Parasitol Res (2008) 103:37–42
DOI 10.1007/s00436-008-0924-5

ORIGINAL PAPER

Genotyping of *Cryptosporidium* isolates from human clinical cases in Poland

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Received: 3 January 2008 / Accepted: 6 February 2008 / Published online: 27 February 2008

Abstract *Cryptosporidium* spp. infection is usually self-limited in immunocompetent hosts but can be severe and life threatening in children and in immunocompromised individuals including those with primary or acquired immunodeficiencies. One hundred and three faecal samples were collected from 35 hospitalised patients with different symptoms and tested for the presence of the parasite. *Cryptosporidium* oocysts were found in four of 35 patients (11.4%) using Ziehl–Neelsen staining of faecal smears and immunofluorescence assay, whereas 12 (34.3%) samples tested positive by nested polymerase chain reaction assay. *Cryptosporidium* DNA was detected in one bile sample but not in a liver tissue biopsy sample collected from a patient who suffered from sclerosing cholangitis. Sequence analysis of oocyst wall protein and beta-tubulin gene fragments revealed three different parasite species (*Cryptosporidium hominis*, *Cryptosporidium meleagridis* and *Cryptosporidium parvum*) in children with primary immunodeficiencies, whereas only *C. parvum* was found in immunocompetent individuals and in those with secondary immunodeficiencies. This study has revealed a high prevalence of *Cryptosporidium* infection in hospitalised patients in Poland and confirmed that molecular techniques enable a more sensitive detection of the parasite.

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Introduction

Cryptosporidium infections are increasingly recognised as a cause of diarrhoea in immunodeficient patients, elderly persons and children worldwide (Neill et al. 1996; Hunter and Nichols 2002; Huang et al. 2004). Cryptosporidiosis which is usually self-limiting in immunocompetent individuals can be severe and life threatening in immunocompromised ones, particularly in AIDS patients and in those with primary immunodeficiencies (PID) (Winkelstein et al. 2003; Cacciò et al. 2005; Thompson et al. 2005). Patients with secondary immunodeficiencies, i.e. those undergoing immunosuppressive treatment before or after transplantation or undergoing chemotherapy during cancer treatment, constitute another group at high risk of acute cryptosporidiosis (Campos et al. 2000; Abdo et al. 2003; Hong et al. 2007). Among different PID, patients with CD40 ligand deficiency (CD40L) and patients with combined immunodeficiency syndromes or interferon gamma deficiency are particularly prone to *Cryptosporidium* infection (Kocoshis et al. 1984; Levy et al. 1997; Wolska-Kuśnierz et al. 2007). Clearance of cryptosporidiosis is impaired in patients with genetic defects of cell-mediated immunity in whom colonisation of the

biliary tract can occur predisposing to the development of sclerosing cholangitis, cirrhosis and cholangiocarcinoma (McLauchlin et al. 2003; Winkelstein et al. 2003).

Detection of *Cryptosporidium* infection in such patients by microscopy, using Ziehl–Neelsen staining of faecal smears and direct or indirect immunofluorescent assays (IFA), is often insufficient. Polymerase chain reaction (PCR)-based methods are more sensitive than microscopy in detecting parasites in patients with immunodeficiencies especially during asymptomatic periods (McLauchlin et al. 2003). Additionally, molecular studies are useful to understand the epidemiology of human cryptosporidiosis as they enable an objective and reliable identification of *Cryptosporidium* species/genotypes (Cacciò et al. 2005).

In Poland, *Cryptosporidium* detection is not routinely performed in clinical practise and only single cases of human cryptosporidiosis have been reported up to date (Siński et al. 1988; Golab et al. 2007; Wolska-Kuśnierz et al. 2007). Unrecognised *Cryptosporidium* infections in immunocompromised patients can be severe and even lead to death (i.e. after the exaggeration of infection following the haematopoietic stem cell transplantation (HSCT) in PID patients) as well as increase the risk of hospital outbreaks (O'Donoghoue 1995; McLauchlin et al. 2003). In this paper we report on the occurrence of *Cryptosporidium* and on the molecular analysis of isolates in three groups of patients: (1) immunocompetent children and adults with symptoms of gastroenteritis, (2) children with different PID and (3) patients with secondary immunodeficiencies, i.e. adults and children with chronic diarrhoeas undergoing immunosuppressive treatment.

