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Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhoea in England between 1985 and 2000

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The characterization of *Cryptosporidium* using DNA extracted from whole faecal samples collected from 2414 humans with diarrhoea in England between 1985 and 2000 where cryptosporidial oocysts were detected using conventional methods is described. Characterization was achieved by PCR/RFLP and DNA sequencing of fragments of the *Cryptosporidium* oocyst wall protein and the 18S rDNA genes. *Cryptosporidium parvum* was detected in 56·1 % of cases, *Cryptosporidium hominis* in 41·7 % and a mixture of *C. parvum* and *C. hominis* in 0·9 %. In the remainder of cases, *Cryptosporidium meleagridis* (0·9 %), *Cryptosporidium felis* (0·2 %), *Cryptosporidium andersoni* (0·1 %), *Cryptosporidium canis* (0·04 %), *Cryptosporidium suis* (0·04 %) and the *Cryptosporidium* cervine type (0·04 %) were detected.

INTRODUCTION

Cryptosporidium is a genus of protozoan parasites that infect a wide range of vertebrates including humans. Human cryptosporidiosis is one of the most common causes of diarrhoeal disease due to enteric protozoa, and results in significant morbidity and mortality in both the developing and developed world. Transmission is through the faecal–oral route, following direct or indirect contact with *Cryptosporidium* oocysts via person-to-person, zoonotic, waterborne, foodborne or airborne contact (Meinhardt *et al.*, 1996; Fayer, 2004).

Molecular biology has provided powerful new tools for characterizing *Cryptosporidium* and has revealed significant variation within the genus (Xiao *et al.*, 2004). *Cryptosporidium* is now recognized as containing assemblages of species which are genotypically heterogeneous but morphologically largely identical (Xiao *et al.*, 2004). In addition to the named species, genotypes are also recognized, some of which have a greater degree of genetic diversity than is found between the named species. However, there is not yet sufficient additional information available for these genotypes to be further classified with species status (Xiao *et al.*, 2004). It is likely that some of these genotypes will be named as separate species in the future. There are marked differences in the host ranges of different *Cryptosporidium* species and genotypes, and there is growing evidence for differences in parasite development, growth rates, drug sensitivity and disease presentation in humans (Hunter *et al.*, 2004a, b). Because of these inter-species and inter-genotypic differences, molecular epidemiological analysis is essential to understand the transmission of these parasites and will be invaluable in the public health investigation of cryptosporidiosis including the targeting of the most appropriate interventions.

We previously reported different *Cryptosporidium* species associated with human cryptosporidiosis in England (Pedraza-Díaz *et al.*, 2001a, b; McLauchlin *et al.*, 2000). In this report, we extend these studies to characterize *Cryptosporidium* species present in more than 2400 faecal samples from humans with diarrhoea in England between 1985 and 2000.

METHODS

Samples of whole faeces were collected from 2414 patients with diarrhoea in England between 1985 and 2000 where hospital laboratories had performed routine microbiological analyses and detected *Cryptosporidium* oocysts using conventional microscopy techniques (Arrowood, 1997). Among the 2414 cases, 589 occurred in seven drinking water borne outbreaks, 51 were associated with seven swimming pool outbreaks and 102 occurred within 37 family outbreaks. The remaining 1672 cases (69 % of all cases) were apparently sporadic. The distribution of *Cryptosporidium parvum* and *Cryptosporidium hominis* within the swimming pool and drinking water associated outbreaks has been published elsewhere (McLauchlin *et al.*, 2000; Pedraza-Díaz *et al.*, 2001a).

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Abbreviation: COWP, *Cryptosporidium* oocyst wall protein gene.

The GenBank/EMBL/DDBJ accession numbers for the sequences described in this study are DQ116568, DQ116569, DQ116570, DQ116571 and DQ116572.

Table 1. *Cryptosporidium* species detected amongst 2414 human cases in England 1985–2000

Cryptosporidium species	No. of patients (%)
C. hominis	1005 (41·7)
C. parvum	1354 (56·1)
C. hominis and *C. parvum*	21 (0·9)
C. meleagridis	22 (0·9)
C. felis	6 (0·2)
C. andersoni	3 (0·1)
C. canis	1 (0·04)
C. suis	1 (0·04)
Cryptosporidium cervine type	1 (0·04)

DNA was extracted from whole faeces by a modification of the 'Boom' method using mechanical disruption in the presence of zirconium beads and guanidinium thiocyanate (McLauchlin *et al.*, 1999). Where amplification was not detected, DNA was further purified using polyvinylpyrrolidone treatment as described previously (Lawson *et al.*, 1997).

