Genetic classification of *Cryptosporidium* isolates from humans and calves in Slovenia

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SUMMARY

To assess the importance of cattle as a source of human cryptosporidial infections in Slovenia, *Cryptosporidium* isolates from calves and humans with cryptosporidiosis were characterized genetically by direct DNA sequencing, targeting a variable region of the 60 kDa glycoprotein (*gp60*) gene. In total, 15 genetic variants, designated 'subtypes', were identified, of which 7 were novel. In humans, *C. hominis* Ia (subtype IaA17R3) and Ib (IbA10G2) and *Cryptosporidium parvum* IIa (IIaA9G1R1, IIaA11G2R1, IIaA13R1, IIaA14G1R1, IIaA15G1R1, IIaA15G2R1, IIaA16G1R1, IIaA17G1R1 and IIaA19G1R1), IIc (IIcA5G3), and IIl (IIlA16R2) were recorded; this is the first record of the latter subtype in humans. In cattle, *C. parvum* IIa (IIaA13R1, IIaA15G2R1, IIaA16R1 and IIaA16G1R1) and IIl (IIlA16R2 and IIlA18R2) were recorded. Of the 15 subtypes identified, subtypes of *C. parvum* IIa were the most frequently encountered (>90%) in both humans and calves. The present findings suggest that zoonotic transmission plays an important role in sporadic human cryptosporidiosis in Slovenia.

Key words: *Cryptosporidium*, subtypes, humans, calves, 60 kDa glycoprotein gene sequences, Slovenia.

INTRODUCTION

Cryptosporidiosis is a frequent diarrhoeal disease of humans and other animals, including cattle. In immunocompetent hosts, the infection is self-limiting, whereas in immunocompromised people and neonatal animals, it has the potential to cause deaths. Since the treatment of cryptosporidiosis is usually limited to supportive therapy, a thorough understanding of the epidemiology and transmission dynamics of *Cryptosporidium* is required in order to allow the control of disease outbreaks (Xiao and Ryan, 2004; Xiao *et al*. 2004).

Thus far, 9 *Cryptosporidium* species/genotypes, including *C. hominis*, *C. parvum*, *C. meleagridis, C. felis, C. canis*, *C. muris, C. suis* and the *Cryptosporidium* cervine and monkey genotypes, are known to be responsible for human cases of cryptosporidiosis, with the former two being the most common species (Pieniazek *et al*. 1999; Katsumata *et al*. 2000; Morgan *et al*. 2000; Pedraza-Diaz *et al*. 2000; Ong *et al*. 2002; Xiao *et al*. 2002, 2004; Mallon *et al*. 2003). *Cryptosporidium hominis* is transmitted anthroponotically, whereas *C. parvum* can be transmitted zoonotically, particularly from cattle, and anthroponotically to humans (Xiao and Ryan, 2004). Cattle can become infected with at least 4 *Cryptosporidium* species/genotypes, including *C. parvum*, *C. bovis*, *C. andersoni* and the *Cryptosporidium* deer-like genotype (Santín *et al*. 2004; Xiao *et al*. 2004; Fayer *et al*. 2006). However, *C. parvum* is the only known zoonotic species in cattle (Xiao and Ryan, 2004).

A number of molecular methods have been developed for the genetic classification of *C. hominis* and *C. parvum* to the subgenotypic level (='subtyping'). These methods provide useful tools for the 'tracking' of infection sources and, consequently, for the surveillance of cryptosporidiosis. One of the most commonly used tools is the sequencing of a 60 kDa glycoprotein (*gp60*) gene, which allows the definition of *C. hominis* Ia, Ib, Id-If and *C. parvum* IIa-IIk (Strong *et al*. 2000; Peng *et al*. 2001, 2003*a*, *b*; Sulaiman *et al*. 2001, 2005; Glaberman *et al*. 2002; Leav *et al*. 2002; Alves *et al*. 2003, 2006; Sturbaum *et al*. 2003; Wu *et al*. 2003; Zhou *et al*. 2003; Chalmers *et al*. 2005; Abe *et al*. 2006; Akiyoshi *et al*. 2006; Trotz-Williams *et al*. 2006; Misic and Abe, 2007; Thompson *et al*. 2007). Except for IIj and IIk, all of the above mentioned 'subtype families' have been detected in humans, whereas exclusively IIa, IId and IIj have been found in cattle.

