original paper

Molecular genotyping of human cryptosporidiosis in Northern Ireland: epidemiological aspects and review

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Abstract

Background *Cryptosporidium parvum* is the most common of the protozoal pathogens associated with gastrointestinal disease in Northern Ireland. Genotyping techniques are valuable in helping to elucidate sources and modes of transmission of this parasite. There have been no reports on the prevalence of genotypes in Northern Ireland, mainly due to a lack of discriminatory genotyping techniques, which recently have become available.

Aim To investigate the genotype of *C. parvum* oocysts isolated from human faeces in sporadic cases of cryptosporidiosis in Northern Ireland.

Methods Thirty-nine isolates of *C. parvum*, representing 79.6% of the total 1998 laboratory reports for the Eastern Health and Social Services Board, were investigated. Following DNA extraction from oocysts the thrombospondin-related adhesive protein 2 (TRAP-C2) locus was amplified by polymerase chain reaction (PCR) and subsequently sequenced.

Results The majority of isolates (87.2%) were classified as bovine genotype II with the remainder (12.8%) being the human genotype I.

Conclusions There is a high prevalence of the bovine genotype II parasite in sporadic cases around the greater Belfast area. Epidemiologically, this suggests that the most frequent mode of transmission may be from animals to humans, but does not suggest a high proportion of human to human spread.

Introduction

Human cryptosporidiosis emerged as an important gastrointestinal infection in the 1990s, due to the ingestion of contaminated water and foodstuffs containing the protozoal parasite, *Cryptosporidium parvum*. Presently, this organism is the most common of the protozoal pathogens associated with gastrointestinal disease in Northern Ireland. Recently, there have been reports of at least three waterborne outbreaks of this organism in Northern Ireland in a 12 month period (May 2000 to May 2001).[1-3]

The organism may be subdivided into two genotypes based on sequence polymorphisms within a number of loci,[4,5] including the human genotype I types, found in humans and non-primates and the bovine genotype II, found in animals as well as humans.[6] Therefore, such genotyping techniques are valuable in helping to elucidate sources and modes of transmission of this parasite within both animal and human populations.

To date, there have been no reports on the prevalence of genotypes within the local population in Northern Ireland, mainly due to a lack of discriminatory genotyping techniques, which recently have become available. The aim of this study is to examine the genotypes of sporadic cases of human cryptosporidiosis in Northern Ireland within a single year. To ascertain the role of animals and humans in the transmission of these parasites within the local population, through the use of novel molecular nucleic acid amplification and sequencing techniques.

Materials and methods

Source and preparation of faecal specimens

Faecal specimens were obtained from patients with a recent history of gastrointestinal infection referred by family practitioners in the community, as well as from hospitalised patients in the greater Belfast area during 1998. Fresh faecal specimens were homogenised in a 1:10 (v/v) ratio with sterile saline (0.9% w/v) and slides prepared for microscopic examination following acid-fast staining, as previously described.[7]

PCR confirmation and molecular genotyping

Oocysts were purified from faecal specimens and cryptosporidial DNA was subsequently isolated by a chelex-100 DNA extraction protocol as previously described.[8] Isolates were confirmed as *C. parvum* following PCR amplification of the 18S rRNA gene followed by restriction fragment length polymorphism (RFLP) analysis. For genotype analysis, PCR amplification was performed targeting the TRAP-C2 in accordance with Sulaiman et al[9] and resulting amplicons were sequenced as described previously.[10] The resulting sequences obtained were compared with those stored in the Genbank Data Base using BLAST alignment software (http://www.blast.genome.ad.jp). In addition, genotypes of *C. parvum* were determined by analysis of the TRAP-C2 sequence in accordance with Sulaiman et al[9] (see Table 1).

Results

Faecal specimens from 39 patients, which had tested positive for Cryptosporidium using acid-fast staining, were confirmed as

C. parvum, by 18S rRNA PCR-RFLP. Following species confirmation, all isolates were genotyped using the TRAP-C2 PCR procedure. The 369bp PCR amplicon obtained from each isolate was sequenced using the ALFexpress II automated DNA sequencer, and subsequently genotyped as either type I (human genotype) or type II (bovine genotype).

