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PCR–IMS detection and molecular typing of *Cryptosporidium parvum* recovered from a recreational river source and an associated mussel (*Mytilus edulis*) bed in Northern Ireland

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SUMMARY

PCR–IMS was used to detect *Cryptosporidium* spp. in environmental water samples in Northern Ireland which had previously tested negative by a conventional IFA staining method. Oocysts of *C. parvum* detected in river water and final treated sewage effluent collected from various sites along the river Lagan were identified as genotype 2 (animal origin) based on polymorphisms observed at the thrombospondin related adhesion protein gene locus. Similarly, genotype I (human origin) oocysts of *C. parvum* were detected in the marine filter feeder mussel, *Mytilus edulis*, collected from the shores of Belfast Lough. Detection of the human genotype of *Cryptosporidium* in mussels destined for human consumption identifies the organism's serious potential as a foodborne pathogen. This work highlights the possible value of monitoring filter feeder systems, in conjunction with specific molecular epidemiological tools, as an alternative monitoring system for the parasite within the aquatic environment.

INTRODUCTION

Cryptosporidium oocysts are frequently present in surface waters, their source most likely to be from agricultural runoff and sewage discharges, often following adverse weather conditions [1–3]. The ubiquitous nature of this protozoal parasite and its potential for waterborne transmission is further facilitated by their small size (3·5–6·0 μm) and their low sedimentation rate (0·5 μm/s) [1]. The potential for cryptosporidial contamination of river water is high in areas where sewage dumping is a common practice and where rivers can be polluted by livestock faecal discharges [4, 5]. The presence of oocysts in river water may also be a source of contamination of the marine environment [5]. It has previously been shown that oocysts can remain viable in seawater for up to three times as long as those found in river or tap water, and that the filter feeding mussel, *Mytilus edulis*, can filter over 100 litres of seawater every 24 h [6, 7]. There have been several recent reports of *Cryptosporidium* oocysts having been detected in molluscs, oysters and clams, although little is known as to the length of time the oocyst can survive within each shellfish [8–12]. It has been suggested, however, that *C. parvum* oocysts can survive in seawater for at least 1 year and can retain their infectivity in filter feeders for up to 14 days [13]. Studies have shown that the eastern oyster (*Crassostrea virginica*), the freshwater clam (*Carbicula fluminea*), and different species of mussels and clams (*Mytilus galloprovincialis* and *Cerastoderma edule*), can remove *C. parvum* oocysts

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from water and accumulate them on their gills and inside haemocytes in the haemolymph [5, 9, 10, 13].

While detection methods for *Cryptosporidium* spp. have improved greatly in terms of sensitivity there are relatively few studies that focus on the identification of different species and even fewer that report on specific types. We have developed a method for the identification of different species of *Cryptosporidium* based upon RFLP analysis of a portion of the 18S rRNA gene [14]. It has also become widely accepted that analysis of sequence polymorphisms in the thrombospondin-related adhesive protein (TRAP-C2) gene can subdivide isolates into animal and human genotypes [15].

We set out to apply the above methods to the analysis of the distribution of *Cryptosporidium* within a single river in Northern Ireland. The river Lagan was selected in this study owing to the numerous sewage and industrial discharges along the length of the river which may contribute significantly to the cryptosporidial oocyst load in this environment. Detection of cryptosporidial oocysts in the river Lagan prompted a study of the mussel beds in Belfast Lough to determine the potential role of the mussel (*Mytilus edulis*) as reservoirs of *C. parvum* oocysts and their possible value as an indicator of river water contamination. The detection of cryptosporidial oocysts in mussel tissue harvested for human consumption has obvious implications for human health.

METHODS

Sample collection

The river was sampled between July and August 1999. Samples were taken at various points along the river, from both river source and final effluent from sewage treatment plants which discharge directly into the river course (Fig. 1). Sampling involved filtering a single 500 l sample of water or final treated sewage effluent, from each sampling site, through a 1 micron yarn filter cartridge (Microwynd), followed by a secondary oocyst IMS concentration step (Dynal UK Ltd). This procedure was carried out in accordance with the UK Water Supply (Water Quality) Regulations, 1999 and the turbidity of all samples processed by IMS did not exceed 2·5% ppv. Previous studies had shown that the sensitivity of the detection assay decreased by a factor of 10 when samples of 5% ppv, as recommended by the manufacturer were used [14].