Currently, information regarding the importance of cattle as a source of human cryptosporidial infections in Slovenia is lacking, since only a small number of human and bovine isolates has been characterized (Stantic-Pavlinic *et al*. 2003). In the present study, we classified more than 70 *Cryptosporidium* isolates from human and bovine cases of cryptosporidiosis from Slovenia by direct DNA sequencing, targeting a variable region of *gp60*. The

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findings suggest that zoonotic transmission may be responsible for most of the human cases of cryptosporidiosis in this country.

MATERIALS AND METHODS

Faecal samples

Thirty-four faecal samples from sporadic human cases of cryptosporidiosis in rural and urban areas of Slovenia, collected at the Institute of Microbiology and Immunology between 2000 and 2006, were used in this study. Twenty-nine of these samples were described in a previous study (Soba *et al.* 2006). Of the remaining 5 faecal samples, 1 was from a human immunodeficiency virus (HIV)-infected patient and 4 were from patients not known to have been immunosuppressed. Four of these 5 patients were hospitalized because of cryptosporidiosis. Also, a total of 51 *Cryptosporidium* oocyst-positive faecal samples from diarrhoeic calves (<8 weeks of age) were included in the study. Bovine faecal samples were collected between 2002 and 2007 on 31 dairy farms throughout Slovenia. These farms were chosen because of problems with diarrhoea in calves. Faecal samples from all diarrhoeic cattle from these farms were examined, but only those positive for *Cryptosporidium* oocysts were included herein. Oocysts were identified microscopically in faecal smears using a direct immunofluorescence test (MeriFluor, Meridian Bioscience, USA). Oocyst-containing faecal samples were stored at +4 °C in 2·5% potassium dichromate until required for molecular analysis.

DNA extraction and genotyping

Faecal samples were washed 3 times in phosphate-buffered saline (PBS, pH 7·2) by centrifugation to remove traces of potassium dichromate prior to DNA extraction. Genomic DNA was extracted from faecal samples using a QIAamp DNA Stool Mini Kit (Qiagen, Germany), according to manufacturer's instructions. The DNA extracts were stored at −20 °C until analysis.

The identification of *Cryptosporidium* in the DNA samples to species was inferred using nested PCR amplification of a region of the nuclear small subunit (SSU; ~830 bp) ribosomal RNA gene, followed by restriction fragment length polymorphism (RFLP) analysis of the secondary PCR product. A primer pair (forward: 5′-TTCTAGAGCTAATACATGCG-3′ and reverse: 5′-CCCTAATCCTTCGAAACAGGA-3′) was used in primary PCR, and another pair (forward: 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and reverse: 5′-AAGGAGTAAGGAACAACCTCCA-3′) in secondary PCR (cf. Xiao *et al.* 1999). The restriction endonucleases *Ssp*I and *Vsp*I were employed, prior to the use of *Mbo*II for the differentiation among *C. parvum*, *C. bovis* and the *Cryptosporidium* deer-like genotype (Xiao *et al.* 2001; Feng *et al.* 2007). All secondary PCR products not representing *C. parvum* were sequenced to establish the species. Genomic DNA from the Iowa strain of *C. parvum* (ATCC PRA-67D) was used as a positive control and H_2O as a negative control in each batch of samples tested. The species of 29 human isolates (SI1-29) had been identified in a previous analysis (Soba *et al.* 2006).