Table 1 shows the sequences of polymorphisms obtained, which enabled each isolate to be genotyped into its respective genotype. Of the 39 clinical isolates genotyped, 35 (89.7%) were identified as being genotype II and four (10.3%) as genotype I.

Discussion

The molecular genotyping of *C. parvum* from human clinical specimens is a novel area of research. The research tools necessary to carry out an epidemiological study of Cryptosporidium isolates have only recently become available. Previously, several studies in the US and the UK have analysed the distribution of genotypes by geographical area.[9,11] Sulaiman et al[9] examined 92 *C. parvum* isolates in the US from human and animal sources from patients in outbreak and non-outbreak settings and concluded that the anthroponotic (human genotype I) accounted for most of the cases. In the UK, McLauchlin et al[11] genotyped 194 samples from patients with diarrhoea diagnosed as having sporadic cryptosporidiosis. They reported that 74 (38%) were genotype I and 120 (62%) were genotype II. There was a greater proportion of infections caused by genotype I in the South Thames region and an excess of infections caused by genotype II in northern England and, in particular, the South and West, as well as the West Midlands.

The authors postulated that the occurrence of these genotypes may reflect the distribution of animals in rural areas (genotype II) and an established genotype I population in the more urban areas. It was also noted that in areas of high genotype II prevalence a greater proportion of surface water was used in the public water supply. Another hypothesis to account for the relatively lower incidence of genotype I organisms may be that genotype I organisms are less virulent than genotype II organisms, hence infected persons are less likely to present to their family practitioner.

More recently, Pedraza-Díaz et al[12] showed that in approximately 2,000 cases of human cryptosporidiosis, the majority (60.7%) were genotype II. However in the UK, four drinking water-associated outbreaks and four swimming pool-associated outbreaks have been shown to be associated predominately with genotype I.[13-17] Furthermore, Pedraza-Díaz et al[12] demonstrated that genotype II were exclusively found in livestock with no occurrence of genotype I in the animals examined.

In Northern Ireland, genotype II parasites accounted for the greatest number of sporadic cases of cryptosporidiosis (87.2%), which is similar to UK data.[11] It would be reasonable to suggest that in areas of intensive livestock farming, such as Northern Ireland, the bovine genotype would be expected to be the most prevalent form of the disease, given that zoonotic transmission of the parasite can occur between cattle and humans. In contrast, the two recent waterborne outbreaks of human cryptosporidiosis occurring in August/September 2000 and April 2001 were reported as being due to human genotype I.[2,3] Cases of genotype I are indicative of person-to-person transmission or the consumption of food or water which has been contaminated with human faeces. Recently, the authors have shown that in the routine monitoring of water supplies for *C. parvum*, only genotype II was detected.[10]

Seasonal or temporal trends have been noted by a number of authors but these vary from country to country including summer in Australia, rainy seasons in Central America and India, spring or late summer in North America and late summer in Germany.[18-21] Studies in the UK and Ireland indicate peak incidence in spring which may indirectly reflect rainfall and farming events such as lambing, calving and agricultural practices such as slurry spreading.[22]

In Northern Ireland, laboratories began to examine faeces for the presence of this parasite in 1990.[23] Total annual laboratory reports for Northern Ireland have fluctuated between 200 in 1990 to 58 in 1992 (see Figure 1). Although there have been marked differences in isolation rates between different health boards, these differences have been suggested to be artefactual, reflecting different testing policies and methodologies adopted by laboratories and not true epidemiological differences.

Results of a recent microbiology audit (unpublished) for the examination of faecal samples demonstrated that, of the seven clinical laboratories questioned, six were using Modified ZN staining, one was using Modified ZN staining plus EIA and another laboratory was using direct microscopy. Furthermore, this audit demonstrated that there were differences in what faeces were tested including only those 'on request' to only faeces from patients aged three months to 15 years, to all faeces, which resulted in a large range tested (0-100%) giving results ranging from 0-6.2% positive, for this organism.

