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The prevalence of *Cryptosporidium* species and subtypes in human faecal samples in Ireland

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SUMMARY

Cryptosporidium is an important cause of diarrhoeal disease worldwide and, as several recent waterborne outbreaks have shown, poses a significant threat to public health in Ireland. We identified the *Cryptosporidium* spp. in 199 positive human stool samples by PCR–RFLP of the 18S rRNA and COWP gene loci. Subspecies were identified in 104 samples by sequence analysis of the 60 kDa glycoprotein (gp60) gene fragment. Overall *C. parvum* was identified in 80 %, and *C. hominis* in 20 % of cases. No other *Cryptosporidium* spp. were detected. *C. parvum* was by far the most common species in the rural, more sparsely populated west of Ireland and exhibited a pronounced spring peak coincident with a peak in the national cryptosporidiosis incidence rate. Our data indicated a trend towards higher proportions of *C. hominis* in older age groups. Ninety-nine per cent of all subtyped *C. parvum* isolates belonged to allele family IIa, of which allele IIaA18G3R1 was by far the most common (63 %). According to a recent study by Thompson and colleagues [*Parasitology Research* (2007), **100**, 619–624] this allele is also the most common in Irish cattle. Subtyping of the *C. hominis* isolates indicated that they belonged to a geographically widely distributed allele (IbA10G2) known to have caused several water- and foodborne outbreaks around the world. The predominance of *C. parvum*, its geographic and seasonal distribution and the IIaA18G3R1 subtype underlines the importance of zoonotic *Cryptosporidium* transmission in Ireland.

INTRODUCTION

Cryptosporidium is one of the most serious causes of waterborne diarrhoea in humans, with neonates and immunosuppressed individuals particularly at risk. Of the 16 *Cryptosporidium* species recognized today, eight have been reported from human cases [1–4]. However, only three are considered important human pathogens: *Cryptosporidium hominis*, *C. parvum* and *C. meleagridis*. *C. hominis* is largely restricted to humans, while *C. parvum* is an important zoonotic agent infecting most, if not all, mammals including humans. It is also a major pathogen of ruminant livestock with peak incidence rates occurring during calving and lambing [5, 6]. The third species, *C. meleagridis* is primarily an avian pathogen. Although common in parts of Latin America [7, 8], it appears to be rare in Northern Europe [6].

Cryptosporidium is transmitted by highly resistant, long-lived oocysts that are passed fully sporulated by the infected host. People become infected through

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direct contact with an infected individual or by ingesting contaminated food or water. The zoonotic species *C. parvum* may also be transmitted via animal-to-person contact.

In Ireland, the combination of high annual rainfall, high livestock stocking densities and the use of unfiltered surface water as drinking water render the public water supply vulnerable to contamination. Since January 2004, when cryptosporidiosis became notifiable in the Republic of Ireland (i.e. cases have to be reported to the medical officer of health), the national communicable disease surveillance agency (the Health Protection Surveillance Centre, HPSC) has reported a total of 431 cases in 2004, 568 in 2005 and 367 in 2006 [9]. These figures correspond to crude incidence rates of 10·2, 13·4 and 8·7/100 000 population respectively [7]. About 605 cases were notified in 2007 [10–13]. As faecal samples are only very rarely sent for laboratory diagnosis, these figures are believed to be a gross underestimate (exact data for Ireland are unavailable but estimates for the United Kingdom state that only about 4·6% of all cases of gastrointestinal disease in the community are sent for laboratory testing [14]). The first large-scale outbreak in Ireland occurred in the spring of 2007 in Galway on the West Coast of Ireland. In addition to about 242 confirmed cases, thousands more were affected by the boil water notice and the economic burden of buying bottled water for a prolonged period of time.

Although the parasite poses a significant public health problem in Ireland, its epidemiology on the island is poorly understood. The species has only been identified in a very small proportion of human cases, and no information whatsoever is available on subtypes that occur in the human population. In this paper we describe the prevalence of *C. parvum* and *C. hominis* in 199 human cryptosporidiosis cases collected in Ireland between 2000 and 2007. In addition, we discuss the distribution of *C. parvum* and *C. hominis* subtypes in the context of previous reports of subtypes identified in humans and neonatal calves on the island of Ireland [1, 15].