Sequence analysis of the 60 kDa glycoprotein (gp60) gene for the classification of 'subtypes' (according to Sulaiman et al. 2005)

The *gp60* gene of *C. hominis* and *C. parvum* parasites was amplified by nested PCR (Alves *et al.* 2003). The secondary PCR products were sequenced from both strands using the forward and reverse primer pair used in the secondary PCR as well as an additional, internal sequencing primer AL3533 (5′-GAGATATATCTTGGTGCG-3′) in an automated sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA). The sequences were assembled using Vector NTI Advance® sequence analysis software (Invitrogen, USA) and subjected to a similarity/identity searches using BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) against sequences in GenBank. Sequences were aligned using the ClustalX program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) and adjusted manually. A neighbour-joining tree was constructed using the Mega program (http://www.megasoftware.net/), the evolutionary distances being calculated by the Kimura 2-parameter model. A *C. meleagridis* sequence (Accession number AF401499) was used as an outgroup. The reliability of groupings was assessed by bootstrapping analysis, using 1000 replicates. The sequences determined in this study were deposited in the European Molecular Biology Laboratory (EMBL) database under the Accession numbers AM937006–AM937018, AM947935 and AM988862–AM988865.

At present, *C. hominis* Ia, Ib, Id-If and *C. parvum* IIa-IIk are classified based on *gp60* sequence data (Strong *et al.* 2000; Peng *et al.* 2001, 2003*a,b*; Sulaiman *et al.* 2001, 2005; Glaberman *et al.* 2002; Leav *et al.* 2002; Alves *et al.* 2003, 2006; Sturbaum *et al.* 2003; Wu *et al.* 2003; Zhou *et al.* 2003; Chalmers *et al.* 2005; Abe *et al.* 2006; Akiyoshi *et al.* 2006; Trotz-Williams *et al.* 2006; Misic and Abe, 2007; Thompson *et al.* 2007). However, *C. parvum* IIj has been used erroneously by a number of workers (Misic and Abe, 2007; Wielinga *et al.* 2008), as this 'subtype family' had already been designated by Thompson *et al.* (2007) (see GenBank Accession number DQ648547). Thus, we propose the use of *C. parvum* IIl instead of IIj for the 'subtype family'

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identified by Misic and Abe (2007) and Wielinga *et al.* (2008).

RESULTS

Cryptosporidium *species*

SSU PCR products of the expected size (~830 bp) were amplified from genomic DNA samples ($n=85$) using the nested PCR. In total, 29 of 34 faecal samples collected from humans had been genotyped previously (Soba *et al.* 2006): *Cryptosporidium hominis* had been identified in 2 of them, *C. parvum* in 26, and *Cryptosporidium* cervine genotype in 1. RFLP analysis of the secondary amplicons from the remaining samples using the endonucleases *Ssp*I, *Vsp*I and separately *Mbo*II showed that all 5 humans and 45 of 51 calves were infected with *C. parvum*, 3 calves with *C. bovis* and 3 calves with *Cryptosporidium* deer-like genotype. *Cryptosporidium bovis* and *Cryptosporidium* deer-like genotype in 6 samples from cattle were established by DNA sequencing of the secondary SSU PCR product. The SSU sequences determined for *C. bovis* and *Cryptosporidium* deer-like genotype were identical to those reported previously for these taxa (see GenBank Accession numbers AY741305 and AY587166; Santín *et al.* 2004; Fayer *et al.* 2006).

Cryptosporidium hominis *and* Cryptosporidium parvum *subtypes*

The secondary *gp60* amplicons from all 78 genomic DNA samples shown to contain *C. hominis* or *C. parvum* were sequenced. The sequence data for *C. hominis* and *C. parvum* were subjected to phylogenetic analysis separately because of the sequence variation in *gp60* between these two species. For *C. hominis*, 2 subtypes defined could be linked to clusters Ia and Ib (Fig. 1A); for *C. parvum*, the 13 subtypes were linked to 3 clusters (IIa, IIc and IIl) (Fig. 1B). Eleven of the 13 *C. parvum* subtypes were from humans, whereas 6 subtypes were from calves. Four subtypes were from both humans and calves. Details on the numbers of samples representing each subtype are presented in Table 1.

