

Research note

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Molecular characterisation of *Cryptosporidium* (Apicomplexa) in children and cattle in Romania

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Abstract: To investigate the transmission of species of *Cryptosporidium* Tyzzer, 1907 in Timis County, Romania, 48 isolates of *Cryptosporidium* coccidia from 11 children, 29 calves and eight pigs were characterised by molecular analysis of two loci (SSU rRNA and 60-kDa glycoprotein gene). Overall, 22 isolates were amplified and sequence analyses revealed that all isolates were *Cryptosporidium parvum* Tyzzer, 1912. Two subtype families were identified, IIA and IID. Subtype IIDA22G1 (n = 4) was the single *C. parvum* subtype found in children. Subtypes found in calves included IIDA27G1 (n = 8), a novel subtype, IIDA25G1 (n = 5), IIDA22G1 (n = 2), IIDA21G1a (n = 1), and IIAA16G1R1 (n = 1). Subtype IIDA26G1 was found in a pig. These results were significantly different from previous Romanian reports, as the five subtypes of family IID identified in this study were never identified previously in this country. Thus, cattle may be a source of *Cryptosporidium* infections for humans and the transmission dynamics of *C. parvum* in Romania is more complex than previously believed.

Keywords: *Cryptosporidium parvum*, man, calves, molecular epidemiology, GP60 variability, eastern Europe

Protists of the genus *Cryptosporidium* Tyzzer, 1907 are important etiological agents of gastrointestinal disease of a wide range of vertebrate animals, including humans (Xiao et al. 2004, Fayer 2010). *Cryptosporidium* infection may be acquired by direct contact with infected persons or animals, and indirectly by ingestion of contaminated water and food (Fayer 2010, Xiao 2010). *Cryptosporidium* includes over 20 species (Xiao et al. 2004, Fayer 2010), with *Cryptosporidium hominis* Morgan-Ryan, Fall, Ward, Hijjawi, Sulaiman et al., 2002, which has anthroponotic transmission and *Cryptosporidium parvum* Tyzzer, 1912 with zoonotic or anthroponotic transmission as the two most common species infecting humans (Xiao 2010). *Cryptosporidium parvum*, *Cryptosporidium bovis* Fayer, Santín et Xiao, 2005, *Cryptosporidium ryanae* Fayer, Santín et Trout, 2008 and *Cryptosporidium andersoni* Lindsay, Upton, Owens, Morgan, Mead et al., 2000 were described from cattle, with an age-related distribution (Santín et al. 2004, Fayer et al. 2007). *Cryptosporidium parvum* is the major

zoonotic species and is the dominant one in pre-weaned calves until two months of age (Santín et al. 2004). Natural infections in pigs by *Cryptosporidium* spp. have also been reported and are commonly attributable to two species (Xiao 2010), *Cryptosporidium suis* Ryan, Monis, Enemark, Sulaiman, Samarasinghe et al., 2004 and *Cryptosporidium scrofarum* Kváč, Kestránová, Pinková, Kvétoňová, Kalinová et al., 2013 (Fayer 2010, Kváč et al. 2013).

The present study was undertaken to genetically characterise *Cryptosporidium* spp. from humans and animals (Timis County, Romania), map the diversity of *Cryptosporidium* species in both populations and identify potential source(s) of human infection.

In this study we used 48 faecal samples from 48 individuals diagnosed as positive for *Cryptosporidium* spp. by a modified Ziehl-Neelsen staining. All faecal samples were collected in Timis County, Romania (2008), including 11 specimens from children under 12 years of age with diarrhea, 29 specimens from calves up to two months old and eight from nine-week-old pigs.

