Molecular characterisation of *Cryptosporidium* isolates from humans in Slovenia

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ABSTRACT

Twenty-nine faecal specimens from Slovenian patients in which *Cryptosporidium* oocysts had been identified were studied. A fragment of the *Cryptosporidium* 18S rRNA gene and a fragment of the *Cryptosporidium* COWP gene were amplified by PCR and sequenced. *Cryptosporidium parvum* was identified in 26 of the 29 specimens, *Cryptosporidium hominis* in two, and *Cryptosporidium* cervine genotype in one. The fact that *C. parvum*, which is associated traditionally with animals, was identified in the majority of human faecal specimens suggests that cryptosporidiosis may have primarily a zoonotic origin in Slovenia.

Keywords *Cryptosporidium* spp., cryptosporidiosis, DNA sequence analysis, oocyst wall protein gene, PCR, 18S rRNA gene

Original Submission: 18 April 2005; **Revised Submission:** 15 November 2005; **Accepted:** 3 February 2006

Clin Microbiol Infect 2006; 12: 918–921
10.1111/j.1469-0691.2006.01465.x

The use of molecular methods in determining the taxonomy of *Cryptosporidium* spp. has led to increased recognition of the diversity of species infecting humans [1]. Human cryptosporidiosis is caused mainly by *Cryptosporidium hominis*, which is found almost exclusively in humans, and *Cryptosporidium parvum*, which is found in most livestock, some wild animals and humans [2,3]. The occurrence of both of these species in humans indicates that anthroponotic and zoonotic transmission cycles can occur in human infections [4]. In addition to *C. hominis* and *C. parvum*, humans are also known to be infected by *Cryptosporidium meleagridis*, *Cryptosporidium muris*, *Cryptosporidium felis*, *Cryptosporidium canis*, *Cryptosporidium suis* and the cervine genotype, which are associated traditionally with animals [3]. The prevalence and significance of the different species and genotypes in humans are not yet clear. Moreover, potential reservoir hosts and transmission pathways for novel species infecting humans have not yet been elucidated. Genotyping of isolates from different parts of the world is therefore essential for a more precise understanding of the epidemiology of *Cryptosporidium* spp. [1,5].

In Slovenia, only five *Cryptosporidium* isolates from human patients have been typed to date, all five of which were *C. parvum* [6,7]. In the present study, isolates from 29 faecal specimens obtained from sporadic cases of cryptosporidiosis, collected at the Institute of Microbiology and Immunology, Ljubljana, Slovenia, between 2000 and 2003 were genotyped. The specimens were obtained from 29 immunocompetent patients who attended health centres and hospitals in various parts of Slovenia because of clinical symptoms consistent with cryptosporidiosis. Five of these patients were hospitalised because of cryptosporidiosis (Table 1); none of the infections was hospital-acquired.

Cryptosporidium oocysts were identified microscopically in faecal smears after staining with modified Ziehl–Neelsen stain, and by use of a direct immunofluorescence test (MeriFluor; Meridian Bioscience, Cincinnati, OH, USA). DNA was extracted from faecal specimens with the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany). A *c.* 830-bp fragment of the *Cryptosporidium* 18S rRNA gene that spanned the hyper-variable region and a 553-bp fragment of the *Cryptosporidium* COWP gene were amplified by nested PCR and PCR, respectively, as described previously [8,9]. PCR products were sequenced in both directions on an ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Overlapping bidirectional sequences were assembled using SeqMan sequence analysis software (DNASTAR Inc., Madison, WI, USA) and were subjected to a BLAST search to determine their identities and to assess their similarities to sequences in GenBank. The sequences were aligned using the ClustalV program. A

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Table 1. Isolate genotypes and clinical and epidemiological data for patients with *Cryptosporidium* infection in Slovenia

