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# A pre-enrichment step is essential for detection of *Campylobacter* sp. in turbid pond water

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**Abstract.** This work aimed to detect *Campylobacter* species from naturally contaminated turbid pond water by PCR. A total of 16 water samples were collected from a turbid village pond. Four methods of DNA extraction were applied to centrifuge pellets from eight 100 ml pond water samples prior to attempted detection of *Campylobacter* by PCR without an enrichment step. These methods were (1) Tris-HCl and sodium dodecyl sulfate followed by phenol:chloroform:isoamylalcohol extraction followed by treatment with DNA clean up kit, (2) proteinase K, (3) Chelex® 100, and (4) boiling. The other eight pond water samples (10 ml and 100 ml) were filtered and filters were incubated overnight in Preston enrichment broth. The centrifuge pellets obtained from enrichment cultures were treated by proteinase K for DNA extraction. Primers CF03 and CF04 for the flagellin genes (*fla*A and *fla*B) of *Campylobacter jejuni* and *Campylobacter coli* were used for amplifying the extracted DNA. The DNA extracted from eight-100 ml pond water samples that were not subject to selective enrichment was never amplified with primers CF03 and CF04, hence *Campylobacter* was not detected. In contrast, the DNA that was from samples that were subjected to a selective enrichment step in Preston broth prior to PCR assay always gave amplified bands of 340-380 bp, therefore the presence of *Campylobacter* was confirmed. Detection of campylobacters from naturally contaminated, turbid, environmental water may not be feasible by direct PCR assay because of low numbers and the presence of high concentration of humic matter and other PCR inhibitors. The enrichment of water samples in selective broth, however, facilitated PCR detection of *Campylobacter* probably by increasing cell number and by diluting PCR inhibitors.

## INTRODUCTION

The routine microbiological detection of *Campylobacter* species from environmental waters by culture methods is laborious, time consuming and usually faces few limitations. Recently, there is growing interest in the development of molecular methods and their application to detect and identify campylobacters from both clinical and environmental samples. Molecular methods such as PCR have been suggested to provide rapid, specific and high sensitive tools for the detection of campylobacters in almost all types of samples (e.g. environmental and clinical) (Abulreesh *et al.*, 2006).

Various PCR protocols, based on the use of various sets of primers were developed and reported to detect campylobacters from artificially contaminated water and wastewater (Alexandrino *et al.*, 2004), food (Winters *et al.*, 1998), and also from naturally contaminated animal faeces (Rodgers *et al.*, 2012). It is important to mention that all above-mentioned reports described the application of PCR assays on artificially contaminated environmental samples. While the goal and real value of developing PCR

protocols is its applicability and efficacy to detect *Campylobacter* species, as well other pathogenic bacteria, from naturally contaminated environmental samples.

The principle aim of the current work, therefore, was to describe a sensitive PCR assay for direct detection of campylobacters from turbid village-pond water.

## MATERIALS AND METHODS

### Site and sampling

The study site was a roadside village pond in East Yorkshire, NE England (National Grid Reference SE 969 454, pond area 2410 $m^2$). The pond harbours permanent population of ducks, receives runoff from adjacent roads, and is amenity site. The water of the pond was turbid and aquatic vegetation is sparse or absent. Surface water was collected into sterile polypropylene bottles. All samples were kept on ice and in darkness during transportation; microbiological and molecular assays were begun on the same day as sampling.

### PCR assay for direct detection of *Campylobacter* in pond water

A PCR assay was applied directly to eight 100 ml pond water samples. Four methods were used to extract genomic DNA from water samples, these were (1) bead-beating method as described by Ogram (1998) using a Wizard DNA Clean Up Resin Kit (Promega, Madison, USA), (2) Proteinase K (Sigma, St. Louis, USA), (3) boiling and (4) Chelex® 100 (Bio-Rad, Hercules, USA). The pellets were prepared by centrifugation of 100 ml of water samples at 10000 RCF for 10 min. The supernatant was discarded and the pellets were washed and re-centrifuged twice in 500 µl phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g $KH_2PO_2$, in 800 ml sterile pure water [pH 7.4]) (Sambrook *et al.*, 1989). A 340-380 bp fragment of the *Campylobacter jejuni* and *Campylobacter coli fla*A/*fla*B gene sequence (Wegmüller *et al.*, 1993) was amplified using primers CF03 (5'-GCT CAA AGT GGT TCT TAT GCN ATG G-3') and CF04 (5'-GCT GCG GAG TTC ATT CTA AGA CC-3') (Invitrogen, Paisley, UK) as described by Abulreesh *et al.* (2005).

### PCR detection limits

In order to determine the least number of *Campylobacter* cells that can be detected by the PCR method employed. DNA was extracted and amplified from sterile pure water and fresh pond water that had both been spiked with known concentrations of *Campylobacter* cells. The *Campylobacter* isolates used for spiking water samples were previously isolated from the pond at South Dalton by membrane filtration and selective enrichment, and confirmed as either *C. jejuni* or *C. coli* by colony-PCR as described by Abulreesh *et al.* (2005).