Last year, the Northern Ireland Public Health Laboratory reported 274 laboratory isolations of Cryptosporidium in stool samples submitted for examination, which represented a combination of sporadic cases, as well as two outbreaks. The majority (69.4%) were submitted by GPs in the community, with the remainder (30.6%) originating from hospital wards. There was an exact male:female ratio of 1:1. High isolations were reported for May, August and September, coinciding with the two reported outbreaks (see Figure 2). In addition, the age range of infected individuals was less than to one year to 97 years, with the majority of cases being reported in the one- to four-year age grouping (see Figure 3). The sporadic cases within these data are consistent with the seasonal trends reported in other areas of the UK, with a definite peak incidence being observed in the spring. Other climatic factors and population trends must be considered in relation to incidence of cryptosporidiosis. Evidence is beginning to emerge to support a correlation between weather conditions, such as rainfall and temperature and cryptosporidiosis incidence rates.

In Britain, previous studies have shown that presentation rates for *C. parvum* were highest in the two- to four-year age group.[24] After adjusting for age and sex, there was a significantly higher infection rate in rural populations in comparison to

Table 1. Alignment of TRAP-C2 nucleotide positions showing polymorphisms among *Cryptosporidium parvum* isolates[6,9]

Isolate	Source	Analysis of TRAP-C2 sequence					Genotype Designation
		Position 51	Position 78	Position 100	Position 147	Position 260	
Type I	Human	G	C	T	C	C/T	I
Type II	Bovine	A	T	G	T	C	II

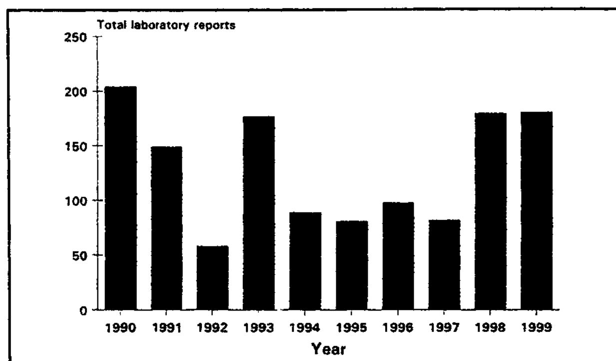

Figure 1. Total laboratory reports of Cryptosporidium in Northern Ireland from 1990-1999.[23]

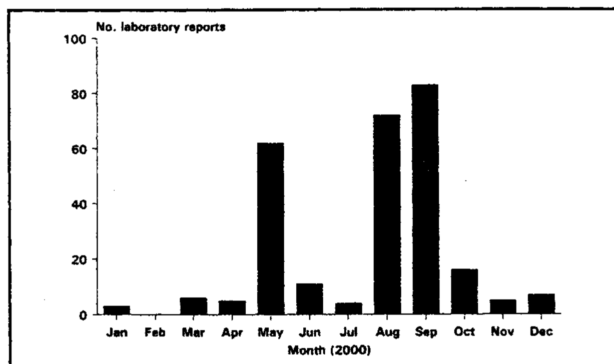

Figure 2. Laboratory reports of Cryptosporidium by month in 2000 issued by the Northern Ireland Public Health Laboratory.

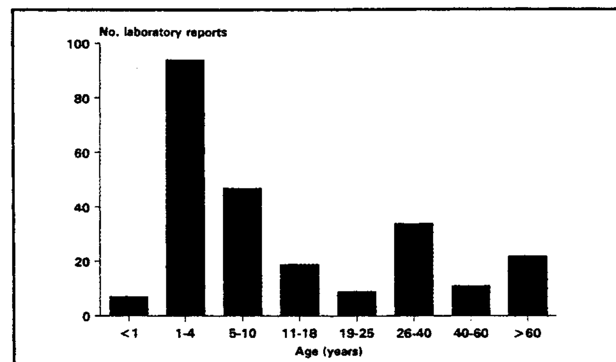

Figure 3. Laboratory reports of Cryptosporidium by age group in 2000 issued by the Northern Ireland Public Health Laboratory.

urban populations ($p \geq 0.001$), as well as regional variation, with the Midlands, the South West and the South East of England having significantly lower rates of infection than the North of England ($0.01<p<0.05$).[24]

Optimum primary diagnostic techniques differ depending on whether the specimen is of human faecal origin or from environmental sources, i.e. water. For human faecal material, the authors recommend that all faeces is examined for the presence of cryptosporidial oocysts, employing the ZN staining technique along with standard light microscopy. Numbers of oocysts can vary. Detection from faeces in a well-marked symptomatic case is usually easier than from water supplies. ZN staining is a relatively cheap, rapid technique, but it relies heavily on the experience of the microscopist in differentiating cryptosporidial oocysts from other parasites.