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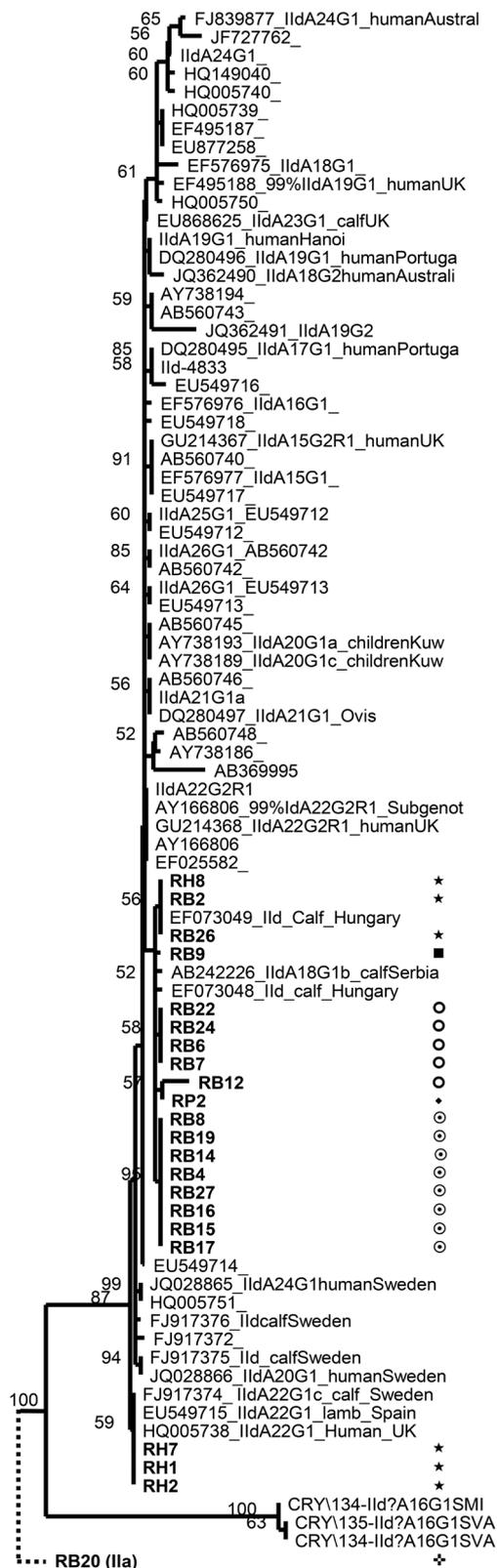


Fig. 1. Phylogenetic relationship between *Cryptosporidium parvum* subtypes characterised in this study and other sequences in GenBank, inferred by a neighbour-joining analysis of GP60 sequences, based on genetic distance calculated by Kimura two-parameter model. Numbers above branches indicate the confidence values obtained by bootstrap analysis with 1 000 replicates. **Abbreviations:** RB – bovine faecal sample; RH – human faecal sample; RP – pig faecal sample; ★ – IIdA22G1; ■ – IIdA21G1a; ○ – IIdA25G1; ◆ – IIdA26G1; ⊙ – IIdA27G1; ⊕ – IIdA16G1R1.

Stool samples from the animals were collected directly from the rectum. Samples were stored in 2.5% potassium dichromate at 4°C. The oocysts from samples were concentrated by a modified water-ether sedimentation method. The DNA was extracted by a MiniBeadBeater/silica method (Alves et al. 2001, 2003).

The identification of *Cryptosporidium* species and genotypes was performed by nested-PCR analysis of an 830 bp fragment of the SSU rRNA gene (Xiao and Ryan 2008). Subtyping analysis was done by nested-PCR analysis of a 400 bp fragment of the GP60 gene (Alves et al. 2003). The secondary PCR products were separated on 1.5% agarose gel and visualised by ethidium bromide staining under UV light.

All secondary SSU rRNA and GP60 products were purified with Jetquick Gel Extraction Spin Kit® (Genomed, Löhne, Germany) and sequenced in both directions on an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA). The sequences obtained in this study were aligned with reference sequences of *Cryptosporidium* spp. from the GenBank database using the MultAlin (<http://multalin.toulouse.inra.fr/multalin/>) software for species/genotypes and subtype identification. The *C. parvum* subtypes were named according to the number of TCA and TCG repeats in the trinucleotide repeat region and the mutations in the nonrepeating regions (Xiao 2010). To evaluate phylogenetic relationship among various *C. parvum* subtypes characterised in this and other studies, a neighbour-joining tree was constructed from the aligned sequences using the Treecom W program, based on genetic distance calculated by Kimura two-parameter model. The reliability of branches was assessed by bootstrap analysis with 1 000 replicates. The partial GP60 sequences generated in this study were deposited in the GenBank database (Acc. Nos. KF500407 and KC469687–KC469694).