A thick suspension of *Campylobacter* cells was prepared and serially diluted. A volume of 1.0 ml from each dilution was added to 100 ml of sterile pure water and fresh pond water. *Campylobacter* concentrations in each spiked sample were calculated using epifluorescence microscopy after acridine-orange staining (Hobbie *et al.*, 1977). Stained bacteria were concentrated at black 0.2 µm polycarbonate filters (Labsales, Cambridge, UK) and at least 600 cells per preparation were counted at x 1250 magnification.

Spiked samples (100 ml) were centrifuged at 10,000 RCF for 10 min and washed as described above. Microbial DNA was extracted from each spiked sample by Proteinase K, boiling, and Chelex® 100 as described above.

### Detection of *Campylobacter* in un-spiked pond water by using PCR after selective enrichment culture

Pond water samples of different volumes (10 ml, 100 ml, 1000 ml) were filtered and the filters were incubated overnight in Preston enrichment broth as described by Abulreesh *et al.* (2005). After enrichment period, 500 µl of each enrichment culture were transferred to a sterile microcentrifuge tube, and spun at 10,000 RCF for 10 min. The supernatant was discarded and the pellets were washed twice in 500 µl of phosphate buffered saline. DNA was extracted from the pellets by the Proteinase K method. Template DNA was

amplified by PCR as previously described. At the same time, a loopful of each enrichment was plated on mCCD agar to confirm the presence of campylobacters in pond water by conventional culture techniques and by PCR applied to colonies on the mCCD agar.

## RESULTS

Four different methods of DNA extraction were performed on pellets prepared from eight 100 ml pond water samples that had suffered natural contamination by campylobacters. Despite the successful extraction of DNA from pond water, no PCR amplification with primers CF03 and CF04 was obtained. Thus, all eight-100 ml pond water samples were recorded as *Campylobacter*-negative by the direct PCR approach. The detection of *Campylobacter* by the conventional enrichment method in two 100 ml samples of pond water, however, yielded positive results (Table 1).

The investigation of detection limits of PCR showed that when the Proteinase K extraction method was used as few as 50 *Campylobacter* cells per 1.0 ml were detected in spiked sterile pure water by direct PCR. The lower limit of PCR detection in spiked pond water following Proteinase K extraction was around 400 *Campylobacter* cells per 1.0 ml.

The DNA extracted by the Proteinase K method from spiked sterile pure water and from spiked fresh pond water was frequently amplified with primers CF03 and CF04. In contrast, the treatment of pellets by boiling as a method of DNA extraction gave fewer positive PCR amplifications. The DNA extracted by Chelex® 100 gave even poorer PCR amplification. Thus DNA from spiked fresh pond water was never amplified, while DNA extracted by Chelex® 100 from sterile pure water amplified only when the highest concentration of spike ($5 \times 10^5$ $ml^{-1}$) was used. A summary of the results of direct PCR detection on pure water and fresh pond water that had been spiked with *Campylobacter* cells is given in Table 2.

PCR amplification of DNA from pellets harvested from Preston enrichment broth culture was always obtained when the enrichments were inoculated with the residue on filters that were from the filtration of 100 ml of pond water. In contrast, PCR amplification was only sometimes obtained when 10 ml samples of pond water were filtered and was never obtained when 1000 ml samples were filtered (Table 3). When PCR amplification was obtained using DNA from the enrichment broth, the DNA from colonies on mCCD agar also gave positive PCR amplification (Table 3).

## DISCUSSION

In the present work, the direct application of PCR to fresh turbid pond water, to detect campylobacters was investigated. DNA that was extracted from eight 100 ml water samples, by four different methods, failed to give PCR amplification with primers CF03 and CF04 (Table 1). This negative result may have been due to the presence of humic

Table 1. The detection of naturally-occuring *Campylobacter* in 100 ml pond water samples by direct PCR and by enrichment culture

| Method used | Total number of samples† | Number of positive samples |
|---|---|---|
| Bead-beating and direct PCR | 2 | 0 |
| Proteinase K and direct PCR | 2 | 0 |
| Boiling and direct PCR | 2 | 0 |
| Chelex® 100 and direct PCR | 2 | 0 |
| Enrichment culture and selective plating | 2 | 2 |

Table 2. Summary of the result of direct PCR applied to sterile pure water and fresh pond water spiked with *Campylobacter*

| Concentration of *Campylobacter* cells added ($ml^{-1}$) | Proteinase K extraction | | Boiling extraction | | Chelex® 100 extraction | |
|---|---|---|---|---|---|---|
| | In sterile pure water | In fresh pond water | In sterile pure water | In fresh pond water | In sterile pure water | In fresh pond water |
| $5x10^5$ | + | + | – | – | + | – |
| $3x10^4$ | + | + | + | + | – | – |
| $3x10^3$ | + | + | + | – | – | – |
| $4x10^2$ | + | + | + | – | – | – |
| 50 | + | – | + | – | – | – |
| 8 | – | – | – | – | – | – |
| 0.9 | – | – | – | – | – | – |

