Short communication

Evaluation of the Cape Town Protocol for the isolation of *Campylobacter* spp. from environmental waters

SM Diergaardt, SN Venter*, M Chalmers, J Theron and VS Brözel
Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002, South Africa

Abstract

Campylobacter is recognised as one of the major causes of bacterial gastro-enteritis world-wide. In addition to poultry products, milk and water have also been implicated as possible sources of infection. Methods for the detection and isolation of this organism have been developed specifically for the medical field and select for *Campylobacter jejuni* and *C. coli*, excluding all other species of *Campylobacter*, whereas the filter-based Cape Town Protocol reportedly yields most *Campylobacter* spp. The Cape Town Protocol was evaluated for possible use in analysis of environmental water samples. It yielded only 0.1% of the total number of actively growing *C. jejuni* and *C. coli* cells, whereas the selective medium mCCDA yielded 10%. Analysis of 60 water samples yielded 221 putative *Campylobacter* isolates, but only four could be confirmed as *Arcobacter butzleri* and none as *Campylobacter*. Our results indicated that neither the Cape Town Protocol nor mCCDA can be used for the direct enumeration or isolation of *Campylobacter* spp. from environmental water samples.

Keywords: Campylobacter, Arcobacter, water-borne pathogens

Introduction

Campylobacter is recognised as one of the major causes of acute bacterial gastro-enteritis world-wide (Allos, 2001; Griffiths and Park, 1990; Lior, 1996). *Campylobacter jejuni*, *C. coli* and *C. lari* are amongst the species known to be pathogenic to humans, but *C. jejuni* is the most commonly isolated species from patients (Engberg et al., 2000; Stanley et al., 1998; Tauxe, 1997; Thomas et al., 1999). In a South African study, *Campylobacter* has been isolated from 22% of diarrhoeic stools, indicating that it is a common cause of diarrhoeal disease (Le Roux and Lastovica, 1998).

There are various routes of infection for *Campylobacter*-related illnesses, but poultry products still remain the primary source implicated in infections of humans, mainly because this organism forms part of the chicken's commensal intestinal microflora (Chan et al., 2001). In addition to poultry, unpasteurised milk and untreated surface water have also been implicated as possible sources of infection (Beumer et al., 1992; Jones et al., 1991; Koenraad et al., 1995). Recent reports by Obiri-Danso and Jones (1999a; 1999b) showed that surface water in the United Kingdom was regularly contaminated with *Campylobacter*, introduced by treated sewage or surface runoff, but that numbers decreased following extended exposure to UVB rays (Obiri-Danso et al., 2001). The role of water in the dissemination of *Campylobacter* therefore remains elusive.

Methods for the detection and isolation of this fastidious organism have been developed with specific application in the medical field. In most cases, selective culture media containing combinations of various different antibiotics, that reportedly inhibit the growth of competitor microflora, are used for the isolation and culturing of selected *Campylobacter* spp. Detection of *Campylobacter* spp. in environmental samples has been based strongly on molecular methods such as PCR (Kirk and Rowe, 1994; Koenraad et al., 1995; Lawson et al., 1998; Oyofo and Rollins, 1993; Waage et al., 1999) and nucleic acid hybridisation (Buswell et al., 1998). All of these methods have been designed to detect or isolate only the thermophilic *Campylobacter* spp., i.e. *C. jejuni*, *C. coli* and in some cases *C. lari*. The only method reported to yield isolates from most of the known members of the genus is based on the ability of *Campylobacter* to move through 0.6 µm pores in mixed ester membrane filters (Steele and McDermott, 1984). This method has been refined by Lastovica and co-workers at the Red Cross Children's Hospital in Cape Town, and is commonly referred to as the Cape Town Protocol. Since no selective agents are used in this method, it is possible to isolate virtually all of the known *Campylobacter* species (Le Roux and Lastovica, 1998). By making use of this method, extensive analysis of stool samples from infected South African patients has revealed that *C. jejuni* and *C. coli* collectively constitute 38% of species isolated, and that an array of other pathogenic *Campylobacter* spp. abound (Le Roux and Lastovica, 1998). A representative analysis of environmental samples for *Campylobacter* should therefore not be too stringent. The Cape Town Protocol represents the only current method for the isolation of all known *Campylobacter* spp.