Marine mussels (*Mytilus edulis*), which are harvested for human consumption, were collected from along the western coast of Belfast Lough (Fig. 1). A total of 16 mussels were collected from the mussel beds in Belfast Lough as part of the regular Environmental Health Department's on going monitoring of commercial shell fish beds. After removal from the site, the barnacles were removed, and the mussels rinsed in distilled water. After drying, the shells were opened and the adductor mussels were cut using a sterile scalpel. The flesh was chopped-up and homogenized in a sterile food blender and an aliquot of tissue homogenate was added to a Dynal ®L10 tube and buffered according to the manufacturer's instructions (Dynal UK Ltd). In each case the turbidity of the mussel tissue homogenate did not exceed 2·5% ppv prior to processing with IMS.

Visual examination of oocysts by IFA

Approximately 50 μl of neutralized oocyst suspension, obtained from IMS secondary concentration step, was transferred to the single 25 mm well of a Dynal (UK) microscope slide. The slide was dried in an oven at 37 °C for 20–30 min and fixed in acetone for 10 min and allowed to air dry. Approximately 20–25 μl of CRYPTO-CEL IF (Cellabs Ltd, UK), fluorescein-labelled monoclonal antibody, diluted in a protein stabilized buffer solution with Evans Blue as counter stain and sodium azide as preservative (Shield Diagnostics, UK) was added to the fixed specimen. The slide was incubated in a humidified chamber at 37 °C for 30 min then washed gently by rinsing in a PBS bath. The slide was drained and excess moisture was carefully removed by wiping around the specimen with a tissue and allowing it to air dry. A drop of mounting fluid and coverslip was added to the slide which was then examined using a fluorescence microscope with filter system for FITC (maximum excitation wavelength 490 nm, emission wavelength 530 nm) and $\times$400–1000 magnification. Oocysts were enumerated and calculated as oocysts per 10 litres of water sample.

Isolation of genomic DNA, PCR and RFLP analysis

Purified oocyst suspensions, obtained from the IMS secondary concentration step were washed three times by centrifuging at 7000 ***g*** for 10 min in double-distilled water (ddH_2O) and resuspending in 200 μl lysis buffer