The phylogenetic analysis of the *gp60* sequence data representing 33 human isolates (using published data for comparison) allowed *C. hominis* samples to be classified as Ia (1/2) and Ib (1/2), and those from *C. parvum* as IIa (29/31), IIc (1/31) and IIl (1/31) (Fig. 1). Nine different subtypes were identified within IIa (Fig. 1B), which differed from one another mostly in the numbers of TCA and TCG repeats (coding for the amino acid serine) and in a single nucleotide polymorphism in a region following the trinucleotide repeats (a change from G to A, resulting in an amino acid substitution – from glycine to aspartic acid). The most common subtype was IIaA15G2R1, detected in 15 of 31 *C. parvum*-infected humans. Of the remaining 8 *C. parvum* IIa subtypes, IIaA15G1R1 was detected in 4 humans, whereas IIaA9G1R1, IIaA11G2R1, IIaA13R1, IIaA14G1R1, IIaA16G1R1, IIaA17G1R1 and IIaA19G1R1 were represented in 1 or 2 humans (Table 1). The sequence of isolate SI32, which had 5 TCA repeats and 3 TCG repeats, had identities of 93–95% with sequences of *C. parvum* IIc (Accession numbers AY166809, AF440621, AF440622, AF440627 and AF164501; Strong *et al.* 2000; Leav *et al.* 2002; Alves *et al.* 2003). However, phylogenetic analysis placed it in the cluster of *C. parvum* IIc sequences (Fig. 1B).

Phylogenetic analysis classified *C. parvum* samples ($n=45$) from cattle as IIa (41/45) or IIl (4/45) (Fig. 1B). Four and 2 subtypes were identified within IIa and IIl, respectively (Fig. 1B). As was found in humans, the most common subtype in calves was IIaA15G2R1, detected in 27 of 45 *C. parvum*-infected calves. This subtype was also the most widely distributed, being present on 19 of the 31 farms studied herein. Subtype IIaA13R1 was detected on 3 farms, whereas subtypes IIaA16R1, IIaA16G1R1, IIlA16R2 and IIlA18R2 were each detected on 1 farm (data not shown). More than 1 diarrhoeic, *C. parvum*-infected calf was detected on 10 of the 31 farms. No sequence variation in *gp60* was detected among samples from cattle on individual farms (data not shown).

DISCUSSION

In Slovenia, there is a paucity of information on the genetic make-up of *Cryptosporidium* from humans and calves (Stantic-Pavlinic *et al.* 2003). In the present study, we studied 2 *C. hominis* and 76 *C. parvum* isolates from 33 humans and 45 calves at the *gp60* gene locus. A total of 15 *gp60* subtypes were identified, and most of the infections in both humans and calves were caused by *C. parvum* IIa which has been reported to be the commonest in cattle (Alves *et al.* 2003, 2006; Peng *et al.* 2003*b*; Brook *et al.* 2007; Geurden *et al.* 2007; Plutzer and Karanis, 2007; Thompson *et al.* 2007; Xiao *et al.* 2007). Therefore, it is not surprising that human infections with *C. parvum* IIa are detected in areas with intensive animal husbandry (e.g. Glaberman *et al.* 2002; Alves *et al.* 2003, 2006; Peng *et al.* 2003*b*; Chalmers *et al.* 2005; Feltus *et al.* 2006; Trotz-Williams *et al.* 2006), as is the case in Slovenia. Ten subtypes within *C. parvum* IIa were identified in the present study, with IIaA15G2R1 being the commonest (60% of *C. parvum* infections in calves and 48% of *C. parvum* infections in humans). Previously, this subtype was shown to be the most prevalent *C. parvum* subtype in calves and humans in the United Kingdom, United States, Portugal, Australia, Japan, Kuwait and Canada, and appears