MATERIALS AND METHODS

Sample collection

Human faecal samples ($n=199$, collected between 2000 and 2007) that had been diagnosed *Cryptosporidium*-positive by the Microbiology staff in 10 hospitals (Cavan General Hospital; Cork University Hospital; University College Hospital, Galway; Midlands Regional Hospital, Westmeath; Midwestern Regional Hospital, Limerick; Our Lady's Hospital for Sick Children, Dublin; Portiuncula Hospital, Galway; St James' Hospital, Dublin; Sligo General Hospital; Waterford Regional Hospital) were sent to the Parasitology laboratories at the University College Dublin School of Agriculture, Food Safety and Veterinary Medicine, for further investigation. These represented roughly 5·5% (2005), 16% (2006) and 10% (2007) of the total incidence reported in these years (no surveillance data were collected in 2000) [9].

Epidemiological data

Where possible the following patient information was collected in conjunction with the diagnostic sample: age, county of residence and date of collection.

Molecular analysis

DNA was extracted according to the methods described by Boom *et al.* [16] as modified by McLauchlin *et al.* [17]. This technique involves breaking up oocysts in a mini bead-beater followed by DNA extraction in guanidine thiocyanate buffer. Prior to PCR amplification all DNA extracts were further purified by PVP (polyvinylpyrrolidone; Sigma-Aldrich Ireland Ltd, Dublin, Ireland) precipitation [18].

Species identification was carried out by nested PCR amplification of the 18S rRNA gene fragment according to Xiao *et al.* [19]. To differentiate *C. parvum* and *C. hominis* from any other *Cryptosporidium* spp. that may infect humans, 2 μl of the amplified product were digested with 2 U *Ssp*I and restriction buffer in a total volume of 5 μl at 37 °C for 1 h. To distinguish *C. parvum* from *C. hominis* the same amount of amplified product was digested with 2 U *Vsp*I under the same conditions. Amplified and digested products were fractionated on 2% agarose gels and visualized by ethidium bromide staining. The PCR–RFLP results based on the 18S rRNA gene fragment were confirmed by a nested PCR for the amplification of the *Cryptosporidium* oocyst wall protein gene fragment (COWP) according to the protocol published by Spano *et al.* [20] and modified by Pedraza-Diaz *et al.* [21]. For restriction fragment analysis, 2 μl amplified product were digested with

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Table 1. *Numbers of* C. parvum *and* C. hominis *identified in samples sent in from different regions around Ireland between 2005 and 2007 and total number of cases reported*

	East	Midlands	Mid-west	North-east	North-west	South-east	South	West	Unknown
2005									
C. parvum		3	7						2
C. hominis	2		4			7†			
Total no. of cases (HPSC*)	38	36	56	62	43	98	105	130	
2006									
C. parvum	1	5			4	30‡	7		
C. hominis		1				5			
Total no. of cases (HPSC*)	7	39	56	28	29	61	74	72	
2007									
C. parvum					6	26		9	
C. hominis	3	5			5[a]	3		0§	
Total no. of cases (HPSC*)	22	34	63	24	25	79	60	298	

HPSC, Health Protection Surveillance Centre.
* Refs [9–13, 25].
† All cases originating from the outbreak in Carlow, 2005.
‡ Some of these cases originated from an outbreak in Portlaw, Co. Waterford, 2006.
§ Unfortunately no samples from the outbreak in Galway were submitted to us, however, a number of *C. hominis* cases that occurred in the neighbouring County Sligo coincided with the outbreak.

2 U *Rsa*I in the appropriate restriction buffer at 37 °C for 4 h. Sequence analysis of the 60 kDa glycoprotein encoding gene fragment (gp60) was used to subtype 104 randomly selected isolates [22, 23].