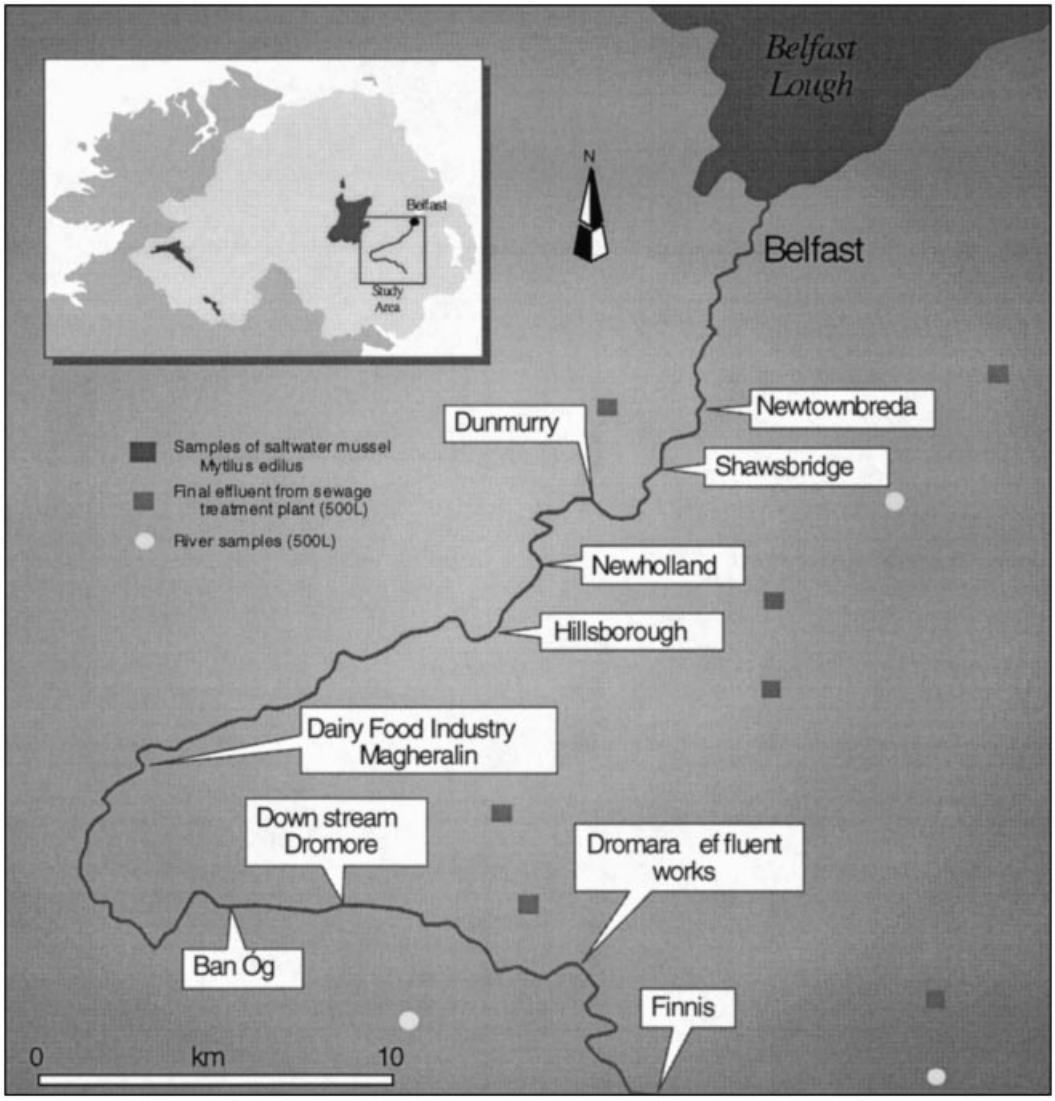

Fig. 1. Map showing *Cryptosporidium* sampling sites in the river Lagan study.

(4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA, pH 7·4), and 40 μl protinease K (2·0 mg/ml) for 1 h at 55 °C. The samples were then subjected to six cycles of freezing in liquid nitrogen for 2 min, followed by thawing at 95 °C for 5 min to release the target DNA which was then purified using a High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Boehringer Mannheim). Briefly, nucleic acids bind specifically to the surface of glass fibres in the presence of a chaotropic salt. Residual impurities such as salts, proteins and other cellular components are removed by a wash step and subsequently nucleic acids are eluted in an elution buffer.

Previously described generic *Cryptosporidium* primers, Low 749F(GCC TTG AAT ACT CCA GCA TG) and 20-mer Low 1300R (GCA GGT TAA GGT CTC GTT CG) were used to amplify a 552 bp PCR amplicon of *Cryptosporidium* spp. [14]. The PCR mixture contained PCR reaction buffer (10 mM Tris–HCl (pH 8·3), 50 mM KCl), BSA 400 μg/ml; 20 pmol each primer; 150 mM each dNTP; 3 mM $MgCl_2$; 2·5 U/ml of *Taq* DNA polymerase (Life Technologies Ltd, Paisley, Scotland), 20 μl of template DNA (70 ng/ml) and 55 μl of LAL reagent water (Biowhittaker, MD). The reaction mixture was overlaid with 75 μl sterile mineral oil (Sigma Chemical Company, St. Louis, MO, USA). PCR was carried out on a thermocycler (Biometra Trio-Thermoblock) for 38 cycles with the following programmed profile; initial denaturation for 3 min at 94 °C and 38 cycles of amplification (annealing for 1 min at 62 °C, extension

Table 1. *Detection of* Cryptosporidium *spp. from the river Lagan study using IMS–IFA, and the generic* Cryptosporidium *detection assay (PCR–IMS)*