Alternatively, in cases of suspected cryptosporidiosis in immunocompromised HIV and HIV/AIDS patients, where faecal smears are negative, consideration should be given to employing immunomagnetic separation and immunofluorescent antibody detection protocols. In addition, all positive faeces from suspected outbreaks should be forwarded to a reference laboratory for molecular genotyping and subtyping for confirmation. As subtyping offers a powerful tool in helping with the epidemiology of this disease, primary laboratories should try to store an aliquot (1ml or 1g) of positive specimen for further downstream analysis, particularly with sporadic cases.

As molecular techniques continuously develop, the tracking of this parasite from animals to humans, from the environment to humans and from human to human has become achievable. Most recently, Xiao et al[4] have demonstrated the value of employing a small double-stranded RNA sequencing method to determine the relatedness of outbreak strains. Employment of such molecular epidemiological techniques may be particularly beneficial in examining human cryptosporidiosis in Northern Ireland, as this province may represent an ideal model system due to its relative geographical isolation within Ireland, the UK and the EU, the homogenous population with limited foreign travel and consumption of water from a limited number of sources with good continuous monitoring facilities. Therefore by combining genotyping and subgenotyping methodologies for human cryptosporidiosis, the sources and routes of transmission of this organism may be further elucidated.

First human cases

The first two cases of human cryptosporidiosis were reported independently in 1976.[25,26] The first was a three-year-old child in rural Nashville, Tennessee who presented with severe gastroenteritis of two weeks' duration. She lived on a farm where there were cattle and domestic animals, but subsequent investigations failed to reveal the source of infection. Cryptosporidial infection was diagnosed by EM examination of an intestinal mucosa biopsy specimen. Infection was shown to produce a non-specific proctitis with infiltration of the lamina propria by acute and chronic inflammatory cells and decreased mucin production by goblet cells. The child recovered spontaneously without sequelae.

The second case was that of a 39-year-old cattle farmer who developed watery diarrhoea during the course of immunosuppressive therapy for an autoimmune disorder. Cryptosporidial infection was diagnosed by jejunal and ileal biopsy, which showed severe mucosal injury with histological changes consistent with those previously described in animals. His symptoms resolved after two weeks. It was speculated that the parasite might be a previously unknown variety for which man is a natural host. Fewer than a dozen cases of cryptosporidiosis were reported over the next five years.[27]

In 1979, two single cases were reported describing a nine-year-old patient with congenital hypogammaglobulinaemia and a 58-year-old immunosuppressed renal transplant recipient with IgA deficiency. Cryptosporidiosis in these patients was confirmed by histological examination of intestinal biopsies. The former patient was found to be co-infected with Giardia lamblia and suffered with unremitting diarrhoea and malabsorption for more than three years. In 1980, cryptosporidiosis was reported for the first time in an immunocompetent adult, who suffered severe watery but self-limiting diarrhoea and vomiting.[28] This was the first time that cryptosporidial infection had been diagnosed in a patient by detection of oocysts in faecal smears.

The emergence of AIDS and AIDS-related infections promoted cryptosporidiosis to the forefront of interest in 1982.[29]

Table 2. Previous reports on the occurrence of Cryptosporidium in human, animal and environmental sources in Ireland from 1985 to present

