett. Appl. Microbiol. 25, 274–278.
Stehr-Green, J.K., Nicholls, C., McEwan, S., Payne, A., Mitchell, P., 1991. Waterborne outbreak of *Campylobacter jejuni* in Christchurch: the importance of a combined epidemiologic and microbiologic investigation. N. Z. Med. J. 104, 356–358.
Stelzer, W., Mochmann, H., Richter, U., Dobberkau, H.J., 1989. A study of *Campylobacter jejuni* and *Campylobacter coli* in a river system. Zentralbl. Hyg. Umweltmed. 189, 20–28.
St-Pierre, K., Lévesque, S., Frost, E., Carrier, N., Arbeit, R.D., Michaud, S., 2009. Thermotolerant coliforms are not a good surrogate for *Campylobacter* spp. in environmental water. Appl. Environ. Microbiol. 75, 6736–6744.
Sylla, B.S., Allard, D., Roy, G., Bourgaux-Ramoisy, D., Bourgaux, P., 1984. A mouse DNA sequence that mediates integration and excision of polyoma virus DNA. Gene 29, 343–350.
Thomas, M.K., Majowicz, S.E., Sockett, P.N., Fazil, A., Pollari, F., Dore, K., Flint, J.A., Edge, V.L., 2006. Estimated numbers of community cases of illness due to *Salmonella*, *Campylobacter* and verotoxigenic *Escherichia coli*: pathogen-specific community rates. Can. J. Infect. Dis. Med. Microbiol. 17, 229–234.
Till, D., McBride, G., Ball, A., Taylor, K., Pyle, E., 2008. Large-scale freshwater microbiological study: rationale, results and risks. J. Water Health 6, 443–460.
Vogt, R.L., Sours, H.E., Barrett, T., Feldman, R.A., Dickinson, R.J., Witherell, L., 1982. Campylobacter enteritis associated with contaminated water. Ann. Intern. Med. 96, 292–296.
Waage, A.S., Vardund, T., Lund, V., Kapperud, G., 1999. Detection of small numbers of *Campylobacter jejuni* and *Campylobacter coli* cells in environmental water, sewage, and food samples by a seminested PCR assay. Appl. Environ. Microbiol. 65, 1636–1643.
Wang, G., Clark, C.G., Taylor, T.M., Pucknell, C., Barton, C., Price, L., Woodward, D.L., Rodgers, F.G., 2002. Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. J. Clin. Microbiol. 40, 4744–4747.