

# Recovery of thermophilic campylobacters from pond water and sediment and the problem of interference by background bacteria in enrichment culture

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## Abstract

The aim of this study was to address problems in the determination of thermophilic campylobacters in turbid pond water and sediment. Thirty sets of three samples of pond water (volumes 10, 100, 1000 ml) or sediment (0.1, 1.0, 5.0 ml) were examined for the presence of thermophilic campylobacters. The different volumes of pond water were processed by membrane filtration followed by selective enrichment. The samples of sediment were subjected directly to selective enrichment. Presumptive isolates were confirmed by Gram stain, cell morphology, presence of oxidase and catalase, growth under microaerobic but not aerobic conditions, and PCR. Confirmed *Campylobacter* species were recovered only from 10 and 100 ml samples of water and from 0.1 and 1.0 ml samples of sediments. The 1000 ml samples of water and 5.0 ml samples of sediment never gave positive isolates. PCR indicated that the confirmed isolates were all either *Campylobacter jejuni* or *C. coli*. Enrichment cultures from 1000 ml filtrations contained the highest number of background bacteria. It is suggested that the processing of large volumes of turbid environmental water samples or of sediment is counterproductive and may not yield positive *Campylobacter* cultures. This is probably due to antagonistic effects of large numbers of background bacteria out-competing campylobacters during the enrichment stage.

Pilot studies to establish appropriate volumes of pond water or sediment samples should be undertaken before routine determination of campylobacters is begun.

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## 1. Introduction

*Campylobacter* is possibly the leading worldwide cause of acute bacterial gastroenteritis (Thomas et al., 1999; Frost, 2001). In England and Wales in

2001 there were around 60,000 reported cases of gastroenteritis caused by *Campylobacter* (Humphrey, 2002). The true incidence of food-borne and water-borne campylobacteriosis may be under-reported since a study on infectious intestinal diseases in the UK showed that there might be around 500,000 cases of *Campylobacter* infection (Humphrey, 2002). *Campylobacter* species that are implicated in water-borne and food-borne human infections are *Campylobacter jejuni* and *C. coli* (Percival et al., 2004).

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Undercooked meat, raw milk and contaminated water are potential sources of *Campylobacter* infection; handling of pets (e.g. dogs and cats) may also be a source of infection (Bolton et al., 1987; Baker et al., 1999; Frost, 2001).

The consumption of untreated or contaminated drinking water is the most common vehicle in large *Campylobacter*-associated outbreaks around the world (Leclerc et al., 2004). In UK, *Campylobacter* has been the main cause of private water supply outbreaks where water was not adequately disinfected (Furtado et al., 1998). *Campylobacters* are widespread in the environment. Sewage effluents, livestock farming and wild birds are established sources of *Campylobacter* to environmental water as well as drinking water (Jones, 2001).

The United Kingdom Public Health Laboratory Service (PHLS) standard protocol for the detection of presumptive campylobacters in all types of water (i.e. drinking and recreational) recommends the membrane filtration of 1000 ml samples followed by overnight incubation in selective enrichment broth (Anon, 1998). The confirmation of presumptive isolates is conventionally based on a range of morphological and biochemical tests. Recently, PCR has been extensively applied to the detection and identification of a wide range of pathogenic bacteria including *Campylobacter* species (Waage et al., 1999). PCR primers that amplify a conserved region of the *flaA* and *flaB* genes have been successfully applied to the detection of *Campylobacter jejuni* and *C. coli* (Oyfo et al., 1992; Wegmüller et al., 1993).

Amenity ponds in parks and villages that harbour large populations of waterfowl are usually of poor microbiological quality and could pose a threat to public health as a source for enteric pathogens. Abulreesh et al. (2004) found that water samples from amenity ponds with waterfowl had high abundance of background bacteria; for example, in a pond at South Dalton, which is one of the ponds examined in the present study, heterotrophic plate counts ranged from 1.6 to  $3.3 \times 10^5 \text{ ml}^{-1}$  while direct counts of bacteria made by epifluorescence microscopy ranged from  $3.4$  to  $7.4 \times 10^7 \text{ ml}^{-1}$ . Also they found that faecal indicator bacteria and *Salmonella* were related to waterfowl populations in amenity ponds but they failed to detect *Campylobacter*. They suspected that the apparent absence of *Campylobacter* was related to the shortcomings of the PHLS standard method for *Campylobacter* determination. The aim of the present work was to develop the UK PHLS (Anon, 1998) protocol for the detection of presumptive thermophilic campylobacters to allow its application to turbid pond water and bottom sediments, and to evaluate PCR for the rapid confirmation of campylobacters from these habitats.