The aim of the work reported here was to evaluate the Cape Town Protocol for the detection of *Campylobacter* spp. from environmental waters.

Materials and methods

Strains and culture media

Clinical strains of *C. jejuni* (MF 1217R) and *C. coli* (NCTC 11283) were obtained from the Red Cross Children's Hospital (Rondebosch, Cape Town). The *Campylobacter* strains were grown on blood agar base No. 2 (Oxoid CM271) supplemented with 10% (v/v)

* To whom all correspondence should be addressed.
☎+27 12 420-4100; fax: +27 12 420-3266;
e-mail: snventer@postino.up.ac.za
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TABLE 1
Recovery of *Campylobacter*-like organisms from different water types with the filter method and on mCCDA (values represent cfu/100 ml)

Water type	Filter method		mCCDA	
	March	July	March	July
Drinking water	2.5×10^0 - 2×10^4	*ND - 1.5×10^0	1.5×10^1 - 6.6×10^4	ND - 5.0×10^0
Groundwater	1.0×10^0 - 2.5×10^3	1.0×10^1	2.3×10^1 - 2.4×10^3	1.6×10^3
Surface water	1.5×10^1 - $1.5 \times ´10^4$	ND - 1.8×10^5	7.0×10^1 - 1.5×10^7	1.2×10^2 - 1.0×10^6
Sewage	1.0×10^3 - 4.5×10^6	3.0×10^1 - 1.7×10^7	2.4×10^4 - 2.5×10^7	3.6×10^2 - 1.8×10^7

* - not detected

sterile horse blood. For the second round of sampling, blood agar base No. 2 was replaced by tryptose blood agar (Oxoid CM233), because it reportedly yields better results (Lastovica, 2001). *Campylobacter* blood-free selective agar base (modified CCDA) (Oxoid CM739) with CCDA selective supplement (Oxoid SR155) was used for selective culturing. All *Campylobacter* cultures were incubated at 37°C in the presence of an Oxoid Gaspack BR038B in order to provide a hydrogen-rich atmosphere. Cells were subcultured every 48 to 74 h. For liquid culture, strains were inoculated into Luria Bertani (LB) broth (Sambrook et al., 1989).

The modified Cape Town Protocol

The Cape Town Protocol, as described by Le Roux and Lastovica (1998), utilises a 0.6 µm mixed ester membrane filter (ME26, Schleicher & Schuell) to facilitate selective passage of *Campylobacter* spp. from diluted stool samples onto tryptose blood agar (Oxoid CM233), supplemented with 10% horse blood. The plates are then incubated in an H_2-enriched micro-aerophilic atmosphere for 48 h at 37°C. The Cape Town Protocol was modified for use with water samples. Suspended cells were concentrated onto 0.45 µm pre-sterilised nitrocellulose membranes by vacuum filtration, the standard microbiological procedure for the concentration of larger water samples. Thereafter the 0.45 µm membrane was aseptically inverted onto the 0.6 µm membrane filter on blood agar plates. The culturable count of spiked samples was determined by plating serial dilutions directly onto blood agar plates and incubating as above.

Analysis of water samples

To isolate possible *Campylobacter* spp. from a range of environmental water sources, 60 water samples (10 drinking, 28 surface, 10 ground and 12 sewage sources) were collected in South Africa on two separate occasions. All samples were transported to the laboratory on ice and analysed within a day of receipt. Different sample volumes (100, 10 and 1 ml) were concentrated by filtration and inverted onto 0.6 µm membrane filters on blood agar. For comparative purposes, duplicate filters bearing concentrated samples were inverted directly onto the blood-free selective agar (mCCDA). All plates were incubated for 1 h in 5% CO_2 at 37°C after which the filters were removed and the plates incubated for a further 48 h.

Identification of *Campylobacter*-like organisms

Campylobacter-like colonies (round, smooth-edged and *ca.* 0.5 to 1.0 mm in diameter) were randomly selected from those isolated on both blood and mCCDA agar plates. The Gram stain (Gerhardt et al., 1994) was performed on all these isolates, and all the Gram-negative spirilloid rods were subjected to an aerobic test. For the aerobic test, all isolates were restreaked onto blood agar plates and incubated under atmospheric conditions at 37°C for 48 h. Isolates that did grow aerobically were regarded as being non-*Campylobacter*.