Sampling site (sample source)	Site code	IMS–IFA	IMS–PCR
Finnis (river)	1	−ve	−ve
Dromara (sewage effluent)	2	−ve	−ve
Dromore (river)	3	−ve	−ve
Ban Og (river)	4	−ve	−ve
Magheralin (river)	5	−ve	−ve
Hillsborough (sewage effluent)	6	−ve	+ve
NewHolland (sewage effluent)	7	−ve	−ve
Dunmurry (sewage effluent)	8	−ve	−ve
Shaws Bridge (river)	9	−ve	+ve
Newtownbredda (sewage effluent)	10	−ve	−ve
Belfast Lough (mussel tissue)	11	−ve	+ve

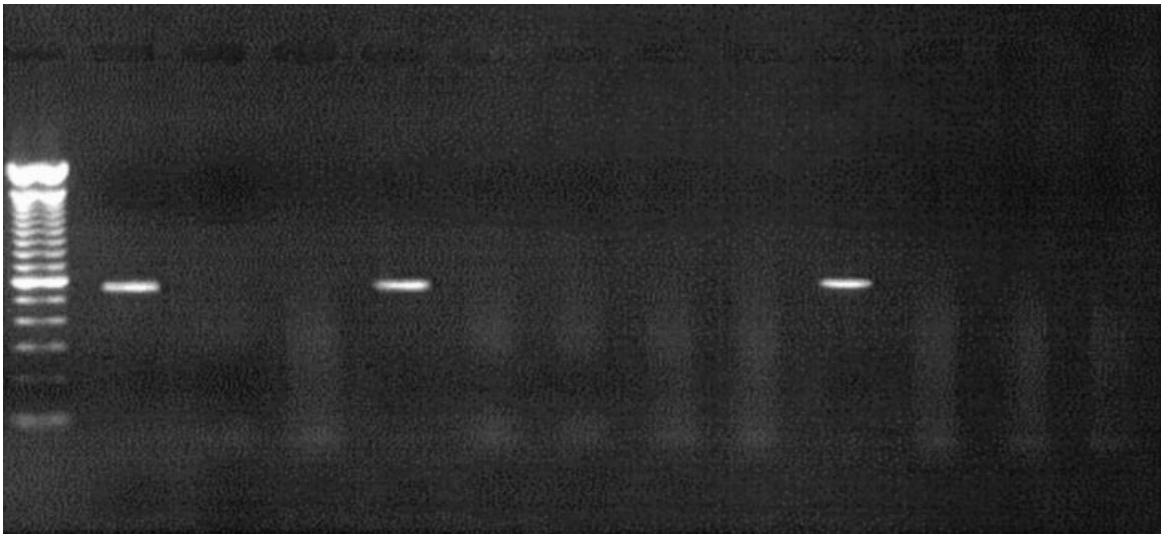

Fig. 2. PCR amplification of the 552 bp fragment of the 18S rRNA gene of *Cryptosporidium* in filtered river water and final treated sewage effluent. Lane 1: Molecular wt DNA ladder 2078-100 bp; Lane 2: Positive control; Lane 3: Newholland*; Lane 4: Newtownbreda*; Lane 5: Hillsborough (+ve)*; Lane 6: Dunmurry*; Lane 7: Magheralin*; Lane 8: Dromore*; Lane 9: Dromora*; Lane 10: Shawsbridge(+ve)†; Lane 11: Ban Og†; Lane 12: Finis†; Lane 13: Negative control (PCR reaction mix with no *Cryptosporidium* DNA).
* Final treated sewage effluent.
† River water samples.

for 1 min at 72 °C, and denaturation at 94 °C). The final extension segment was extended to 10 min and the samples were then cooled and stored at 4 °C. Endonuclease restriction of the generic *Cryptosporidium* PCR products was carried out using endonucleases *Mae* I (Boehringer Mannheim) and *Vsp* 1 (Life Technologies) according to the manufacturer's specifications.

A second gene fragment, a 369-bp region of the thrombospondin-related adhesive protein (TRAP-C2) of *C. parvum*, was also amplified with the following primers: 5′-CAT ATT CCC TGT CCC TTG AGT TGT-3′ and 5′-TGG ACA ACC CAA ATG CAG AC-3′ using previously described PCR conditions [15]. PCR products and restriction fragments were resolved on 1·5% agarose (Life Technologies, UK) and 2% Polyvinylpyrrolidone (PVP) (Sigma, UK), and the results were visualized by ethidium bromide staining using the Synapse Grabber Software Package (Synoptics Ltd, UK).