Positive (purified *C. parvum* DNA) and negative controls (master mix without a DNA template) were included in each batch of PCR amplification reactions. The resulting PCR products were purified using the QIAquick PCR purification kit (Qiagen, Crawley, UK) and sequenced in both directions (GATC Biotech AG, Konstanz, Germany). The sequences were compared with published sequences using NCBI Blast and aligned with the ClustalW sequence alignment programme. Within each Gp60 allele family (i.e. Ib, IIa and IId), subtypes were identified using the nomenclature proposed by Sulaiman *et al.* [24]. In short, the subtypes are coded according to the number of trinucleotide repeats (TCA and TCG) in the microsatellite region, A14–A21 indicating the number of TCA repeats and G1–G4 indicating the number of TCG repeats. R1 and R2 are used to indicate the number of ACATCA repeats immediately after the trinucleotide repeat sequences. Gp60 fragment sequences for which there were no identical matches in GenBank were deposited under accession numbers EU272171 to EU272175.

Statistical analysis

The relative numbers of *C. parum* and *C. hominis* isolated from male and female patients were compared using χ^2 analysis.

RESULTS

In total, 50, 31, 58 and 60 samples were examined in 2000, 2005, 2006 and 2007, respectively. *C. parvum* accounted for 94% (in 2000), 39% (in 2005), 81% (in 2006) and 68% (in 2007) of all samples that were successfully genotyped. All the remaining samples were identified as *C. hominis*. In a small percentage of samples (between 2% and 20% of the annual total examined) PCR amplification was unsuccessful. This was either because they contained PCR inhibitors or because they had been wrongly identified as being *Cryptosporidium* positive.

All samples examined from 2000 had been collected in the west of Ireland. Just two of 50 isolates from that year were *C. hominis* (4%). The number of samples containing *C. parvum* and *C. hominis* obtained from different regions around the country between 2005 and 2007 are shown in Table 1. In 2005, the largest proportion of samples that were typed originated

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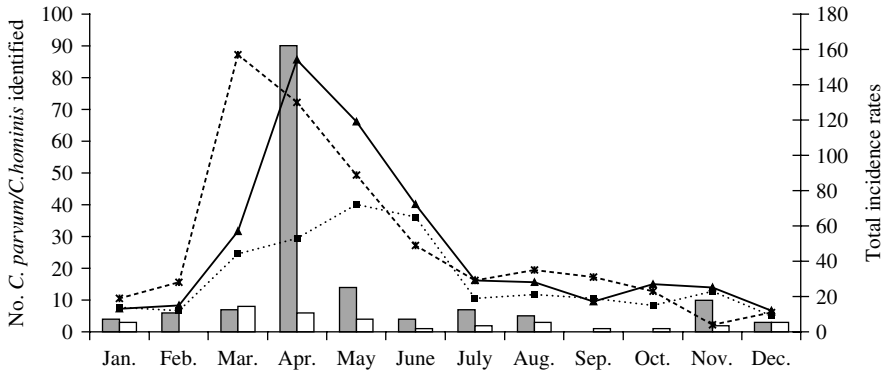

Fig. 1. Seasonal distribution of *C. parvum* () and *C. hominis* (□) in 2005, 2006 and 2007, and total numbers of reported cases in 2005 (—▲—), 2006 (· · · ■ · · ·) and 2007 (- - × - -) [9–13].

from the mid-west ($n=11$, 44%) and south-east ($n=7$, 28%). In contrast, national incidence data from 2005 showed that most cases occurred in the west, the south and the south-east of the country. Just over half of all isolates collected in the mid-west and all of the samples from the east and south-east were identified as *C. hominis*. Sixty-six percent of all samples examined in 2006 were collected in the south-east ($n=35$). According to HPSC data this region had the third highest incidence of *Cryptosporidium* that year [9]. Between 14% and 17% of isolates from the south-east and the midlands were *C. hominis*. In 2007, unprecedented incidence rates caused by the outbreak in Galway, were reported from the west of the country. As in the previous years, incidences were also high in the mid-west, the south-east and the south of the country. Again most samples were received from the south-east ($n=29$, 51%). All of the samples collected in the east ($n=3$) and the midlands ($n=5$), 75% of the samples from north-west ($n=3$), 15% of samples from the west ($n=2$) and 10% of the isolates sent from the south-east ($n=3$) were identified as *C. hominis*.