Identification of the isolates by sequencing of their 16S rDNA genes

Genomic DNA of randomly selected isolates that did not grow aerobically was extracted using the boiling method as described by Lemanceau et al. (1995). A 1495 bp region of the 16S rDNA gene of these isolates was amplified using the universal 16S rDNA primers, fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3'), as described by Weisburg et al. (1991). Amplicons were purified with a Geneclean™Kit (Bio 101, Inc; Vista, CA) as per the manufacturer's instructions, but using silica instead of glassmilk (Boyle and Lew, 1995). Sequencing was performed on an ABI PRISM 377 Sequencer (Perkin Elmer) using the BigDye™Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequences were edited by Sequence Navigator and the identities were determined by searching known sequences in GenBank using a basic BLAST search of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/ BLAST).

Results

The modified Cape Town Protocol was first evaluated under laboratory conditions by using four sterilised water types (tap, ground, surface water and sewage) spiked with 48 h old cultures of *C. coli* or *C. jejuni* to a final concentration of 10^9 cfu/ml. The number of colonies recovered using the Cape Town Protocol was then compared to the culturable count. The Cape Town Protocol yielded a recovery of less than 0.1% of the total culturable number of *Campylobacter* cells introduced into the various environmental water types. This held true for both *C. coli* and *C. jejuni* in all the different water types tested, and therefore necessitated detailed investigation of the reasons underlying the dismal recovery rate. By contrast, placing of the inverted 0.45 µm membrane filters directly onto the mCCDA medium yielded a 10% recovery of the culturable population of the laboratory-grown *Campylobacter* (Diergaardt, 2001).

Evaluation of the Cape Town Protocol

The various stages of passage for *Campylobacter* isolates in the modified Cape Town Protocol were investigated quantitatively using *C. coli*. It was determined that laboratory cultures did not pass through either 0.45 µm or 0.22 µm pore size filters as no culturable cells were detected in the filtrate, and therefore all cells were retained on the filters. *C. coli* did not adhere irreversibly to the nitrocellulose filter as most of the cells were recovered from the filter when inverted directly and left on blood agar for 60 min. Longer exposure did not lead to improved release. Movement of cells through the 0.6 µm pores was not hampered by a lack of capillary fluid as addition of liquid on the filters did not enhance recovery. Cell movement was also not limited by a lack of cellular energy, because the addition of glycine and citric acid to the sample did not improve recovery (Diergaardt, 2001).

Analysis of water samples

Counts of *Campylobacter*-like organisms (CLO) obtained during both sampling rounds were fairly high in surface waters and even higher in sewage samples (Table 1). Four to five colonies were selected at random from plates of the highest dilution showing growth and 221 CLOs, i.e. Gram-negative spirilloid rods, growing in 5% CO_2, were isolated (91 by means of the Cape Town Protocol and 130 by means of mCCDA). These 221 isolates were subjected to the aerobic test and only eight isolates failed to grow, four from the filter method and four from mCCDA.

Identifications of isolates based on their partial 16S rDNA sequences

The 16S rDNA genes of the isolates were partially sequenced. The sequence data revealed that none of the eight isolates belong to the genus *Campylobacter* (Table 2). Four of the isolates were identified as *Arcobacter butzleri*, three from the filter method and one from mCCDA. The fourth isolate obtained by the filter method was identified as a *Bacillus* spp. Two of the isolates from mCCDA were identified as *Acinetobacter* spp. and the other one as a *Microvirgula* spp. In order to determine whether possible *Campylobacter* spp. had been discarded by the aerobic test, seven aerobic isolates were selected at random and identified according to their 16S rDNA sequences. None of these isolates were identified as either *Campylobacter* or *Arcobacter*, but comprised various Gram-negative bacteria and actinomycetes (Table 3).

TABLE 2
Identity of the eight isolates obtained from environmental water samples using the modified Cape Town Protocol (CTP) as determined by partial sequencing of their 16S rDNA genes

Water type	Medium	Identity
Surface water	mCCDA	*Acinetobacter*
Surface water	CTP	*Bacillus*
Surface water	CTP	*Arcobacter butzleri*
Surface water	mCCDA	*Arcobacter butzleri*
Surface water	CTP	*Arcobacter butzleri*
Drinking water	mCCDA	*Microvirgula aerodenitrificans*
Treated sewage	mCCDA	*Acinetobacter*
Raw sewage	CTP	*Arcobacter butzleri*

TABLE 3
Identity of randomly selected isolates that grew aerobically as determined by partial sequencing of their 16S rDNA genes

Water type	Identity
Drinking water	*Paracraurococcus* spp.
Drinking water	*Paracraurococcus* spp.
Surface water	*Burkholderia* spp.
Surface water	*Cellulomonas* spp.
Sewage	*Acinetobacter* spp.
Sewage	*Acinetobacter* spp.
Sewage	*Paracraurococcus* spp.