PCR was applied to DNA samples for amplification of a fragment of the *Cryptosporidium* oocyst wall protein (COWP) gene (Spano *et al.*, 1997; Pedraza-Díaz *et al.*, 2001a), and selected samples were further tested by analysis of a fragment of the 18S rRNA gene (Johnson *et al.*, 1995; Bornay-Llinares *et al.*, 1999). Post-PCR analyses were performed either by RFLP analysis of the COWP gene fragment using *Rsa*I, or by cloning the PCR products into the TOPO plasmid vector with the TOPO-TA cloning kit (Invitrogen) and sequencing in both directions using an ABI377 automated sequencer and BigDye terminator chemistry with M13 primers, or using a CEQ 2000 Dye Terminator Cycle Sequencing (DTCS) Quick Start kit and a CEQ 2000XL automated capillary sequencer (Beckman Coulter) with Ptag primers. Sequence analysis was performed either using the Genetics Computer Group (GCG) program package (University of Wisconsin) and CLUSTALW, or with GeneBuilder, CLUSTAL, Bionumerics version 2.5 (Applied Maths, Hortrijk, Belgium) and BioEdit Sequence Alignment Editor version 5.0.0.

RESULTS AND DISCUSSION

DNA extracts from 2414 samples were initially screened by PCR/RFLP analysis of the COWP gene fragment. Selected samples, including those showing no or unusual products, were further analysed by PCR amplification and sequencing of the 18S rDNA fragment as well as, where possible, sequencing of the COWP fragment. Using PCR/RFLP analysis of the COWP fragment alone, *C. parvum* and *C. hominis* were detected in the majority of the cases (98·6 %), and *Cryptosporidium meleagridis* in a further 22 (0·9 %) samples (Table 1). The COWP fragment was not amplified from the remaining 12 samples.

To investigate the reliability of RFLP analysis of the COWP fragment for identification of *C. parvum*, *C. hominis* and *C. meleagridis*, the COWP and 18S rRNA gene fragments amplified from 71 samples were sequenced. Identification was confirmed for 22 *C. hominis* and 30 *C. parvum* samples and no sequence variation from previously deposited sequences was detected within either of the two gene fragments (Table 2).

The COWP gene fragment was also characterized by sequencing from 19 of the samples where *C. meleagridis* had been identified. Eighteen of the 19 COWP fragments showed no DNA sequence variation from previously deposited *C. meleagridis* sequences at this locus. Sequence analysis of the 18S rDNA fragment was not performed for six of the *C. meleagridis* samples. The identification as *C. meleagridis* was further confirmed in 12 of these samples by an absence of sequence variation at the 18S rDNA locus as compared to previously deposited sequences (Table 2). Analysis of DNA from the final sample where *C. meleagridis* was detected by PCR/RFLP analysis showed 99 % identity (504/506 bp) with two mismatches to the sequence of the COWP fragment (AF266266) and 99 % identity (391/392 bp) with one mismatch to the sequence of the 18S fragment (AF112574) previously designated type 2A (Glaberman *et al.*, 2001).

Analysis of the 18S rDNA fragment amplified from the 12 samples from which the COWP gene fragment could not be amplified revealed the presence of: *Cryptosporidium felis* (six samples), 100 % identical (313/313 bp) to AF087577 (Bornay-Llinares *et al.*, 1999); a *Cryptosporidium* more similar to *Cryptosporidium andersoni* than to *Cryptosporidium muris*

Table 2. Confirmation of *Cryptosporidium* identification by DNA sequencing of COWP and 18S rDNA fragments

Species	No. of patients	Sequence analysis applied to:
C. hominis	22	All by both COWP and 18S rDNA fragments
C. parvum	30	All by both COWP and 18S rDNA fragments
C. meleagridis	19	All by COWP and 13 by 18S rDNA fragment
C. felis	6*	All by 18S rDNA only
C. andersoni	3*	All by 18S rDNA only
C. canis	1*	All by 18S rDNA only
C. suis	1*	All by 18S rDNA only
Cryptosporidium cervine type	1*	All by 18S rDNA only

*No amplification of COWP fragment detected.

(three samples), with 99 % identity (389/390 bp with 1 gap) to *C. andersoni* AF093496 (Xiao *et al.*, 1999a) and 98 % identity (383/390 bp with 2 gaps) to *C. muris* AF093498 (Xiao *et al.*, 1999a); *Cryptosporidium canis* in one sample (Pedraza-Díaz *et al.*, 2001b); *Cryptosporidium suis* in one sample, 99 % identical (392/394 bp) to AF115377 (Xiao *et al.*, 1999b); and *Cryptosporidium* cervine type in one sample, 100 % identical (390/390 bp) to isolate 563 AF297512 (Perz & Le Blancq, 2001).