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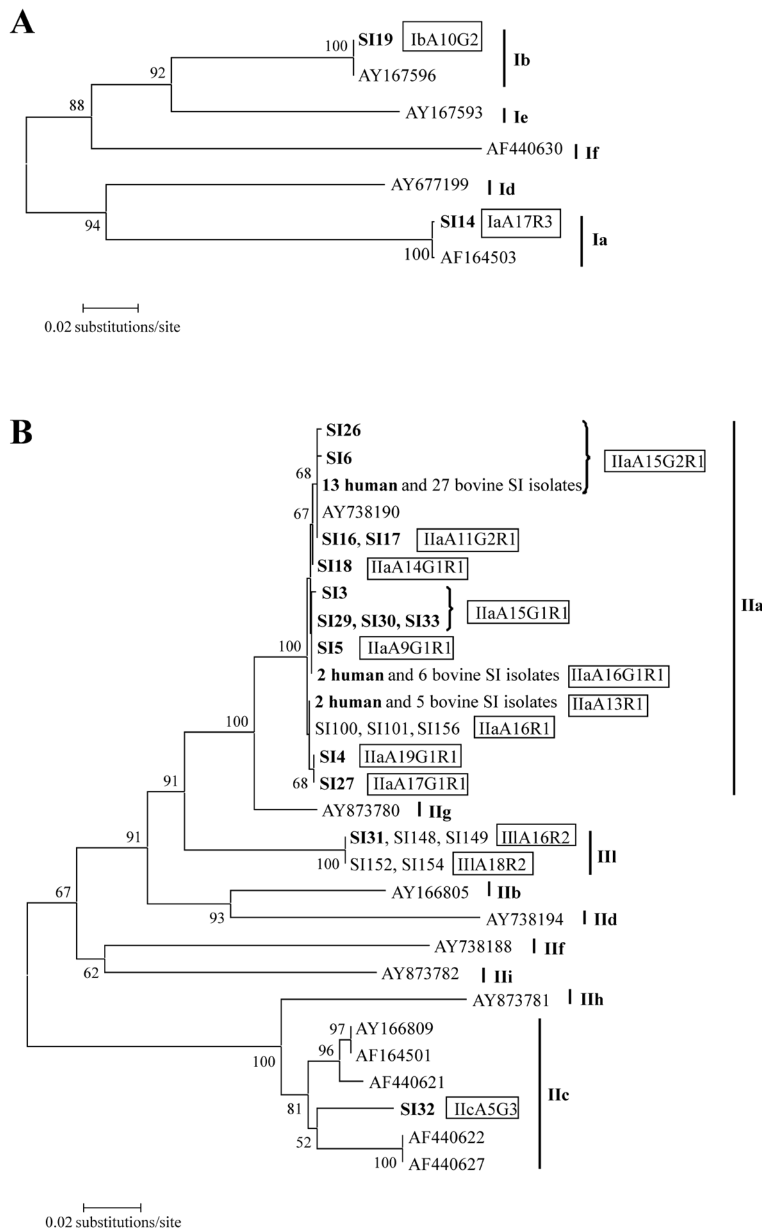

Fig. 1. Phylogenetic relationship of *Cryptosporidium hominis* (A) and *C. parvum* (B) isolates from humans and calves examined in the present study and sequences previously deposited in GeneBank as inferred from neighbour-joining analysis of the partial *gp60* gene. Human isolates examined in the present study appear in boldface. Codes in boxes following isolates examined in the present study are the names of subtypes. Values on branches are percentage bootstrap values using 1000 replicates. Bootstrap values greater than 50% are shown.

to be frequently linked to zoonotic cryptosporidiosis (Glaberman *et al.* 2002; Peng *et al.* 2003*b*; Wu *et al.* 2003; Chalmers *et al.* 2005; Sulaiman *et al.* 2005; Abe *et al.* 2006; Alves *et al.* 2006; Trotz-Williams *et al.* 2006; Xiao *et al.* 2007). In the present investigation, 2 other *C. parvum* IIa subtypes, IIaA16G1R1 and IIaA13R1, were identified in both human and bovine isolates and, therefore, may also be zoonotic. Subtype IIaA13R1 (identified herein in 2 human and 5 bovine isolates) was novel, whereas subtype IIaA16G1R1 (identified herein in 2 human and 6 bovine isolates) has been reported previously from calves in the United States, Canada, Serbia and Montenegro, Hungary and The Netherlands (Peng *et al.* 2003*b*; Trotz-Williams *et al.* 2006; Plutzer and Karanis, 2007; Misic and Abe, 2007; Wielinga *et al.* 2008). There are no previous reports of IIaA16G1R1 infecting humans. Subtypes IIaA11G2R1, IIaA17G1R1 and IIaA19G1R1, identified in 2, 1 and 1 *Cryptosporidium* isolates from humans, respectively, have been identified previously in calves in the United Kingdom, Hungary and also Slovenia (Stantic-Pavlinic *et al.* 2003; Brook *et al.* 2007; Plutzer and Karanis, 2007; Thompson *et al.* 2007), indicating that these 3 subtypes may also be exchanged between humans and farm animals. Herein, 2 additional new subtypes (IIaA9G1R1 and IIaA14G1R1) were each identified in 1 isolate