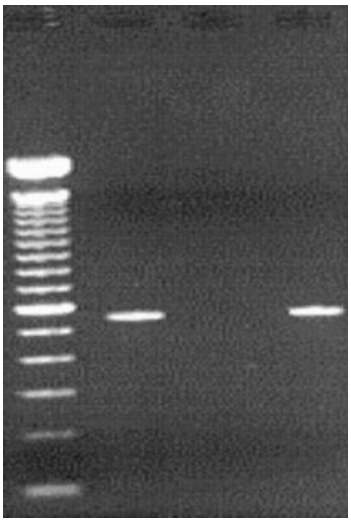

Fig. 3. PCR amplification of the 552 bp fragment of the 18S rRNA gene of *Cryptosporidium* saltwater mussels from Belfast Lough. Lane 1: Molecular wt DNA ladder 2078-100 bp; Lane 2: Positive control; Lane 3: Negative control (PCR reaction mix without *Cryptosporidium* DNA); Lane 4: Site 11 (*Mytilus edulis*, mussel tissue).

Sequencing and genotyping of *Cryptosporidium parvum* by TRAP-C2

TRAP-C2 gene PCR products were purified using QIAquick PCR Purification Kit (Qiagen UK Ltd) and prepared for sequencing with the Thermo Sequenase Fluorescent labelled Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech UK Ltd). Products were sequenced using dye terminator chemistry on an ALF Express II automated DNA analyser (Amersham Pharmacia Biotech UK Ltd). Sequence identity was confirmed using the sequence similarity software BLAST (Basic Local Alignment Search Tool) served by Genomenet. All sequences were aligned and compared with the published 369 bp sequences of the *C. parvum* TRAP-C2 gene using the Clustal Pairwise Alignment Method (Windows 32 Megalign 3.99).

RESULTS AND DISCUSSION

Recognition of cryptosporidiosis as a potentially waterborne disease led to investigations of the prevalence of the parasite in waters. The National *Cryptosporidium* Survey Group (NCSG) in the UK, comprised of five English water service companies, reported *Cryptosporidium* oocysts present in 55·2% of 691 river samples with a mean concentration of 0·38 oocysts/litre (unpublished data).

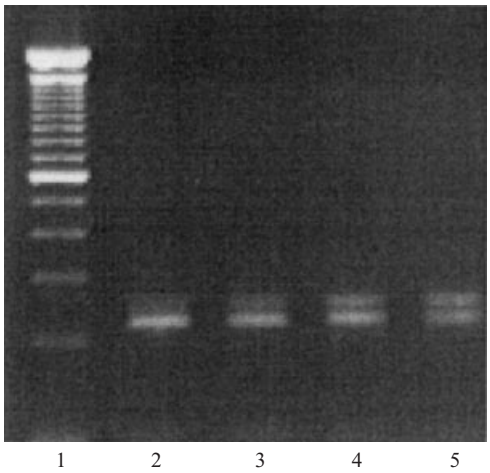

Fig. 4. Post amplification endonuclease restriction of all positive 552 bp PCR amplicons. Lane 1: Molecular wt DNA ladder 2078-100 bp; Lane 2: Site 6 (Hillsborough sewage effluent); Lane 3: Site 9 (Shaws Bridge river sample); Lane 4: Site 11 (Mussel tissue 1); Lane 5: Site 11 (Mussel tissue 2).