Amplification of *Cryptosporidium* DNA was successfully achieved for 14 (two specimens from children and 12 from calves) of the 48 specimens diagnosed as positive for *Cryptosporidium* spp. by microscopy. The subsequent sequencing and alignment of all our nucleotide sequences with others of known identity deposited in GenBank database showed that all sequenced isolates belonged to *C. parvum*. Of the 14 isolates, six had 100% homology with the *C. parvum* nucleotide sequence DQ656355, six had 100% similarity with the *C. parvum* nucleotide sequence AB513881 and two had 100% homology with the *C. parvum* nucleotide sequence JN247404.

PCR products of the GP60 gene were obtained for 22 (four specimens from children, 17 from calves and one from a pig) of the 48 *C. parvum* isolates. The alignment of the sequences with reference sequences from GenBank confirmed that *C. parvum* was the only species found in the samples. The GP60 sequences of the 22 isolates confirmed six subtypes belonging to two zoonotic subtype families, IId and IId. Four of the IId subtypes, IIdA21G1a, IIdA22G1, IIdA25G1 and IIdA26G1, are known subtypes, with GenBank Acc. Nos. AB560746, EU549715, EU549712 and EU549713. A fifth subtype, IIdA27G1, was a novel subtype identified in this study. The IId subtype characterised in this study (IIdA16G1R1) had 100% homology to sequence AY149615 deposited in the GenBank database.

The remaining sequences (subtypes of subtype family IId) had only 99% homology to the sequences in GenBank with at least one polymorphism in the non-repeating region. The sequences from RB4, RB8, RB14, RB15, RB16, RB17, RB19 and RB27,

Table 1. Distribution of *Cryptosporidium parvum* GP60 subtypes in humans and animals in Timis County, Romania.

Subtypes	Host		
	Man (n = 4)	Calf (n = 17)	Pig (n = 1)
IlaA16G1R1	0	1	0
IIdA21G1a	0	1	0
IIdA22G1	4	2	0
IIdA25G1	0	5	0
IIdA26G1	0	0	1
IIdA27G1	0	8	0

all identified as the new subtype IIdA27G1, exhibited intra subtype sequence polymorphism. According to the nomenclature proposed by Sulaiman et al. (2005) and Xiao (2010), we assigned these new subtypes as IIdA21G1b (RB9), IIdA22G1b (RH1, RH2, RH7), IIdA22G1c (RH8, RB2, RB26), IIdA25G1b (RB6, RB7), IIdA25G1c (RB22), IIdA26G1b (RP2), IIdA27G1a (RB4, RB14, RB16, RB19) and IIdA27G1b (RB8, RB15, RB27). Moreover, the multiple alignment of GP60 sequences obtained shows that although all sequences of subtype family IId are identical to each other in the non-repeating region, sequences of seven isolates stand out from others due to the existence of at least one polymorphism in the non-repeating region. Phylogenetic analysis of the sequences confirmed that *C. parvum* isolates belonged to two subtype families (Ila and IId) and showed the existence of two genetically distinct types of sequences in the subtype IIdA22G1 (Fig. 1). One of them was described in three human isolates (RH1, RH2 and RH7), whereas the second type of sequence was detected in one human (RH8) and two bovine isolates (RB26 and RB2).

Subtype family IId was prevalent and widely distributed in the present study. Of the 22 isolates, 96% (21/22) belonged to this subtype family, being detected in humans (4 isolates), calves (16) and pigs (1). In contrast, subtype family Ila was less prevalent: of the 22 isolates, only one was detected in a calf. As shown in Table 1, IIdA22G1 was the only subtype detected in humans (4 isolates). In contrast, multiple subtypes were seen in calves: IIdA27G1 was the predominant (8 isolates from a total of 17 samples), followed by subtypes IIdA25G1 (5 isolates) and IIdA22G1 (2), whereas subtypes IlaA16G1R1 and IIdA21G1a were rare (only 1 isolate). The only specimen characterised in pigs had the subtype IIdA26G1.