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Table 1. Distribution of *Cryptosporidium hominis* and *C. parvum* subtypes in humans and calves in Slovenia as inferred from *gp60* sequence data

Subtype	Host		Isolate code[1]
	Human	Bovine	
IaA17R3	1	0	**SI14**
IbA10G2	1	0	**SI19**
IIaA9G1R1	1	0	**SI5**
IIaA11G2R1	2	0	**SI16, SI17**
IIaA13R1	2	5	**SI22, SI24**, SI112, SI135, SI145-147
IIaA14G1R1	1	0	**SI18**
IIaA15G1R1	4	0	**SI29, SI30, SI33** **SI3**[2]
IIaA15G2R1	15	27	**SI2, SI7-13, SI15, SI20, SI21, SI25, SI28**, SI103-106, SI108-111, SI113-115, SI117-126, SI128, SI129, SI136, SI150, SI151, SI155 **SI6**[3] **SI26**[4]
IIaA16R1	0	3	SI100, SI101, SI156
IIaA16G1R1	2	6	**SI1, SI34**, SI137, SI138, SI140-143
IIaA17G1R1	1	0	**SI27**
IIaA19G1R1	1	0	**SI4**
IIcA5G3	1	0	**SI32**
IIlA16R2	1	2	**SI31**, SI148, SI149
IIlA18R2	0	2	SI152, SI154
Total	33	45	

[1] Human isolates appear in boldface.
[2] With a change of A to G downstream of the trinucleotide repeats.
[3] With a change of C to T downstream of the trinucleotide repeats.
[4] With a change of C to T shortly after the trinucleotide repeats.

from a human, and another (IIaA16R1) was identified in 3 isolates from infected calves. The zoonotic potential of these 3 subtypes, as well as that of IIaA15G1R1 (identified in 4 human isolates and, previously, in 2 isolates from children in Kuwait (Sulaiman *et al.* 2005)), remains to be determined.

Cryptosporidium parvum IIl was identified in 1 human (subtype IIlA16R2) and 4 bovine isolates (subtype IIlA16R2 and a new subtype IIlA18R2); it has been identified previously in cattle from Serbia and Montenegro and The Netherlands (Accession numbers AB242225, AB242227 and EF576957; Misic and Abe, 2007; Wielinga *et al.* 2008) but erroneously named as IIj. This designation had already been used by Thompson *et al.* (2007) for a genetic variant of *C. parvum* from a calf in Northern Ireland (see GenBank Accession number DQ648547). This study is the first published report of *C. parvum* IIl in humans. The finding of the subtype IIlA16R2 in human and bovine hosts suggests that at least 1 subtype of *C. parvum* IIl is zoonotic, representing a potential human health threat.

Cryptosporidium parvum IIc, which has not yet been found in animals, is commonly detected in infected humans in many countries (Leav *et al.* 2002; Alves *et al.* 2003, 2006; Xiao *et al.* 2003; Xiao and Ryan, 2004; Sulaiman *et al.* 2005), indicating that it is probably anthroponotic. *Cryptosporidium parvum* IIc was identified in 1 human isolate herein and was, as expected, not detected in infected calves, suggesting that, although rare, anthroponotic transmission of *C. parvum* can occur in Slovenia. *Cryptosporidium parvum* IIc determined in the present study was genetically distinct from all of the *C. parvum* IIc subtypes reported previously. This information is consistent with findings from previous studies, which have shown that sequence divergence among *gp60* subtypes within *C. parvum* IIc is greater than within other genotypes, although no variation in the number or type of trinucleotide repeats had been detected for IIc (they all have 5 TCA repeats and 3 TCG repeats) (Strong *et al.* 2000; Sulaiman *et al.* 2005).