The seasonal distribution of the total numbers of *C. parvum* and *C. hominis* identified in the samples received between 2005 and 2007 (no faecal collection dates were available for 2000) is shown in Figure 1 together with the overall cryptosporidiosis incidence reported by the HPSC for each year. A very pronounced spring peak in the number of *C. parvum* coincided with peaks in the total annual incidences of cryptosporidiosis. A second, much smaller peak appears to occur in late autumn. While small numbers of *C. parvum* cases were identified throughout the rest of the year, none was detected in September and October in either of the 3 years. Small numbers of *C. hominis* cases were detected throughout the year.

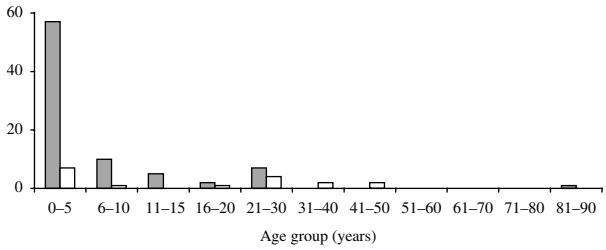

Fig. 2. Age distribution of *C. parvum* () and *C. hominis* (□) cases ($n=99$).

There appears to be a slight accumulation of this species during the spring months but numbers were too low to identify definite trends.

Information on the age of the patient was only available in 50% ($n=99$) of all samples. Sixty-five percent of all stool samples examined had been collected from children aged <6 years, 81% of all isolates originated from children aged <16 years. A plot of the the age distribution of *C. hominis* and *C. parvum* (Fig. 2) indicated a trend towards higher proportions of *C. hominis* in older age groups.

A total of 104 randomly selected faecal samples were subtyped on the basis of the gp60 locus (25 *C. hominis* and 79 *C. parvum* isolates). All *C. hominis* isolates belonged to the same gp60 subtype which was homologous to a previously described DNA fragment logged under the accession number AY167596. According to the nomenclature described by Sulaiman *et al.* [24] they were identified as gp60 subtype IbA10G2. Of the *C. parvum* isolates, 78 belonged to the allele family IIa and one to the allele family IId. The IIa alleles fell into 11 subtypes of which IIaA18G3R1 was by far the most prevalent (Fig. 3). All IIa isolates had only one ACATCA repeat following the trinucleotide repeat region (designated

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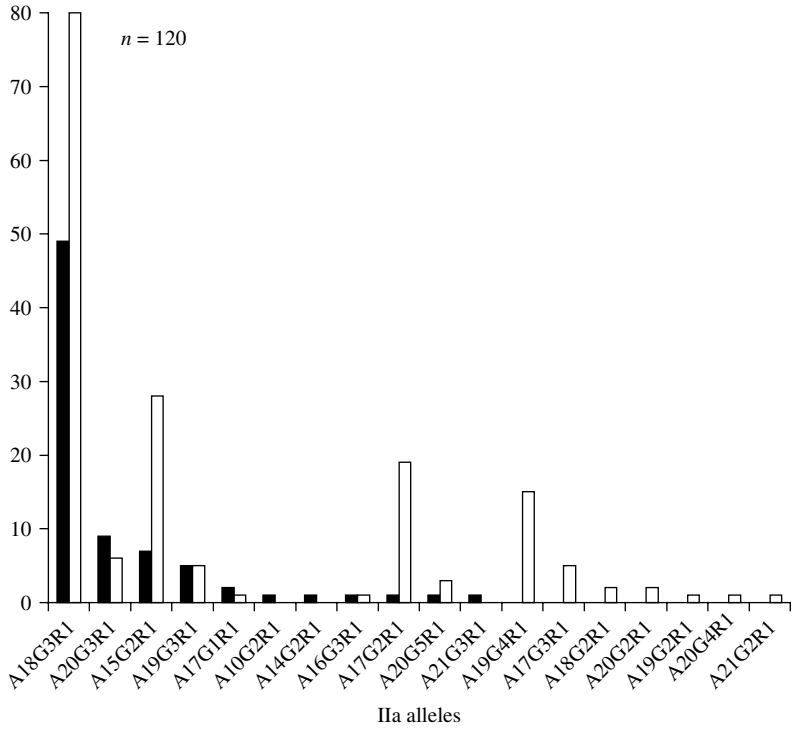

Fig. 3. Frequency of various *C. parvum* IIa alleles in 79 human isolates (■) characterized in the present study compared to 216 neonatal calf samples (□) genotyped by Thompson *et al.* [1] in Northern Ireland.