Materials and methods

Clinical cases

During a 5-year period (2002–2007), 103 samples were collected from 35 patients in three hospitals: the Children's Memorial Health Institute in Warsaw, the Children's Hospital in Otwock and the Institute of Oncology in Warsaw. Patients were divided into three groups on the basis of their immunological status. The first group consisted of 13 immunocompetent children and one adult who suffered from recurrent abdominal pains and/or recurrent or chronic diarrhoea. The second group consisted of 17 children with different PID (six with CD40L deficiencies, six with severe combined immunodeficiencies (SCID), two with X-linked lymphoproliferative syndrome, one with primary lymphopenia CD40 and two with immunodeficiencies of unknown background). Patients in this group were either asymptomatic or showed signs of sclerosing cholangitis and hepatitis and diagnosis for *Cryptosporidium* was performed due to regular screening recommendation for PID. The third group consisted of patients with secondary immunodeficiencies and chronic diarrhoea and included two adults undergoing cancer treatment and two children after liver and/or kidney transplantation.

Faecal samples and, in one case of PID with sclerosing cholangitis and chronic hepatitis, bile and liver tissue samples were stored at +4°C for no longer than 5 days prior to DNA extraction.

Detection of *Cryptosporidium* oocysts by microscopy

Faecal or bile specimens from patients were collected once, twice or more times and examined for *Cryptosporidium* oocysts by a modified Ziehl–Neelsen acid-fast technique (Henriksen and Pohlenz 1981) and by IFA using a commercial kit (Merifluor *Cryptosporidium*/*Giardia*, Meridian Diagnostics, Cincinnati, OH, USA) and following the manufacturer's instructions. Oocysts were concentrated prior to IFA using modified Sheather's sucrose flotation (Bajer et al. 2002).

Molecular typing of *Cryptosporidium* spp.

Oocysts disruption and DNA purification were carried out using two methods: (1) extraction from whole faeces using the FastPrep 120 instrument and the FastDNA Spin Kit (Bio 101 Inc., Carlsbad, CA, USA) as described by da Silva et al. (1999) and (2) extraction from concentrated or unconcentrated samples using Genomic Mini AX Stool kit (A & A Biotechnology, Gdynia, Poland). Concentration was performed by modified Sheather's sucrose flotation or by overnight decantation in phosphate-buffered saline (pH 7.2) at +4°C. Purified DNAs were stored at −20°C until analysis. The same methods were applied to the bile and liver biopsy specimens.

Amplification of an N-terminal fragment of the *Cryptosporidium* oocyst wall protein (COWP) gene was performed using the previously described nested PCR protocol (Spano et al. 1997; Pedraza-Diaz et al. 2001a). In the primary PCR, primers BCOWPF (5′-ACCGCTTCTCAACAACCATC TTGTCCTC-3′) and BCOWPR (5′-CGCACCTGTTCCC ACTCAATGTAAACCC-3′) were used to produce a ~769-bp fragment. In the nested PCR reaction, primers Cry15 (5′-GTAGATAATGGAAGAGATTGTG-3′) and Cry9 (5′-GG ACTGAAATACAGGCATTATCTTG-3′) were used to produce a ~550-bp fragment.

Amplification of a fragment of the beta-tubulin gene was performed using a nested PCR protocol. In the primary reaction, the full-length gene was amplified as described by Cacciò et al. (1999) whereas in the nested reaction the forward primer (5′-ACCAGATTGGTGCTAAATTC-3′) and

the reverse primer (5′-CTGCAAAATACGATCTGGGA-3′) were used to amplify a ~550-bp fragment.

