SHORT ARTICLE

Unusual *Cryptosporidium* species recovered from human faeces: first description of *Cryptosporidium felis* and *Cryptosporidium* 'dog type' from patients in England

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DNA was extracted from faecal samples collected from 1680 patients in which *Cryptosporidium* oocysts were recognised by light microscopy. DNA from faeces from five of these patients failed to amplify by PCR three gene fragments – the *Cryptosporidium* oocyst wall protein (COWP) gene, the thrombospondin-related adhesive protein of *Cryptosporidium*-1 (TRAP-C1) gene and the thrombospondin-related adhesive protein of *Cryptosporidium*-2 (TRAP-C2) gene – with primers designed from *C. parvum* sequences. However, DNA from these five patients did amplify cryptosporidial 18S rDNA gene fragments and a heat-shock protein (HSP70) gene fragment was also amplified from four of them. The purpose of this study was to characterise further the *Cryptosporidium* associated with infection in these patients. DNA sequence analysis of 18S rDNA genes showed that four of these patients were infected by *C. felis*, and the remaining one by an as yet un-named *Cryptosporidium* species designated the 'dog type' (*C. dt*). Infection by *C. felis* was further confirmed in all four patients by DNA sequence analysis of the HSP70 gene. Oocysts present in all five samples reacted strongly with two anti-cryptosporidial oocyst monoclonal antibodies, except for the *C. dt*, which was tested with only one of the antibodies. Two of the patients infected by *C. felis* had underlying illness; one 8-year-old male had an undefined severe inherited underlying condition, and the second patient, a 32-year-old male, was HIV positive. Two of the remaining three patients (two females aged 1 and 2 years, respectively) were apparently immunocompetent (one infected with *C. felis* and one with the *C. dt*). No information was obtained for the fifth patient. The patient infected by *C. dt* had a recent history of travel to Africa. This is the first report of infection with these two *Cryptosporidium* species in immunocompetent patients, and in any patient in the UK.

Introduction

Cryptosporidia are species of protozoan parasites that infect various mammals, birds, reptiles and fish [1]. Traditionally, the different species of this genus have been identified on the basis of host range, oocyst morphology and site of infection. However, DNA sequence analysis shows the genus to be complex; it may contain >20 different species, which cannot be distinguished reliably by traditional methods [1].

Molecular techniques show *Cryptosporidium parvum* to be the major cause of cryptosporidiosis in humans and to comprise two different genotypes [1] that may constitute separate species [2]. However, other *Cryptosporidium* species have also been associated with human infections: *C. felis*, *C. meleagridis* and an as yet un-named *Cryptosporidium* species designated the dog-type (*C. dt*) have been detected in the faeces of HIV-positive individuals, [3,4]. *C. meleagridis* has also been found in the faeces of immunocompetent individuals [5].

the *Cryptosporidium* oocyst wall protein (COWP) gene [6] have been described previously. These techniques were applied to faecal specimens from 1680 patients, and the COWP gene fragment was amplified from 95.2% of the samples [6]. Specimens from some patients failed to amplify the COWP gene fragment, but did amplify fragments of the 18S cryptosporidial rDNA gene, and it was suggested that these may represent species of *Cryptosporidium* other than *C. parvum* [6]. The purpose of the present study was to characterise these cryptosporidia further and to give brief clinical information on the infected patients.

Materials and methods

The faecal specimens collected were from diarrhoeal patients in which *Cryptosporidium* oocysts were detected by hospital laboratories by conventional microscopy [7]. Brief clinical and epidemiological details were obtained from the original request forms submitted with the specimen, and further information was obtained by interview of the patient (or where appropriate the patient's parents) with the use of a standard questionnaire.

Specimens were stored at 4°C without preservatives and re-examined by light microscopy in the PHLS Food Safety Microbiology Laboratory by modified Ziehl-Neelsen (MZN) acid-fast and immunofluorescence staining [2]. Immunofluorescence staining was performed with two monoclonal antibodies (MAbs) against *Cryptosporidium* oocyst cell surface: one produced 'in-house' (designated MAB-C1 [2]) and one produced commercially (Cellabs, Brookvale, Australia). Oocyst disruption and DNA purification were performed as described previously [2].

PCR amplification of the 18S rDNA fragment 1 [8, 9], 18S rDNA fragment 2 [2, 10], COWP [6, 11], TRAP-C1 [12], TRAP-C2 [13] and HSP70 [14] gene fragments were performed as described previously and were analysed by electrophoresis in agarose 1% ethidium bromide gels and recorded by UV transillumination with type 667 film (Polaroid, St Albans).

For DNA sequencing, PCR products were purified in Microspin S-400 HR (Pharmacia Biotech, St Albans) and cloned with the TOPO-TA Cloning kit (Invitrogen). Plasmid DNA was purified with the Promega Wizard SV kit (Promega) and clones were sequenced on an ABI377 automated sequencer with BigDye™ terminator chemistry with the M13 set of primers at the Single Reaction DNA Sequencing Service (Cambridge Bioscience, Cambridge, UK). Sequences were analysed with the Genetics Computer Group (GCG) program package (University of Wisconsin, WI, USA).