R1). The IId allele was identified as subtype IIdA26G1 (identical to logged sequence AY738185) [24].

DISCUSSION

Among the clinical isolates characterized during this study, *C. parvum* was predominant in all years except in 2005. In that year samples from an outbreak caused by *C. hominis* in County Carlow in the south-east [26] were over-represented in our sample selection. Overall *C. parvum* was identified in 80%, and *C. hominis* in 20% of all cases. They were the only two species identified. This predominance of *C. parvum* was also observed in other European countries such as France [27], Switzerland [28], Portugal [23], and Ireland's closest neighbour, the United Kingdom [4, 6, 21] including Northern Ireland [5]. The notable exception to this was Spain where human cases with *C. hominis* outnumbered those with *C. parvum* [29]. Moreover, *C. hominis* was found to be the predominant species in studies carried out in the Americas, Africa, Australia, and Asia [30]. Most if not all, larger community-scale outbreaks that have occurred on the island of Ireland over the last number of years have been waterborne. Of these, four were attributed to *C. hominis* [two outbreaks in the greater Belfast area in 2000/2001 with 117 and 230 confirmed cases [15]; the incidence in Carlow mentioned above (26 cases) and the large-scale outbreak in Galway in 2007 (242 cases)], and three to *C. parvum* (in Belfast in 2000 with 129 confirmed cases [15]; Westmeath, 2002 (26 cases) [31]; Waterford, 2006 (8 cases) [32], respectively). While the number of community-size outbreaks caused by the two species was similar, the number of people affected by outbreaks due to *C. hominis* was almost four times higher than the number of cases resulting from *C. parvum* outbreaks. Our results indicated that among sporadic cases the incidence of the two species was reversed with *C. parvum* being up to four times more common than *C. hominis*. This has also been observed in the United Kingdom [6, 21]. Waterborne outbreaks due to *C. parvum* tend to coincide with lambing or calving [5, 6]. In contrast, outbreaks due to *C. hominis* are reported to occur throughout the year. Interestingly, however, all *C. hominis* outbreaks in Ireland in the recent past occurred in the spring. Moreover, outbreaks due to either species occur in both rural and urban areas. As McLauchlin *et al.* [6] pointed out, the source of water and the proportion of surface water used in public water supply is much more important for determining the predominant route of contamination than the community that is

served by the water supply. Considering the island's mild and wet climate ideal for the survival and distribution of oocysts combined with the fact that most drinking water catchments are intensively used for agriculture, it is to be expected that contamination of reservoir water bodies with *C. parvum* is quite common. That waterborne outbreaks due to *C. parvum* are relatively rare and that fewer people are affected by them when they do occur may be due to a level of background immunity that already exists in a predominantly rural population.

The overall incidence data of cryptosporidiosis in Ireland released by the HPSC [9–13, 25] shows an uneven distribution across the country, with fewer cases in the east and north-east and an increase in numbers towards the south-east, the midlands and the west coast. This distribution is reflected in a decline in population density from east to west and an increase in the importance of agriculture. While *C. hominis* was more commonly identified in samples from the east of the country, the numbers were unfortunately too small to draw conclusions. However, our results indicate that in the west of the country, *C. parvum* is by far the most common cause of sporadic cases.

It is generally agreed that the spring peak in human cryptosporidiosis is due to a sharp increase in environmental pollution with *C. parvum* oocysts during lambing and calving [5, 6]. This was borne out by the large numbers of *C. parvum* cases identified during the spring months in this study. In the United Kingdom, the national *Cryptosporidium* incidence has a bimodal distribution, with the autumn peak thought to be in part due to a second calving event later in the year, although both species are detected during this time [6]. Interestingly in Ireland, the autumn peak is absent [9], although there appeared to be a slight increase in *C. parvum* cases in late autumn. It may be that autumn calving is less practised than in the United Kingdom or that autumn calves are housed earlier limiting animal-to-human contact. Sporadic *C. hominis* cases occurred throughout the year. It is generally thought that they are more common in patients with a history of foreign travel [6, 21]. Probably as a result of this they tend to be more prevalent among older patients as was observed in the present study. It may also be the case that adults are more likely to seek medical attention when infected with *C. hominis* because of its greater pathogenicity [33].