Cryptosporidium species were first identified by a PCR–restriction fragment length polymorphism (RFLP) method (Spano et al. 1997; Xiao et al. 2000). The nested PCR COWP amplicons were digested with the endonuclease *Rsa*I and the resulting fragments were separated by electrophoresis in 2% agarose gel.

COWP and beta-tubulin PCR products were purified and sequenced on both strands and sequences were edited and assembled using the software SeqMan 7.0. BLAST comparisons were run against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Results

Prevalence of *Cryptosporidium* infection in three groups of patients

In the first group, six out of 14 (42.9%) immunocompetent patients were positive for *Cryptosporidium* on the basis of nested PCR, whereas Ziehl–Neelsen staining revealed no positive samples in this group.

In the second group, five of 17 children with PID were determined as *Cryptosporidium* positive (29.4%) on the basis of Ziehl–Neelsen staining of faecal smears and nested PCR. Nested PCR detected twice as many positive samples as Ziehl–Neelsen technique in specimens from infected individuals.

In the third group of four patients with secondary immunodeficiencies, only one *Cryptosporidium* infection was confirmed, solely on the basis of nested PCR, in an individual undergoing immunosuppressive chemotherapy.

Taken together, 12 *Cryptosporidium* infections were detected in 35 patients (34.3%). *Cryptosporidium* DNA was detected in the single bile sample but not in the liver tissue biopsy sample collected from a patient with PID who suffered from sclerosing cholangitis (Fig. 1).

Distribution of *Cryptosporidium* species in the three groups of patients

All six *Cryptosporidium* isolates derived from immunocompetent patients (group 1) were *Cryptosporidium parvum* according to PCR–RFLP analysis and to sequence analysis of COWP products (Fig. 1). The homology to the *Cryptosporidium parvum* homologous sequence (GenBank accession number AF266273) ranged between 99.78% and 100%.

The same result was obtained for the isolate derived from the patient of the Institute of Oncology (group 3) where PCR–RFLP analysis and sequencing of COWP product showed the presence of *C. parvum* (100% homology to GenBank AF266273).

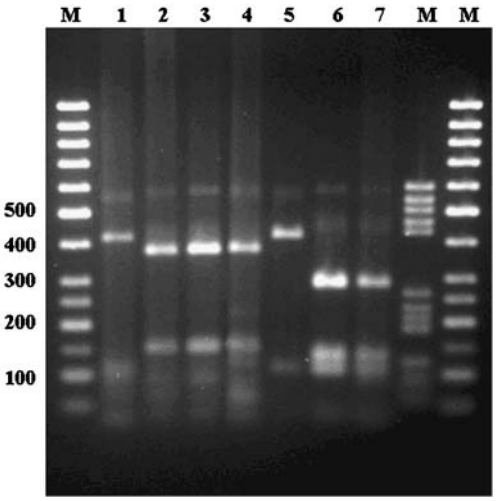

Fig. 1 Electrophoretic separation of COWP amplification product after digestion with the endonuclease *Rsa*I. Lanes *M* molecular weight markers (50-bp ladder and marker V from Boerhinger), lane *1 C. parvum* in immunocompetent child B.D. (group 1), lane *5 C. parvum* in patient J.W., Institute of Oncology (group 3) (fragments of 413, 106 and 34 bp), lanes *2–4 C. meleagridis* in patient E.N. with PID (fragments of 372, 147 and 34 bp), lanes *6* and *7 C. hominis* in stool (*6*) and bile sample (*7*) from patient H.Ch. with PID (fragments of 284, 129, 106 and 34 bp)

The highest diversity of *Cryptosporidium* species was observed in the second group, i.e. in children with PID. Among five positive cases, three different parasite species were identified by PCR–RFLP analysis and sequencing of COWP amplicons (Fig. 1). The prolonged infection in patient E.N., who had CD40L deficiency, was caused by *Cryptosporidium meleagridis* (100% homology with GenBank sequence AF266266). This species assignment was further confirmed by sequencing of a beta-tubulin gene fragment that revealed full homology with *C. meleagridis* isolates of human origin (GenBank accession numbers AF323574 and AF323575).