Table 2. *Basic local alignment search tool results (BLAST) for TRAP-C2 sequences obtained from the river Lagan study*

Isolate no.	Location	% similarity	Species/genotype
Clone9001	Shaws Bridge	214/227 (94)	*C. parvum*/2
Clone4001	Hillsborough	189/198 (95)	*C. parvum*/1
Clone3001	Mussel tissue	192/199 (96)	*C. parvum*/1
Clone2001	Mussel tissue	190/199 (95)	*C. parvum*/1

Previous studies have compared the analysis of oocysts in various environmental water samples using IFA and PCR [16]. Of 14 river water samples tested from the Milwaukee outbreak (1993), 21·4% were positive by IFA compared with 42·8% positive by PCR. Similarly, cistern waters, wastewaters and coastal waters tested 16·7, 54·2 and 8·7%, respectively, positive by IFA compared with 66·7, 58·3 and 34·7%, respectively, by PCR [16]. Variation in the results was attributed to differences in method sensitivity, and the presence of empty oocysts, which would be undetectable by PCR but seen under a microscope. IMS–PCR has been shown to routinely detect as few as 10 oocysts per 10 ml environmental filtrate sample [14]. Added to this, PCR-endonuclease restriction (ER) also has the ability to differentiate between *Cryptosporidium* spp. and to identify that species which is pathogenic to man [14].

The river Lagan was selected for this study as it has the highest population density surrounding any river in Northern Ireland. Not only does the catchment