Source	Year	Location	Description	Reference
(i) Human	1985	West of Ireland	41/935 (4.4%) children aged three weeks to 12 years positive for *C. parvum*. 23 rural; eight farms had a recent outbreak of diarrhoea among calves	[22]
	1987	Dublin	*C. parvum* detected in children under 14 years in 4% of 1,621 admissions to a large Dublin isolation fever unit	[31]
	2000	Co Down	Water-borne outbreak involving 192 cases	[3]
	2000	Lisburn/Poleglass	Water-borne outbreak involving 117 cases	[1,2]
	2001	Belfast/Co Antrim	Water-borne outbreak involving 110 cases	[3]
(ii) Animal	2001	Dublin	Asymptomatic shedding of Cryptosporidium oocysts by red deer	[36]
	2000	Co Kildare	*C. parvum* prevalence in cattle/horses 12% and 67% respectively	Tom Buckley (personal communcation)
(iii) Shellfish	1997	Co Sligo	Detection of Cryptosporidium oocysts in marine mussels	[37]
	1999	Belfast Lough	Detection of *C. parvum* genotype I in mussels	[38]
(iv) Water	1997	Co Sligo	Detection of *C. parvum* in river water	[37]
	2000	Greater Dublin area	Five sampling sites demonstrated oocysts on at least one occasion; max no./site = 6/L. Maximum load noted after heavy rainfall	[39]
	1996-1999	Northern Ireland	474 waters investigated by IFA; 380 and IMS-IFA; 94 of which 3% positive. 214 samples also investigated by PCR techniques of which 5.1% positive. Samples classified as genotype II following sequence analysis of the TRAP-C2 amplicon	[10]

Of the first 58 cases of cryptosporidiosis described in humans by 1984, 40 (69%) were in immunocompromised patients who contracted severe, often reversible, diarrhoea. Of these 40 patients, 33 (83%) had AIDS and 55% of the 40 immunocompromised patients died. The organism was initially believed to be an opportunistic pathogen but it became increasingly apparent that there were cases of cryptosporidiosis amongst otherwise healthy individuals. Over 1,000 subsequent reports have documented cryptosporidiosis in humans in 95 countries in all continents except Antarctica.[30]

Options for the treatment of cryptosporidiosis remain a dilemma. In the case of infection in the non-immunocompromised and healthy individual, clinical management of the patient should follow standard treatment of infectious diarrhoeal diseases, including prevention of dehydration and maintenance of electrolyte balance. Given the reported low infectious dose of this infection (132 oocysts),[31] it is important to stress to patients the importance of good personal hygiene to prevent secondary cases occurring within the household.

Treatment of the disease in immunocompromised and AIDS patients is more complicated, with relatively few conventional antiparasitic agents showing clinical efficacy. Of these, three agents have shown some potential, paromomycin,[32] paromomycin in combination with azithromycin[33] and nitazoxanide.[34] However, preliminary studies with polyclonal, antibody-based immunotherapies may represent a more structured approach to the treatment of such infections. In these, high titres are developed, which when given orally in for example hyperimmune bovine colostrum, specifically knockout key stages of the pathogenic mechanisms within its life cycle, including attachment to the host epithelium in the gut. Furthermore, extension of established genome sequencing projects to include *C. parvum* may help elucidate targets to which specific interventions may be developed.

Thus, as treatment options are limited more emphasis is now being placed on the primary prevention of oocysts acquisition. Prevention strategies should therefore be directed at previous evidence-based reports, including maintenance of good personal hygiene following farm visits or with pets, as well as in households with primary cases. More importantly, there should be a re-evaluation of risk association with drinking water supplies and appropriate controls introduced where indicated to reduce the risk of acquisition from such sources.

In Ireland, there have been several reports on the isolation of *C. parvum* from humans, animals and environmental sources as outlined in Table 2. At present, cryptosporidiosis is not a specific disease which is notifiable under the Infectious Disease Regulations 1981 and as revised in 1985, 1988 and 1996, except where it is included in the 'gastroenteritis for children under two years' category. However, in a recent review paper which is currently in consultation (Review of the current list of notifiable diseases and of the process of notification), it is proposed to include *C. parvum*, as a notifiable organism for Directors of Microbiology. Currently, the frequency and genotypes of *C. parvum* that occur in the Irish population are undefined. Additionally, it is unclear how much of the epidemiology is shared between Northern Ireland and the Republic of Ireland. Further work is therefore required to examine the molecular characteristics of strains isolated on the entire island in order to help elucidate sources and routes of transmission. This would mean effective controls could be introduced to reduce the incidence of this pathogen.

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