Cryptosporidium hominis was identified in stool and bile samples in patient H.Ch. who had primary lymphopenia CD40. Sequence analysis of the COWP fragment revealed 100% homology with GenBank sequence AF248741 (Fig. 1). *Cryptosporidium* DNA was not detected in liver specimen taken from this patient. In two other PID children, both with CD40L deficiency, PCR–RFLP and sequencing of the COWP fragment revealed the presence of *C. parvum* (with 99.79–100% homology with GenBank accession number AF266273). Of note, one of the patients was the brother of patient E.N. who was infected with *C. meleagridis*. Species identification was further confirmed by sequencing of beta-tubulin gene fragment that revealed 100% homology with *C. parvum* (GenBank accession number Y12615).

For the last patient in this group (patient M.W., with SCID and symptoms of cholestasis), it was impossible to determine the *Cryptosporidium* species involved.

Duration of *Cryptosporidium* infection and resolution of symptoms

In immunocompetent patients (group 1) and in those with secondary immunodeficiency (group 3), the parasite was detected only once during the sampling period. For the six children in group 1, the treatment was successful and all of them recovered. The course of *C. parvum* infection in the adult patient undergoing chemotherapy was not monitored.

In the group of children with PID (group 2), all five infections were chronic, lasting from 3 to 29 months despite applied treatment (Table 1). Two patients within this group died, one due to complication after HSCT (*C. parvum* infection) and the second (H.Ch.) due to B-cell lymphoma infiltration of the gall bladder.

C. meleagridis infection in patient E.N. was monitored both by microscopy and PCR and, despite different treatment methods, lasted for up to 29 months. After a third and successful HSCT, the patient recovered as confirmed by negative results of microscopy and nested PCR on the COWP gene. The other *C. parvum*-positive child also recovered after successful HSCT (Table 1).

Discussion

The aim of the present study was to investigate *Cryptosporidium* infection in three groups of patients at high risk including immunocompetent individuals with symptoms of cryptosporidiosis, children with PID and individuals with secondary immunodeficiencies and symptoms of gastroenteritis. Using two sensitive PCR-based methods, an overall prevalence of 34.3% of *Cryptosporidium* infection was observed in these patients. Of three groups of patients, the highest prevalence was found in children with symptoms of cryptosporidiosis and in those with PID and cholangitis (43% and 29%, respectively). One case of *Cryptosporidium* infection was also identified in a patient from the Institute of Oncology (group 3). Similar high rates of *Cryptosporidium* infection in immunocompetent children with chronic diarrhoea were confirmed recently in Poland (Golab et al. 2007) and in other countries worldwide (Ajjampur et al. 2007; Llorente et al. 2007; ten Hove et al. 2007; Sanad and Al-Malki 2007). High prevalence of *Cryptosporidium* infection in individuals with primary and secondary immunodeficiencies, exceeding even 82% in patients with AIDS and chronic diarrhoea, is commonly found in clinical practise (Winkelstein et al. 2003; Sanad and Al-Malki 2007).