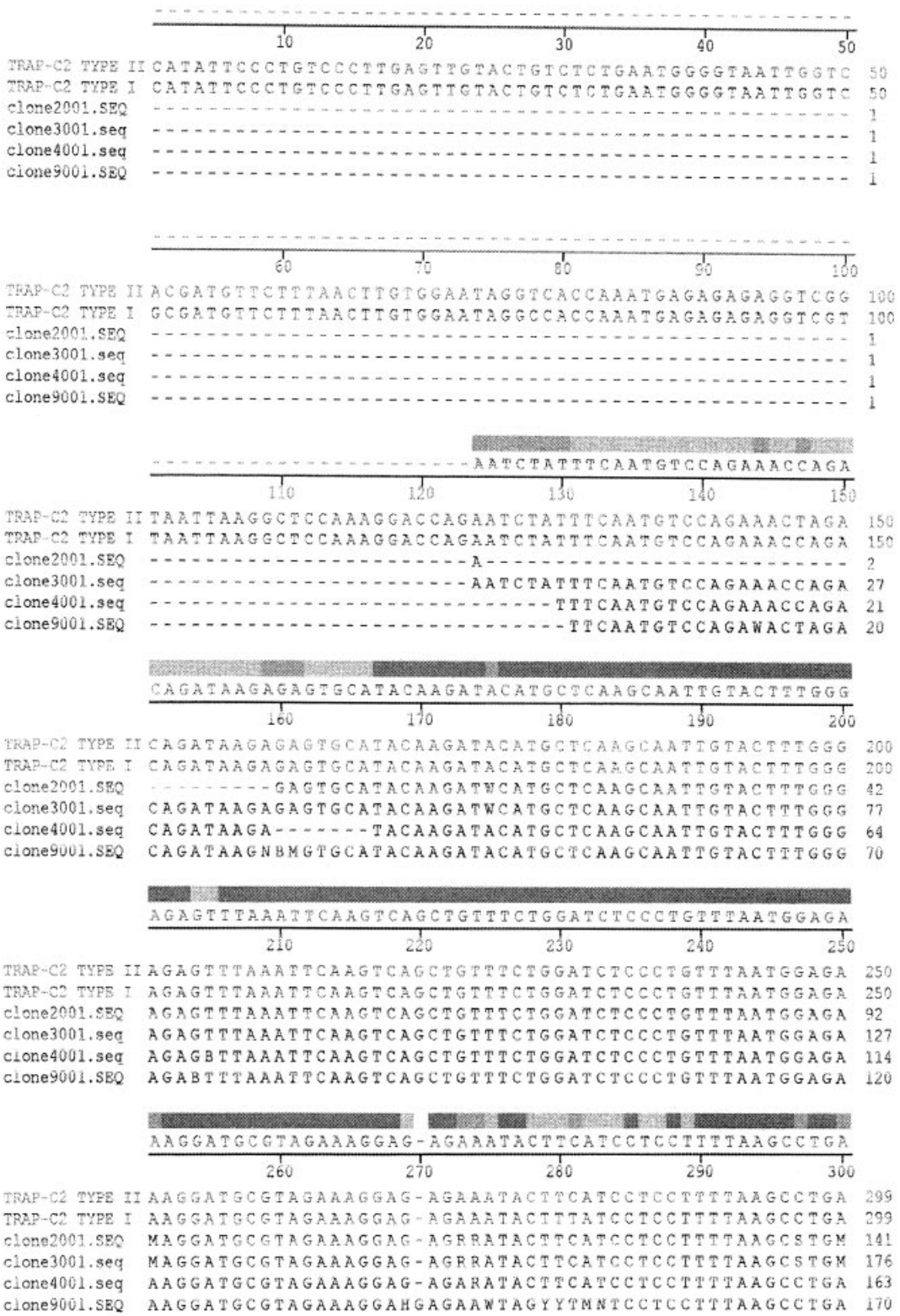

Fig. 5. For legend see facing page.

contain the city of Belfast – the economic and industrial centre – but also some of the most productive agricultural land in Northern Ireland. The Lagan rises as a spring on Slieve Croob some 30 km to the south west of Belfast. For the first few kilometres of its length the Lagan falls steeply through un-

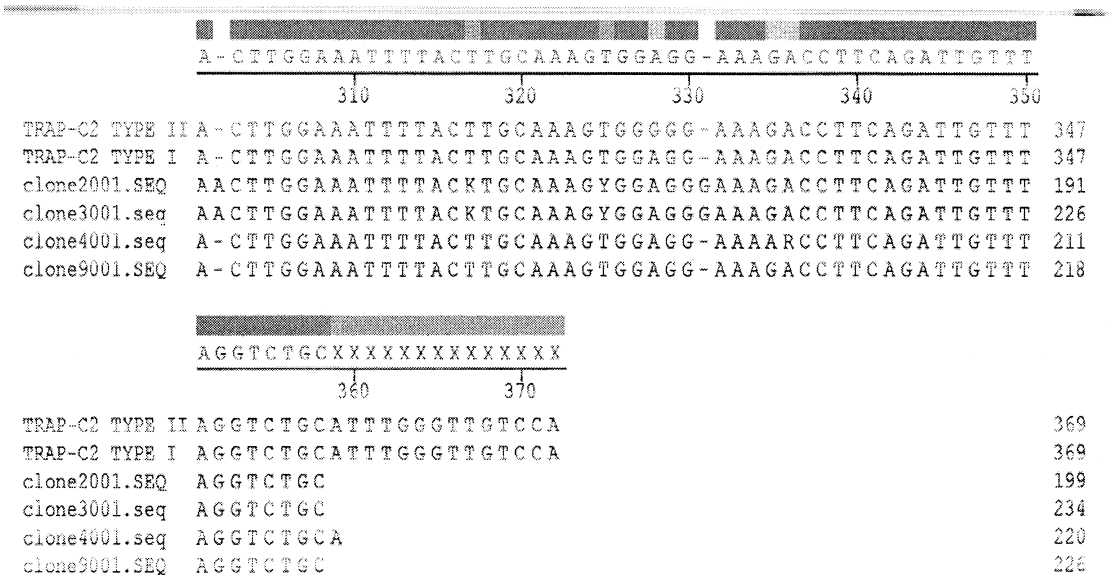

Fig. 5. Alignment of the TRAP-C2 sequences obtained from the Lagan study with published sequences of *C. parvum* genotype 1 and genotype 2.

improved mountain pastures and quickly develops a meandering character with a shingle bed. There are numerous sewage and industrial discharges along the length of the river which eventually flows into Belfast Lough, an area of intense economic and industrial activity. The river is heavily used for a range of recreational activities including swimming and white water canoeing. In addition to its recreational values, the Lagan is also used for stock watering and as a small but significant public water supply. There are a dozen boreholes that are used for public supply and these are all located in the lower reaches of the river course. Belfast Lough contains mussel beds used for human consumption. Because bivalve molluscs such as oysters and clams are filter feeders, feeding on plankton and a variety of microflora, and are also important seafood, they pose a potential public health risk when they concentrate pathogenic bacteria and viruses in waters [17].

Cryptosporidium spp. was not detected in any river or final effluent samples examined by IMS–IFA in this study. *Cryptosporidium*, however, was detected using PCR–IMS in the river sample taken at Site 9, and at Site 6 in the final treated effluent from Hillsborough sewage facility (Table 1). The *Cryptosporidium*-specific 552 bp PCR product was obtained from samples collected at both Site 9 and Site 6 (Fig. 2).