+ Indicates detection of *Campylobacter* by direct PCR; – indicates not detected

Table 3. Detection of campylobacters in un-spiked pond water by PCR after selective enrichment

| | Volumes filtered† | | |
|---|---|---|---|
| | 10 ml n:p | 100 ml n:p | 1000 ml n:p |
| PCR with Proteinase K extraction from pellets after selective enrichment | 4:2 | 4:4 | 4:0 |
| Confirmation by selective plating after enrichment‡ | 4:2 | 4:4 | 4:0 |

† Un-spiked pond water was filtered and filters were incubated overnight in Preston enrichment broth
‡ This represents positive confirmation of colonies on mCCD agar by both conventional biochemical/ morphological methods and by PCR
n = total number of samples
p = total number of positive samples

substances that have an inhibitory effect on the DNA polymerase enzyme, and/or colloidal material that has a high affinity for DNA (Wilson, 1997). Alternatively, the negative result may be because low numbers of campylobacters were present or because they were absent in the samples. Since one of the four protocols used for DNA extraction included the use of the wizard DNA clean up kit, the inhibition should have been partially removed. It follows that the negative results with PCR were probably due to the absence or low numbers of *Campylobacter*. However, since filtration, enrichment and selective plating of 100 ml samples from the same pond yielded confirmed *Campylobacter* isolates (Table 1) this strongly suggests that *Campylobacter* was present but in such low numbers that it was not readily detectable by PCR.

The direct PCR detection of 50 *Campylobacter* cells in 1.0 ml of spiked pure water is relatively close to that reported by Kirk & Rowe (1994), who achieved a PCR detection limit of 10-20 *Campylobacter* cells $ml^{-1}$ in spiked pure water. When PCR was applied to spiked turbid pond water, however, the lowest concentration of *Campylobacter* cells detectable by direct PCR was higher, at around 400 cells $ml^{-1}$ (Table 2). This result

supports the suggestion that *Campylobacter* cells in low numbers in turbid environmental waters are not easily detectable by direct PCR.

Extraction with Proteinase K was much the most successful method. With the boiling method, DNA was amplified from fewer samples (Table 2). This result supported the suggestion of Mohran *et al.* (1998) that some *Campylobacter* strains are resistant to lysis by boiling. When the DNA was extracted by Chelex® 100, PCR almost always gave a negative result (Table 2). The successful use of Chelex® 100 for the extraction of prokaryotic and eukaryotic DNA has been reported (Brasher *et al.*, 2002). However, Chelex® 100 is actually a chelating resin that has affinity for polyvalent metal ions that is usually applied to remove PCR inhibitors and to protect extracted DNA from degradation (Malrony & Helmuth, 2003). It appears not to be appropriate for extraction of DNA from campylobacters.

In the current study, when 10 ml and 100 ml samples of turbid pond water were filtered, and the filters were incubated overnight in a selective enrichment culture, the DNA subsequently extracted almost always amplified with primers CF03 and CF04 (Table 3), giving a product size of 340-380 bp. Thus, the results showed that selective enrichment culture followed by PCR is a reliable method for the relatively rapid detection of campylobacters in naturally contaminated turbid environmental waters. This conclusion agrees with many authors who have used PCR after an enrichment step to detect *Campylobacter* in spiked estuarine and river waters, naturally contaminated and spiked sewage (Waage *et al.*, 1999; Pitkänen *et al.*, 2009), naturally contaminated and spiked food samples (Sails *et al.*, 2003; Singh *et al.*, 2011), and from naturally-contaminated human faecal samples (Vanniasinkam *et al.*, 1999). In general, enrichment incubation after sample collection has been shown to provide higher level of PCR detection by increasing the number of target cells. Thus, the results reported in this study support the suggestion that the combination of selective enrichment culture followed by a PCR assay is a more rapid method, with higher detection ability, than conventional enrichment culture followed by confirmation using biochemical and morphological tests (Koenraad *et al.*, 1995).

Another potential disadvantage of direct PCR assay on environmental samples without an enrichment step is the possibility that naked DNA fragments or DNA from dead cells might be detected (Mandrell & Wachtel, 1999). The presence of dead *Campylobacter* cells in environmental waters may suggest that the water has been contaminated, but no longer poses any public health threat (Theron & Cloete, 2004). Since the detection of viable *Campylobacter* cells in environmental waters is the point of concern to public health authorities, the application of an enrichment step prior to PCR assay will allow only the detection of viable cells.

In conclusion, detection of campylobacters from naturally contaminated, turbid, environmental water may not be feasible by direct PCR assay because of low numbers and the presence of high concentration of humic matter and other PCR inhibitors. The enrichment of water samples in selective broth, however, facilitated PCR detection of *Campylobacter* probably by increasing cell number and by diluting PCR inhibitors.

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