Isolate code	Year of collection	Patient age (years)	Gender	Type of region	Symptoms	Hospitalisation	Species, genotype (18S)	Species, genotype (COWP)
SI 1	2002	32	F	Urban	Diarrhoea	No	*C. parvum* (A)	*C. parvum*
SI 2	2002	12	F	Rural	NA	Yes	*C. parvum* (A)	*C. parvum*
SI 3	2003	8	F	Rural	NA	No	*C. parvum* (A)	*C. parvum*
SI 4	2002	31	M	Rural	NA	No	*C. parvum* (A)	*C. parvum*
SI 5	2003	3	M	Rural	Diarrhoea	No	*C. parvum* (A)	*C. parvum*
SI 6	2002	23	F	Rural	Enterocolitis	No	*C. parvum* (A)	–[a]
SI 7	2002	23	F	Rural	Diarrhoea	No	*C. parvum* (A)	*C. parvum*
SI 8	2002	1	M	Rural	NA	Yes	*C. parvum* (A)	*C. parvum*
SI 9	2002	23	M	Urban	NA	No	*C. parvum* (A)	*C. parvum*
SI 10	2002	18	F	Urban	NA	Yes	*C. parvum* (A)	*C. parvum*
SI 11	2002	28	F	Urban	Gastroenterocolitis	No	*C. parvum* (A)	*C. parvum*
SI 12	2002	6	F	Urban	NA	Yes	*C. parvum* (A)	*C. parvum*
SI 13	2000	18	F	Rural	Enterocolitis	Yes	*C. parvum* (A)	*C. parvum*
SI 14	2000	NA, child	M	Rural	NA	No	*C. hominis*	*C. hominis*
SI 15	2000	9	F	Urban	Enterocolitis	No	*C. parvum* (A)	*C. parvum*
SI 16	2001	8	F	Rural	NA	No	*C. parvum* (A)	*C. parvum*
SI 17	2001	8	F	Rural	NA	No	*C. parvum* (A)	*C. parvum*
SI 18	2001	11	F	Urban	Gastroenterocolitis	No	*C. parvum* (B)	–[a]
SI 19	2001	29	F	Urban	NA	No	*C. hominis*	*C. hominis*
SI 20	2001	1	M	Rural	NA	No	*C. parvum* (A)	*C. parvum*
SI 21	2002	11	M	Urban	Abdominal pain	No	*C. parvum* (A)	*C. parvum*
SI 22	2002	1	M	Rural	NA	No	*C. parvum* (A)	*C. parvum*
SI 23	2002	1	F	Rural	Diarrhoea	No	Cervine	Cervine
SI 24	2002	3	M	Rural	Enterocolitis	No	*C. parvum* (A)	*C. parvum*
SI 25	2002	6	M	Rural	NA	No	*C. parvum* (A)	*C. parvum*
SI 26	2002	6	M	Rural	Enterocolitis	No	*C. parvum* (A)	*C. parvum*
SI 27	2002	4	M	Rural	Gastroenterocolitis	No	*C. parvum* (A)	*C. parvum*
SI 28	2003	2	M	Rural	Enterocolitis	No	*C. parvum* (A)	*C. parvum*
SI 29	2003	52	F	Urban	NA	No	*C. parvum* (A)	*C. parvum*

M, male; F, female; NA, information not available; (A), type A subunit sequence; (B), type B subunit sequence.
[a]Amplification was unsuccessful.

neighbour-joining tree was constructed from the 18S rRNA gene fragment information by using the TreeconW program, and evolutionary distances were calculated by Kimura two-parameter analysis. The 18S rRNA and COWP gene sequences of five representative patient isolates (Fig. 1) have been deposited in the European Molecular Biology Laboratory (EMBL) database, under accession numbers AJ849457–AJ849465.

All 29 specimens gave the expected *c.* 830-bp amplicon for the 18S rRNA gene. The comparison of the 18S rRNA gene sequences with published reference sequences by multiple sequence alignment and phylogenetic analysis (Fig. 1) showed that the 29 isolates fell into three main groups. The first group comprised 26 *C. parvum* isolates, SI 1–13, 15–18, 20–22 and 24–29 (Fig. 1). Sequence analysis showed that these isolates were of two different subunit types; 25 isolates (SI 1–13, 15–17, 20–22 and 24–29), which were identical to one another, had *C. parvum* type A subunit sequences, while the remaining isolate (SI 18) had a *C. parvum* type B subunit sequence. The second group comprised *C. hominis* isolates SI 14 and SI 19 (Fig. 1). The third group was represented by a single isolate (SI 23) whose sequence was identical to the published sequence of the *Cryptosporidium* cervine genotype identified in lemurs (AF442484) [10] (Fig. 1), which has only been reported once previously in humans [11]; however, this organism could emerge as an important human pathogen following increasing contact between humans and wildlife [12].

PCR amplification of the COWP gene fragment was successful for 27 of the 29 isolates, yielding amplicons of 553 bp. Following sequencing, 24 amplicons (SI 1–5, 7–13, 15–17, 20–22 and 24–29) proved to be *C. parvum* sequences, two (SI 14, SI 19) were *C. hominis* sequences, and one (SI 23) was a *Cryptosporidium* cervine genotype sequence. This sequence was identical to that of the isolate from lemurs described by da Silva *et al*. [10].

In developed countries, most cases of cryptosporidiosis occur in children aged 1–4 years, perhaps because of increased exposure as they explore their environment [13]. However, in the present study, there was a slightly greater proportion of cases in the group aged 5–14 years than in the group aged 1–4 years. Moreover, there were more cases involving children aged ≤14 years than there were adult cases. However, in Slovenia, while cryptosporidiosis is mainly a disease of pre-school and school-aged children, it is also a disease of adults.

Genetic characterisation of the *Cryptosporidium* isolates revealed that in Slovenia, as in other