Cryptosporidium was not detected in any mussel tissue examined using IMS–IFA but was, however, detected in 2 of the 16 mussel tissues examined by IMS–PCR. Figure 3 shows the 552 bp PCR amplicon obtained which indicates the presence of *Cryptosporidium* species. The lack of IMS–IFA positives in this study may be explained by the fact that a modified PCR protocol was developed using both Polyvinylpyrrolidone (PVP) and Bovine Serum Albumin (BSA) to overcome PCR inhibition. All positive PCR samples were speciated with the newly developed *Cryptosporidium* PCR assay and all were identified as *C. parvum*, the human pathogen. The positive 552 bp PCR amplicons obtained at Site 6, Site 9 and the mussel tissues from Belfast Lough, each digested with *Mae*I restriction enzyme to give the two band digestion profile of 252 and 300 bp, indicative of *C. parvum* (Fig. 4). This preliminary PCR–RFLP characterization and species identification confirmed the identity of *Cryptosporidium* detected at Site 9, Site 6 and in the mussel tissue at Site 11, as being *C. parvum*. Further molecular characterization of these isolates using the sequence polymorphisms of the TRAP-C2 gene was carried out to elucidate the possible anthroponotic and zoonotic transmission pathways for *C. parvum* in the river Lagan study area. All the isolates obtained produced the TRAP-C2 369 bp PCR amplicons. A BLAST similarity search of the TRAP-C2 gene sequences obtained from the Express II automated DNA analyser confirmed that the species detected in each case was *C. parvum*.

The Lagan TRAP-C2 isolate sequences were aligned with published TRAP-C2 sequences of

C. parvum genotype 1 (GeneBank ascension number AF082523) and genotype II (GeneBank ascension number X77586) using the Clustal Pairwise Alignment Method (Windows 32 Megalin 3.99). On the basis of nucleotide differences observed at positions 147 and 280, clone9001 from site 9, was identified as genotype II, the bovine genotype. Clone4001 and clone3001 from sites 6 and 11, respectively, were both identified as genotype 1, the human genotype as was Clone2001 isolated from the mussel tissue (Table 2, Fig. 5).

The detection of *C. parvum* oocysts in the river Lagan prompted a survey of the Lough's mussel beds, which are harvested for human consumption. Previous studies have suggested that if oocysts of *C. parvum* contaminated estuarine waters where oysters were harvested for human consumption, the oocysts could be taken up by the oysters, potentially posing a threat of infection to persons who eat raw oysters [18, 12]. Other studies involving clams (*Corbicula fluminea*) have shown that the average clam can filter 2·5 l/h and that in a natural situation in the Trinity River, Clear Fork, USA (average depth, 0·25 m; current flow, 18·5 m/min), the entire water volume overlying *C. fluminea* beds was filtered every 16 min [19]. The salt mussel, *Mytilus edulis*, which is harvested in Belfast Lough can filter over 100 l of seawater every 24 h [7].

Genotype 1 oocysts of *C. parvum* were detected in the mussels collected from Belfast Lough. This finding indicates a possible food poisoning threat to anyone ingesting these mussels. Nothing is known of survival of oocysts under typical mussel cooking conditions. Because oocysts of this species are infectious for humans but can be rendered non-infectious by heating to temperatures above 72 °C, it is further recommended that mussels be cooked before being eaten. The presence of genotype 1 in the mussel tissue implicates human sewage as the source of oocyst contamination, as genotype 1 oocysts are involved in an anthroponotic transmission cycle exclusive to humans [15]. This is the first study of its kind to establish human sewage as the source of oocyst contamination in saltwater mussels through molecular characterization of the human genotype. These findings also establish that the filter feeder mussel *Mytilis edulis* can effectively remove and retain oocysts of *Cryptosporidium* from faeces in contaminated estuarine waters. This indicates the possible value of filter feeder systems as an alternative biological monitoring system for the parasite within the aquatic environment.

ACKNOWLEDGEMENTS

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