Results

DNA was extracted from faecal samples obtained from 1680 patients and shown to contain cryptosporidial oocysts. Extracts from the faeces of five patients (designated A–E) failed to amplify the COWP, TRAP-C1 or TRAP-C2 gene fragments, but did amplify 18S rDNA gene fragments 1 and 2, except for the DNA from patient C, which did not amplify fragment 2. The 18S rDNA fragment 1 was sequenced from all five patients (AF323563–AF323567) and found to be identical to the sequence for *C. felis* (AF087577) in patients A–D, and for the *C. dt* (AF087576) in patient E. Amplification of the HSP70 gene fragment was also achieved from patients A–D. Sequence analysis showed that these fragments (AF323568–AF323571) were identical to the sequence for *C. felis* (AF221538), confirming the results obtained for the 18S rDNA fragment 1. Examination of the faecal samples from the five patients by conventional light microscopy showed that the oocysts from all five samples were morphologically indistinguishable from *C. parvum* by MZN and initially reacted strongly by immunofluorescence with MAB-C1. All samples were tested by immunofluorescence with the Cellabs MAb at least 1 year later, and all were strongly positive except for the *C. dt* (patient E), with which no oocysts were detected by either the MAB-C1 or the Cellabs antibodies.

Brief clinical details were obtained for all patients, and further information about two of the cases was obtained by questionnaire (Table 1). Patients B (*C. felis*) and E (*C. dt*) were apparently immunocompetent, and no

Table 1. Brief clinical details of patients infected by *C. felis* and the *Cryptosporidium* dog type

Patient number	Age	Sex	Underlying illness	Date of collection	Recent travel history
<i>C. felis</i>					
A	8	M	Yes*	Sept. 1998	NK
B	2	F	No	Dec. 1998	No
C	32	M	HIV positive	Dec. 1998	NK
D	19	F	NK	Sept. 1999	NK
<i>Cryptosporidium</i> dog type					
E	1	F	No	April 1999	Africa

NK, not known.

*Undefined severe inherited underlying condition.

other pathogen was identified in their faeces. Patient A had an undefined severe inherited underlying condition, and patient C was HIV positive. Apart from a recent history of foreign travel with swimming pool use in Africa (patient E), no other risk factors were identified, including exposure to animals or consumption of contaminated drinking water, food or beverages.

Discussion

Previous studies have shown *C. parvum* to be the major species of *Cryptosporidium* in human infection [1]. However, the identification of additional species that affect man [3, 4] is important and highlights the need for further molecular studies to enhance understanding of the disease. To achieve this, further characterisation of a wider range of cryptosporidia is needed, together with a re-evaluation of the taxonomy of the genus. Phylogenetic analysis based on DNA sequencing of 18S rDNA and a 70-kDa heat-shock protein (HSP70) support the characterisation of *C. felis* and the *C. dt* as distinct from *C. parvum* and as separate species [1, 14, 15]. Both *C. felis* and the *C. dt* have been described in the faeces of HIV-positive individuals [3, 4] and this current report is the first to describe infection of immunocompetent patients and of patients in the UK with these two *Cryptosporidium* species.

As cryptosporidia do not multiply in the environment, and infection results from either direct or indirect exposure to an infected animal [16], an understanding of the host range of different *Cryptosporidium* species is essential for the identification of reservoirs of infection and ultimately control of the disease. To date, *C. felis* has been isolated from the faeces of cats, a cow and human patients [3, 4, 9, 17, 18], and *C. dt* has been recovered from dogs and human patients [3, 19]. However, because of the similarities in the physical appearance of oocysts produced by different species of *Cryptosporidium* (see below) and the, as yet, limited application of molecular analysis to larger series of samples from a wide range of potential hosts, the true distribution of these parasites remains to be established.

The reasons for the inability to amplify fragments of the COWP TRAP-C1 and TRAP-C2 genes with the existing primers reported here is not known. Difficulties in amplification of the COWP gene from *C. meleagridis* DNA were most likely to be due to a single base mismatch in one of the primer sequences and were recently reported from this laboratory [5]. A similar explanation is most likely for the PCR amplification failures in the present study. However, this study demonstrates that a combined analysis with PCR procedures for COWP and 18S rDNA gene fragments will identify unusual *Cryptosporidium* species in human faeces and will require further characterisation by DNA sequencing for identification.

The lack of reaction between the *C. dt* and Cellabs MAB may be due to spontaneous degradation of the oocysts, as oocysts could not be detected on re-testing the sample with the antibody MAB-C1 *c.* 1 year after the initial tests. This sample behaved differently from *C. parvum* oocysts similarly stored in whole faeces, which retained morphology and strong reaction with both the MABs over much longer periods of time (unpublished data).

The identification of different species of *Cryptosporidium* in environmental samples relies on the recognition of oocyst morphology in several different staining procedures, including reactions with MABs [20]. The reaction of these antibodies with *C. felis* and the *C. dt* is reported here, and it has been shown previously that both these antibodies react with *C. parvum* (genotypes 1 and 2), *C. meleagridis* and *C. wairi* [2, 5, unpublished data]. These five different *Cryptosporidium* species all produce oocysts of similar size [1] and hence it is not possible to distinguish them reliably by immunofluorescence microscopy. As these species are detected in human infections in very different proportions (see below), there are considerable problems with assessing the public health importance of oocyst detection in water (together with foods or beverages) based solely on the current detection methods using microscopy.

Previous studies have shown that *C. parvum* is the major species involved in human infection [16]. This study, together with other data from this laboratory from >2000 cases of cryptosporidiosis, allows an estimation, for the first time, of the distribution of different *Cryptosporidium* species that cause human disease in the UK [2, 5, 6, 21, unpublished data]. Approximately 97% of cases in which *Cryptosporidium* oocysts are detected in human faeces are due to *C. parvum*, 1% to *C. meleagridis*, 0.2% to *C. felis* and <0.1% to the *C. dt*: the remainder have not yet been characterised. This distribution varies markedly in immunocompromised patients where a greater proportion of non-*C. parvum* species and both *C. parvum* genotypes 1 and 2 occur together (unpublished data). This study, together with other recent advances in the molecular analysis of cryptosporidia, highlights the hitherto uncharacterised complexity of human cryptosporidiosis.

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