In this study, *C. hominis* Ia (subtype IaA17R3) and Ib (subtype IbA10G2) were each identified in 1 human isolate. *Cryptosporidium hominis* Ib and subtype IbA10G2 have been recorded in Europe both in sporadic cases and outbreaks (Glaberman *et al.* 2002; Chalmers *et al.* 2005, 2008; Alves *et al.* 2006; Cohen *et al.* 2006; O'Brien *et al.* 2008). Moreover, infections with *C. hominis* subtypes

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other than IbA10G2 are usually linked to travel outside of Europe (e.g. Alves *et al.* 2006; Chalmers *et al.* 2008). As no information was available on foreign travel for the patient infected with the novel *C. hominis* subtype IaA17R3, it is not possible to infer whether this subtype is endemic in humans in Slovenia or whether it was acquired in a foreign country.

The lack of sequence variation in *gp60* among samples from diarrhoeic, *C. parvum*-positive calves from individual farms suggests that a single subtype is endemic on each farm. This finding is similar to a study on farms around Belgrade in Serbia and Montenegro (see Misic and Abe, 2007) but contrasts with the situation on several farms in Michigan, Ontario and other parts of the USA, where multiple subtypes have been detected on individual farms (Peng *et al.* 2003*b*; Trotz-Williams *et al.* 2006; Xiao *et al.* 2007). This difference probably reflects management practices on farms, including the purchase of calves and the mixing of groups of different origins and/or ages. While the exchange of calves between farmers and/or the introduction of new animals is common in the USA (Peng *et al.* 2003*b*), dairy farms in Slovenia usually represent 'closed systems', and calves are almost never purchased from other farmers or traders, explaining the presence of a single *C. parvum* subtype per farm. Similarly, in the UK, Brook *et al.* (2007) demonstrated multiple *C. parvum* subtypes on a commercial beef farm onto which calves were introduced and on which calves were also raised, while on 'closed' dairy farms only one subtype was present. Besides genetic diversity of *Cryptosporidium* within a single farm, management issues are also likely to contribute to genetic diversity among farms. Indeed, much greater genetic diversity of *C. parvum* was recorded among farms in Slovenia than in the United States, Canada and Portugal (Alves *et al.* 2003, 2006; Peng *et al.* 2003*b*; Trotz-Williams *et al.* 2006; Xiao *et al.* 2007). Substantial genetic diversity within *C. parvum* has also been detected among farms in Northern Ireland (Thompson *et al.* 2007), where management practices on farms are similar to those in Slovenia.

In conclusion, the results of this study provide useful insights into the epidemiology of *Cryptosporidium* infecting humans and cattle in Slovenia. The low percentage of human infections attributable to *C. hominis* and *C. parvum* IIc indicates that anthroponotic transmission may not contribute as significantly to sporadic human cryptosporidiosis in Slovenia, as has been inferred in other areas of the world (Peng *et al.* 2001, 2003*a*; Leav *et al.* 2002; Alves *et al.* 2003, 2006; Xiao and Ryan, 2004; Xiao *et al.* 2004; Wielinga *et al.* 2008; Chalmers *et al.* 2008; O'Brien *et al.* 2008). Most cases of human cryptosporidiosis examined in the present study were caused by 'zoonotic' *C. parvum* subtypes, suggesting that zoonotic transmission plays an important role in human cryptosporidiosis in this country. Although these conclusions need to be confirmed through further studies, they seem to reflect the agricultural context in Slovenia. Measures to reduce the risk for human infections from cattle will be important. However, the origin of some novel *C. parvum* subtypes found exclusively in humans remains to be determined. Further molecular epidemiological studies are required to clarify the zoonotic potential and host affiliation of such *C. parvum* subtypes.

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