Amplification and sequencing of COWP and beta-tubulin gene fragment revealed the presence of three *Cryptosporidium* species in the patients in this study. The most common was *C. parvum* which was detected in ten samples. All six positive children in group 1 were infected with this species. On the contrary, in other studies in immunocompetent children, the most common species (>80%) was *C. hominis* (Gatel et al. 2006; Ajjampur et al. 2007). However, *C. parvum* infection was detected only once during the sampling period and the children showed full recovery. A larger diversity of *Cryptosporidium* was observed in group 2, i.e. in children with different PID. In this group, *C. parvum* was detected in two cases and *C. hominis* and *C. meleagridis* were identified in the remaining two cases (Wolska-Kuśnierz et al. 2007). In other studies, up to eight different *Cryptosporidium* species/genotypes were identified in immunocompetent and immunodeficient children, including the mouse and cervine genotypes of *C. parvum* as well as *C. meleagridis*, *Cryptosporidium felis*, *Cryptosporidium canis* and *Cryptosporidium muris* (Pedraza-Diaz et al. 2001b; Xiao et al. 2001; Soba et al. 2006; Ajjampur et al. 2007; Gatel et al. 2007; Llorente et al. 2007). In contrast with the first group of patients, those within group 2 suffered from disseminated infection with symptoms of inflammation of the bile duct and gall bladder. Two patients in this group died, one due to complication after HSCT and the other one due to cholangiocarcinoma which was very likely caused by prolonged inflammation of bile ducts caused by cryptosporidiosis (Levy et al. 1997; Wolska-Kuśnierz et al. 2007). Successful amplification of *Cryptosporidium* DNA from a bile sample confirmed the extra-intestinal dissemination of *C. hominis* infection in this fatal case. Sclerosing cholangitis associated with *Cryptosporidium* infection is a well-known clinical manifestation found in PID patients, i.e. among patients with the X-linked hyper-IgM syndrome (Levy et al. 1997; Winkelstein et al. 2003). Severe and

Table 1 Duration of *Cryptosporidium* infection in children with PID

Cryptosporidium species/(life history of patient)	Period of study	Duration of infection (months)
C. parvum 1[a]	March 2004–February 2005	11
C. parvum 2 (HSCT)	May 2005–August 2006	15
C. hominis (H.Ch.)[a]	October 2004–January 2005	3[b]
C. meleagridis (E.N.) (HSCT)	March 2004–August 2006	29
Cryptosporidium spp. (HSCT)	May 2006–September 2006	4

HSCT Successful haematopoietic stem cell transplantation
[a] Died
[b] Late diagnosis for *Cryptosporidium* features of sclerosing cholangitis lasting 2 years

disseminated course of *Cryptosporidium* infections in PID was also previously described in UK (McLauchlin et al. 2003). HSCT is a method of choice in treatment of cryptosporidiosis in patients with PID, giving them opportunity for recovery even from an advanced disseminated infection (Dimicoli et al. 2003).

An adult patient, who was receiving immunosuppressive treatment at the Institute of Oncology, was infected with *C. parvum* and suffered from prolonged and severe diarrhoea.

In summary, the course of cryptosporidiosis was more severe among patients with PID resulting in disseminated infection and sclerosing cholangitis. Patients with secondary immunodeficiency suffered from acute cryptosporidiosis and among immunocompetent children mild infection was observed.

Chronic *Cryptosporidium* infections were monitored in children with PID and lasted from 3 to 29 months (Table 1), confirming their inability to clear the infection despite a range of treatment methods (Wolska-Kuśnierz et al. 2007). *Cryptosporidium* infections were cleared at last after successful HSCTs in two patients. One case of chronic infection was caused by *C. meleagridis* and thus represents, to the best of our knowledge, the first report of a long-lasting *C. meleagridis* infection in humans.

In agreement with a previous study (McLauchlin et al. 2003), our data have shown that microscopy is less sensitive than nested PCR in the detection of the parasite in asymptomatic cases. No positive cases were detected by Ziehl–Neelsen staining and IFA in immunocompetent children and in a patient from the Institute of Oncology (groups 1 and 3), probably due to the low intensity of infection. In children with PID, five were *Cryptosporidium* positive according to PCR assay and only four of these were also positive by microscopy. However, only half of all samples positive by PCR were also positive by microscopy. Therefore, molecular diagnostic tools should be considered the method of choice in screening possible *Cryptosporidium* infections of PID. Stool samples from such patients should be checked regularly, at least three times per year, even in the absence of diarrhoea or other symptoms.

Acknowledgements We gratefully acknowledge the financial support by State Committee for Scientific Research (KBN), through the Faculty of Biology, Warsaw University intramural grant BW no. 1601/53 (AB) and KBN grant no. 2PO4C09827 (ES).

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