Discussion and conclusions

The Cape Town Protocol is a useful method for the isolation of *Campylobacter* spp. from clinical samples as it reportedly recovers all known species of *Campylobacter* (Le Roux and Lastovica, 1998; Engberg et al., 2000). By contrast, our results show that it is an inadequate method for the isolation and enumeration of *Campylobacter* spp. from water as it is of insufficient stringency to exclude a host of non-*Campylobacter* spp. commonly occurring in aqueous environments.

The modified Cape Town Protocol appeared promising in the initial stages of this study as a host of isolates showing the typical *Campylobacter*-like morphology was obtained. Colonies appeared round, smooth-edged and *ca.* 0.5 to 1.0 mm in diameter, and cells were generally spirilloid Gram-negative rods. Of the 221 CLOs obtained (91 through the Cape Town Protocol and 130 through mCCDA), none could be identified as *Campylobacter*. Four were identified as *A. butzleri*. The identification procedure is lengthy and requires extensive expertise, so that most laboratories would score colonies fitting the accepted description for *Campylobacter* as positive, leading to a vast over-estimation of *Campylobacter* spp. in the respective water samples. The widely-used selective medium, mCCDA, was also insufficiently stringent to suppress the growth of a host of non-*Campylobacter* spp. This may, however, be overcome by the addition of a selective enrichment step during initial isolation.

No *Campylobacter* spp. were isolated from the various water samples. Possible factors contributing to the apparent absence of *Campylobacter* in the waters tested could be the warm climate and long daylight hours accompanied by high incandescent light experienced in South Africa. *Campylobacter* does not survive in water at high temperatures for very long periods, and is easily damaged by exposure to UVB rays (Korhonen and Martikainen, 1991; Obiri-Danso et al., 2001). We expected to obtain more *Campylobacter* isolates during July (winter in the southern hemisphere), but this was not the case. Only one of the eight candidate *Campylobacter* spp. was obtained during the July sampling period. It was isolated from sewage and identified as *Acinetobacter*. Obiri-Danso and Jones (1999a; 1999b) were able to isolate *Campylobacter* from environmental water in the UK with mCCDA, a method very similar to the one we used in parallel to the Cape Town Protocol. Our results therefore indicated that *Campylobacter* occurred below the detection limit in the water samples tested. It is, however,

possible for *Campylobacter* spp. to survive in biofilms in an aqueous environment.

The isolation of four *A. butzleri* was surprising. Members of the genus *Arcobacter* share the same general features with *Campylobacter*. They can reportedly be differentiated from *Campylobacter* by their ability to grow aerobically at 30°C (Ursing et al., 1994; Vandamme et al., 1991), although none of the *Arcobacter* isolated during this study were able to grow aerobically at 37°C. Another distinguishing feature of this organism is its ability to grow at lower temperatures (15°C) (Ursing et al., 1994; Vandamme et al., 1991), thus making it more suitable to survive in the environment. It has been implicated as the causal agent for diarrhoeal illnesses in humans (Engberg et al., 2000; Lerner et al., 1994; On, 1996) and it has been isolated from environmental as well as drinking water reservoirs previously (Jacob et al., 1998; Rice et al., 1999; Vandamme et al., 1991; Wesley et al., 1995; Wesley et al., 2000).

The data indicate that neither the Cape Town Protocol nor the selective medium mCCDA can be used for the direct isolation of *Campylobacter* spp. from environmental water samples. Whilst the Cape Town Protocol is an excellent method for the isolation of *Campylobacter* spp. from clinical samples where these organisms are present in very high numbers in an infected individual, it is unable to prevent the passage and growth of a variety of bacteria commonly occurring in water. In order to clarify the role of water in *Campylobacter* infections, the development of a selective culturing method suitable for the isolation of Campylobacters from samples containing a diverse microbial flora, is required.

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