## 2. Materials and methods

### 2.1. Sites and sampling

The study sites were three roadside village ponds in East Yorkshire, NE England. All three ponds have resident semi-tame ducks, and sometimes geese, and receive run-off from adjacent roads. All three ponds are amenity sites. The ponds are at Brantingham (National Grid Reference SE 941 296, pond area 240 m<sup>2</sup>), Little Weighton (SE 988 338, 640 m<sup>2</sup>), and South Dalton (SE 969 454, 2410 m<sup>2</sup>). The waters of these ponds are turbid and aquatic vegetation is sparse or absent.

Water and sediment samples were collected in the morning during August–December 2003. Surface water was collected into sterile polypropylene bottles; superficial sediment was scooped from shallow water areas, with depth of less than 10 cm, and transferred to sterile 30 ml universal bottles. All samples were kept on ice and in darkness during transportation; microbiological assays were begun on the same day as sampling.

### 2.2. Detection and confirmation of thermophilic campylobacters

Membrane filtration techniques were employed for the detection of thermophilic campylobacters in water samples, following the standard methods for the examination of water as used by the Public Health Laboratory Service (Anon, 1998). Water samples (10, 100, 1000 ml) were filtered through 0.45 µm, diameter 47 mm, cellulose nitrate and mixed ester membrane filters (Labsales, Cambridge, UK). Membranes were transferred to 150 ml of Preston enrichment broth (Oxoid, Basingstoke, UK), containing 5% lysed horse blood (Oxoid) and growth supplement consisting of ferrous sulphate (0.125 g l<sup>-1</sup>), sodium metabisulphite (0.125 g l<sup>-1</sup>) and sodium pyruvate (0.125 g l<sup>-1</sup>) (FBP) (Oxoid), and incubated for 22 h at 37 °C, followed by 22 h at 42 °C. Microaerobic conditions were achieved by incubation in screw-top bottles with minimal air-space (less than 1.0 ml). Broth cultures were then subcultured onto blood-free *Campylobacter*-selective modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Oxoid) and incubated in a microaerobic atmosphere (N<sub>2</sub> 85%, O<sub>2</sub> 5%, CO<sub>2</sub> 10%) using Oxoid CampyGen, at 37 °C for 48 h.

Aliquots of 1.0 ml of ten times diluted sediment, and 1.0 ml, and 5.0 ml of undiluted sediment samples were transferred to sterile 30 ml universal bottles. Preston enrichment broth was added and the procedure was then continued as described above.

Presumptive colonies were subjected to the following confirmatory tests: Gram stain with 0.85% carbol fuchsin as counter stain; cell morphology; presence of

oxidase and catalase; growth on blood agar at 37 °C under microaerobic but not aerobic conditions and finally PCR.

### 2.3. Determination of heterotrophic plate counts and coliform counts

Spread plate counts of culturable heterotrophic bacteria and coliforms in *Campylobacter* enrichment cultures were made on Nutrient Agar (Oxoid) and MacConkey Agar (Oxoid) respectively. Ten replicate plates were inoculated with 0.1 ml sub-samples of serially diluted enrichment broth and were incubated at 25 °C for 48 h for the heterotrophic plate counts and at 37 °C for 24 h for the coliform counts.

### 2.4. Direct counts of total bacteria

Counts of total bacteria in *Campylobacter* enrichment cultures were performed using epifluorescence microscopy after acridine-orange staining (Hobbie et al., 1977). Stained bacteria were concentrated on black 0.2 µm polycarbonate filters (Labsales) and at least 600 cells per preparation were counted at ×1250 magnification.

### 2.5. PCR assay

PCR was performed on the presumptive colonies to confirm that they were thermophilic campylobacters. DNA was extracted by suspending a loop of presumptive *Campylobacter* colony in 100 µl sterile, pure water and boiling for 5 min. The suspension was then centrifuged for 5 min at 1260 *g* and 10 µl of the supernatant were used as target DNA. Primers CF03 and CF04 (Invitrogen, Paisley, UK) from the *Campylobacter jejuni flaA* and *flaB* gene sequence were used (Wegmüller et al., 1993). Their sequences are: CF03, 5'-GCT CAA AGT GGT TCT TAT GCN ATG G-3' (forward); CF04, 5'-GCT GCG GAG TTC ATT CTA AGA CC-3' (reverse).