Ever since it has become obvious that great biological and genetic heterogeneity exists within some *Cryptosporidium* spp., particularly *C. parvum*, isolates have been typed to subtype level at numerous loci. By this approach it is hoped to identify the most important transmission routes in an area and aid the sourcing of future outbreaks. In the present study we typed just over half of the human isolates to subspecies level at the gp60 locus. This gene codes for a sporozoite surface glycoprotein, is highly polymorphic and contains a microsatellite region. Worldwide the most common *C. parvum* gp60 alleles identified in humans are IIa and IIc (formerly known as Ic) [24]. IIa was also the most common human *C. parvum* allele identified in our study. As it is by far the most predominant allele in cattle [1, 23, 34], its predominance in human cryptosporidiosis stresses the importance of zoonotic transmission in Ireland. The most prevalent IIa subtype in our study, IIaA18G3R1, was also the most frequently identified human subtype in a study carried out in Northern Ireland in 2000/2001 [15]. Glaberman *et al.* [15] typed *C. parvum* isolates from an outbreak in the greater Belfast area and from several sporadic cases originating from the west coast of Ireland. The IIaA18G3R1 subtype was also the most prevalent *C. parvum* subtype identified in neonatal calves in Northern Ireland (55·6%) [1] (Fig. 3). Of the other IIa subtypes identified in our study, IIaA15G2R1, IIaA17G2R1 and IIaA19G3R1 were also detected in a recent study of human cryptosporidiosis samples collected in Australia [35]. Subtype IIaA15G2R1 has also been reported from human cases in such widely dispersed places as Portugal [23], Kuwait [24], Canada [36] and the United States [34]. Moreover, this subtype and IIaA17G2R1 were prevalent among neonatal calves in Northern Ireland [1]. On the other hand, another common calf IIa allele (IIaA19G4R1) that was absent in our study was confined to a particular area in Northern Ireland. The authors suggested that all calves infected with this subtype may have had a common source of infection [1]. The only other *C. parvum* allele we detected in one human sample was IId. This allele was as common as IIa among children in Kuwait [24], but apart from that has only been found in a small number of patients and cattle in Portugal [23] and a single HIV + human isolate from Switzerland [35]. The anthroponotic allele IIc, which is the most prevalent *C. parvum* allele in the Americas and Africa [30] and others described elsewhere (e.g. IIb, IIf) were not detected in Ireland.

All *C. hominis* isolates belonged to the same allele Ib, and within this allele to the same subtype, IbA10G2. Interestingly two community-based out-

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breaks in Northern Ireland in 2000–2001 and several sporadic cases from the north-west of England were ascribed to the same Ib subtype [15]. Reports from all over the world indicate that Ib has a wide geographic distribution and has been the cause of several water- and foodborne outbreaks worldwide [15]. The dominance of a single *C. hominis* gp60 allele in the Republic and Northern Ireland contrasts with the large variety of *C. hominis* subtypes (belonging to alleles Ia, Ic, Id, Ie, If) identified in Portugal, India, Canada and Australia [23, 35–37]. This homogeneity may be the result of Ireland's geographic isolation. It is possible that IbA10G2 is the only endogenous *C. hominis* subtype or that it was introduced at some time in the past and has since become established on the island. If so it is probably only a matter of time until other exotic *C. hominis* subtypes are introduced by returning holiday-makers.

To conclude, it appears that in terms of the overall numbers of people affected, zoonotic transmission of *C. parvum* is more important in sporadic cryptosporidiosis while *C. hominis* is most prevalent in outbreak situations. Further studies of subtypes that occur in humans and livestock are necessary to better identify the *C. parvum* subtypes that are most important to human health and to clarify the role of animals as the source of disease outbreaks.

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DECLARATION OF INTEREST

None.

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