The ages of the patients from whom the least common *Cryptosporidium* species/genotypes were recovered are shown in Table 3; none were associated with outbreaks. Two of the patients from whom *C. felis* was recovered were immunocompromised (Pedraza-Díaz *et al.*, 2001b); however, none of the other patients were known to be immunocompromised, and no other enteric pathogens were detected by routine microbiological procedures. Two patients had a history of recent foreign travel: one of the patients with *C. andersoni* had recently returned from the Canary Islands and the patient with *C. canis* had returned from Africa.

This study summarizes analyses of a large series of cases of cryptosporidiosis. Data on confirmation by sequencing of identification of *Cryptosporidium* species by RFLP analyses of the COWP gene fragment validates this as a strategy suitable for national surveillance of human infections since the method accurately identified the most common species associated with human infection. However, we would not recommend this alone for analyses of samples from other sources (e.g. faeces of animals, water or food) since *Cryptosporidium* species with additional variation in the COWP gene are likely to be present and this RFLP method may give misleading results.

The results of this study confirm the importance of *C. parvum* and *C. hominis* (previously named *C. parvum* genotypes 2 and 1 or calf and human types, respectively) as the major causes of cryptosporidiosis in the general population. Similar data have been reported for other European countries including the Czech Republic (Hajdusek *et al.*, 2004), Denmark (Enemark *et al.*, 2002), France (Guyot *et al.*, 2001), the Netherlands (Homan *et al.*, 1999), Northern Ireland (Lowery *et al.*, 2001), Switzerland (Glaeser *et al.*, 2004; Fretz *et al.*, 2003) and Scotland (Mallon *et al.*, 2003) albeit that these other studies were on a much smaller scale and included a maximum of 135 patients (Mallon *et al.*, 2003). Chalmers *et al.* (2002) reported on 3100 isolates that were collected in England and Wales after collection of the isolates described here. The majority of their isolates were either *C. parvum* or *C. hominis* (the numbers of these were not stated), 21 were *C. meleagridis* and two could not be identified (Chalmers *et al.*, 2002).

The presence of a *Cryptosporidium* species other than *C. parvum* and *C. hominis* in 1·4 % of patients also confirms our previous report (Pedraza-Díaz *et al.*, 2001b). *C. canis*, *C. felis* and *C. meleagridis* have been reported in immunocompetent patients elsewhere (Xiao *et al.*, 2001). Infection with *C. suis* (Xiao *et al.*, 2002; Cama *et al.*, 2003) and a *Cryptosporidium* parasite similar to *C. andersoni* (Guyot *et al.*, 2001) has been reported in human patients with HIV. The finding here of a heterogeneous group of *Cryptosporidium* species in humans without immunocompromising illness suggests that 'unusual' species may play a role in human infections and highlights the need to improve detection methods for a better understanding of the disease in humans.

The 18S rDNA fragment of the *Cryptosporidium* cervine type found in this study was identical to that initially described as genotype 3, which was amplified from a white-tailed deer in a wildlife survey in Lower New York State (Perz & Le Blancq, 2001) and from storm water samples collected from a stream in the watershed area of New York State (Xiao *et al.*, 2000). Analysis of the 18S rDNA sequence showed two deletions from the group of isolates described as genotype 3 above as compared with the cervine type previously reported in human infections (Ong *et al.*, 2002), which was identical to the cervine type genotype 3 isolate 524 (AF297511) from a white-tailed deer (Perz & Le Blancq, 2001). Furthermore, the sequences from the human samples reported as the 'cervine genotype' had 100 % sequence identity in the 18S rDNA gene fragment to that from an isolate subsequently described from captive lemurs (da Silva *et al.*, 2003). Both isolates from humans and captive lemurs amplified an N-terminal portion of the COWP gene with primers Cry15/Cry9 and had 100 % sequence homology (Ong *et al.*, 2002; da Silva *et al.*, 2003). The isolate described here as the cervine type did not amplify the same fragment of the COWP gene using the nested PCR procedure (Pedraza-Díaz *et al.*, 2001a). It is likely that the human cases previously reported as the *Cryptosporidium* cervine type were the same as that described as the lemur type, and differed slightly to the *Cryptosporidium* species from a human case reported here.

Table 3. Characteristics of the patients associated with the more unusual types/species of *Cryptosporidium*

Cryptosporidium species	Age in years
C. felis	2
	2
	8
	19
	30
	32
C. andersoni	11
	43
	NK
C. canis	1
C. suis	2
Cryptosporidium cervine type	4

NK, Not known.

In summary, we report here the distribution of *Cryptosporidium* species amongst 2414 patients with diarrhoea in England between 1985 and 2000. Although *C. parvum* and *C. hominis* were identified in the majority of patients, other species were detected in a small proportion of cases including the immunocompetent. This report provides valuable baseline data for identification of changes in the distribution of *Cryptosporidium* species.

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