The PCR reaction mixture (50 µl total volume) contained: 0.25 µmol l<sup>-1</sup> of each primer; 75 mmol l<sup>-1</sup> Tris-HCl; 20 mmol l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 2.0 mmol l<sup>-1</sup> MgCl<sub>2</sub>; 0.01% (v/v) Tween<sup>®</sup> 20; 0.2 mmol l<sup>-1</sup> each of dATP, dCTP, dGTP, and dTTP; 1.25 units of Thermoprime Plus DNA Polymerase (ABgene, Surrey, UK). DNA amplification used the following temperature cycle: denaturation at 94 °C for 4 min, 30 cycles at 95 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 5 min. A total of 10 µl of PCR products was analysed by 1.5% (w/v) agarose gel (BioLine, London, UK) electrophoresis and made visible by ethidium bromide (0.5 µg ml<sup>-1</sup>) staining and UV transillumination.

### 2.6. Statistical analysis

To test the null hypothesis that enrichments from different volumes filtered did not have different concentrations of background bacteria the Kruskal–Wallis non-parametric test was used.

## 3. Results

Thirty sets of three samples of pond water (volumes 10, 100, 1000 ml) or sediment (0.1, 1.0, 5.0 ml) were examined for the presence of thermophilic campylobacters. For pond water samples, presumptive thermophilic campylobacters were recovered only from 10 and 100 ml samples. Presumptive *Campylobacter* isolates were never recovered following the filtration of 1000 ml samples (Table 1). For sediment samples, presumptive *Campylobacter* isolates were only recovered following the enrichment of 0.1 and 1.0 ml samples. Presumptive thermophilic campylobacters were never recovered from 5.0 ml samples (Table 1).

Heterotrophic plate counts, coliform counts and acridine-orange direct microscopic counts (AODC) of total bacteria were performed on enrichment cultures derived from the filtration of water samples. The counts were made at the end of enrichment-culture incubations (44 h). All *Campylobacter*-negative cultures (from 1000 ml filtration) had higher levels of heterotrophic bacteria and coliforms than positive cultures (10, 100 ml)

Table 1  
The recovery of thermophilic campylobacters from pond water and sediment

Location of pond	Volume of water filtered		
	10 ml n:p (%)	100 ml n:p (%)	1000 ml n:p (%)
South Dalton	6:5 (83)	6:6 (100)	6:0 (0)
Little Weighton	6:2 (33)	6:2 (33)	6:0 (0)
Brantingham	6:2 (33)	6:2 (33)	6:0 (0)
Total for all ponds	18:9 (50)	18:10 (56)	18:0 (0)
	Volume of sediment used for inoculation		
	0.1 ml n:p (%)	1.0 ml n:p (%)	5.0 ml n:p (%)
South Dalton	4:2 (50)	4:2 (50)	4:0 (0)
Little Weighton	4:0 (0)	4:1 (25)	4:0 (0)
Brantingham	4:0 (0)	4:0 (0)	4:0 (0)
Total for all ponds	12:2 (17)	12:3 (25)	12:0 (0)

*n* = total number of samples; *p* = number of positive samples (percentage of positive samples in brackets). All presumptive isolates were confirmed as *Campylobacter*.

Table 2

Heterotrophic plate counts, coliform counts and acridine-orange direct counts (AODC) in *Campylobacter* enrichment cultures prepared using pond water samples

	Volume of water filtered			<i>P</i>
	10 ml Mean (range) <i>n</i>	100 ml Mean (range) <i>n</i>	1000 ml Mean (range) <i>n</i>	
Heterotrophic plate count $\times 10^6$ cfu ml <sup>-1</sup>	0.55 (0.013–0.98) 4	1.1 (0.66–1.6) 4	6.4 (1.8–11) 4	<0.05
Coliforms $\times 10^6$ cfu ml <sup>-1</sup>	0.13 (0.12–0.14) 4	0.69 (0.66–0.72) 4	1.6 (1.4–1.8) 4	<0.05
AODC $\times 10^9$ ml <sup>-1</sup>	0.036 (0.032–0.038) 4	0.083 (0.071–0.089) 4	5.8 (5.5–6.1) 4	<0.05

*P* is the probability that there is no difference between the number of bacteria in enrichment culture inoculated with the residue from filtration of different sample volumes (Kruskal–Wallis test), *n* = number of samples.

(Table 2). The count of total bacteria in *Campylobacter*-negative enrichments was many fold higher than in positive cultures (Table 2). The Kruskal–Wallis test showed that there was significant difference in heterotrophic plate counts, coliforms, and total bacteria between enrichments set up using different volumes filtered (Table 2).

All 24 presumptive isolates from pond water and sediment were found to be Gram negative, gull-wing shaped, both oxidase and catalase positive, and they grew on blood agar incubated at 37 °C under micro-aerobic, but not aerobic conditions. Thus according to conventional criteria they all were confirmed as *Campylobacter* species. DNA extracted from all 24 confirmed isolates was successfully amplified by PCR. The amplified products were between 340 and 380 bp in length. This reinforced the confirmation that these isolates were thermophilic campylobacters.

#### 4. Discussion

Thermophilic campylobacters are thought to be ubiquitous in aquatic environments, but their detection can be difficult. This is because the organism may be present in low numbers and/or sub-lethally injured. To overcome these problems, the detection of *Campylobacter* from environmental waters involves initial concentration on a membrane filter followed by incubation of the filters in an enrichment broth (Hunter, 1997; Percival et al., 2004).

In the present study, a combination of a selective enrichment procedure and selective plating was used. This approach has been reported to increase the recovery of campylobacters from environmental waters (Rosef et al., 2001). Preston enrichment broth and mCCDA selective agar were used as selective media in the current study; the successive use of these two media was suggested to be useful for the determination of thermophilic campylobacters from environmental sam-

ples (e.g. water), where *Campylobacter* numbers are low (Corry et al., 1995). The initial incubation of enrichment cultures at 37 °C followed by selective incubation at 42 °C and selective plating seems to significantly enhance the recovery of sub-lethally injured thermophilic campylobacters from environmental waters (Humphrey and Muscat, 1989).

Since the number of thermophilic campylobacters in environmental water is believed often to be low, the examination of a large water volume seems to be an obvious way of increasing recovery (Bolton et al., 1982; Hänninen et al., 2003). Therefore, the standard PHLS protocol recommends the filtration of 1000 ml of water. The results obtained in the present study, however, showed that the filtration of a large volume (1000 ml) of turbid pond water never yielded *Campylobacter* isolates (Table 1). These were clearly false-negative results, since the filtration of smaller volumes (10 or 100 ml) of water from the same sites frequently gave positive results (Table 1). Since the *Campylobacter*-negative cultures from 1000 ml filtration had high levels of heterotrophic bacteria, coliforms and total bacterial populations (Table 2), it is likely that high levels of background bacteria competing for nutrients prevented the growth of *Campylobacter* during the enrichment procedure. Similarly, Aquino et al. (1996) found that the majority of *Campylobacter*-negative cultures from heavily contaminated poultry products, had high levels of faecal indicators and lactobacilli, which may have hindered the recovery of campylobacters. Thus, the seeding of enrichment culture with the residue from a large volume of turbid water (1000 ml), may lead to overloading by background bacteria and the out-competition of *Campylobacter* to the extent that it is unable to grow to detectable levels. Fricker (1987) reviewed various methods that are used to isolate *Campylobacter* from environmental samples and suggested that overloading by background microflora may prevent the growth of campylobacters during the enrichment stage; this suggestion is supported by our results.

Similar observations were obtained from our examination of sediment samples. A large volume (5.0 ml) of sediments did not give any presumptive campylobacters (Table 1). The concentration of bacteria in aquatic sediment has been reported to be around a hundred times higher than in overlying water (Cavallo et al., 1999), thus we suggest that the use of a large inoculum of microbially rich sediment to assess the presence of campylobacters may lead to false-negative results, probably because of competition by the background microflora.

It may be suggested that in the processing of large volumes of turbid water (1000 ml), the use of a larger volume of enrichment broth (500 or 1000 ml) per sample would prevent the interference of background bacteria and allow *Campylobacter* to grow to detectable levels. However, for a laboratory carrying out routine investigations, such a procedure would be both expensive and impractical, especially when smaller volumes of enrichment broth (150 ml) give positive results provided that appropriate volumes of water are processed, as is shown in this study.

The *Campylobacter* flagellum is believed to be a significant virulence factor (Percival et al., 2004). In this study, we used a PCR protocol that amplifies the intergenic sequence between the *Campylobacter* flagellin genes, *flaA* and *flaB*, as a rapid confirmatory test on *Campylobacter* presumptive isolates. We choose the primers CF03 and CF04 (Wegmüller et al., 1993) because of their specificity for detecting *Campylobacter jejuni* and *C. coli*. Our results showed that the amplification of DNA extracted from all 24 presumptive *Campylobacter* isolates, and also from a clinical isolate, produced PCR fragments of 340–380 bp in length. Thus, all presumptive isolates were confirmed as *Campylobacter* spp. and we conclude that they were either *C. jejuni* or *C. coli* on the basis of the work described by Wegmüller et al. (1993) and Waage et al. (1999), who showed that the amplification with CF03 and CF04 gives 340–380 bp fragments with *C. jejuni* and *C. coli*.

## 5. Conclusions

Standard methods for the detection of *Campylobacter* spp. recommend the processing of water samples of large volume. Our results suggest, however, that the filtration of large volumes of turbid environmental water, or of sediments, may be counterproductive and may not yield presumptive campylobacters. Pilot studies to establish appropriate volumes of environmental water or sediment samples should be undertaken before routine determination of *Campylobacter* is begun.

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