Genetic characterisation of *Cryptosporidium* isolates from Timis County, Romania, revealed that *C. parvum* was the only species present in children. Our results are in agreement with several studies conducted in European countries (France, Portugal, Slovenia, Switzerland, UK), which have shown a common occurrence of *C. parvum* in humans (McLauchlin et al. 2000, Morgan et al. 2000; Alves et al. 2001, 2003, Guyot et al. 2001, Matos et al. 2004, Soba and Logar 2008). In contrast, *C. hominis* is more prevalent in other regions of the world, especially in developing countries, Australia and USA (Xiao 2010).

In calves, all isolates were characterised as *C. parvum*, as in previous studies conducted in the same region (Imre et al. 2011). Our results correlate with those on genotyping of *Cryptosporidium* spp. in calves of the same age, in which *C. parvum* was considered responsible for most infections (Xiao 2010). The high

prevalence of *C. parvum* detected in this study is consistent with the age of the calves, which may serve as major reservoirs of the most important zoonotic species, *C. parvum*.

In pigs, a single isolate was identified as *C. parvum*, whereas the species most often detected in pigs are *C. suis* and *C. scrofarum* (see Kváč et al. 2013). *Cryptosporidium parvum* has been only detected in a few studies (Kváč et al. 2009, Xiao 2010, Garcia-Preseido et al. 2013, Nĕmejc et al. 2013).

The present study reveals the existence of some genetic diversity in *C. parvum* in Romania. Overall, isolates of *C. parvum* were from two subtype families, Ila and IId, and six subtypes, namely IlaA16G1R1, IIdA21G1a, IIdA22G1, IIdA25G1, IIdA26G1 and IIdA27G1. All subtypes were only seen in calves, except subtype IIdA22G1 in both calves and humans, and subtype IIdA26G1, in a pig. Interestingly, the subtype family Ila was only seen in a single isolate of *C. parvum*, being identified as the subtype IlaA16G1R1. This subtype, which is an important etiological agent for bovine cryptosporidiosis (Xiao 2010), is predominant in countries neighbouring Romania (71% in Hungary, but only 21 samples examined; 33% in Serbia and Montenegro, 18 samples; 13% in Slovenia, 6 samples) (Plutzer and Karanis 2007, Misić and Abe 2007, Soba and Logar 2008), suggesting that in these countries as well as in Romania, this subtype is an important etiological agent for bovine cryptosporidiosis (Plutzer and Karanis 2007, Soba and Logar 2008, Imre et al. 2011). Besides calves, this subtype has also been found in lambs, pigs and humans and thus can be considered as a zoonotic pathogen (Soba and Logar 2008, Kváč et al. 2009, Imre et al. 2013). In our study, the subtype IlaA15G2R1, described as the most widely distributed in calves worldwide (Xiao 2010), was not identified.

Amongst the *C. parvum* IId subtype families characterised, five different subtypes were detected for the first time in Romania. Interestingly, some of these subtypes, namely IIdA21G1a, IIdA25G1 and IIdA26G1, have been found only occasionally in small ruminants (Xiao 2010), except for subtype IIdA22G1, which has also occasionally been implicated in bovine cryptosporidiosis in some European countries (Belgium, Germany, Hungary, Sweden – Silverlås et al. 2010, 2012, Xiao 2010). Apart from calves, this subtype was also detected in children with gastrointestinal symptoms in the present study. Subtype IIdA22G1 was also previously seen in humans in Portugal and in the United Kingdom (Alves et al. 2006, Chalmers et al. 2011). Results of phylogenetic analysis of the GP60 gene revealed the existence of two types of sequence within this subtype. One of these types from three human isolates (RH1, RH2 and RH7) was related to, but not identical with, isolates from lambs in Spain (GenBank Acc. No. EU549715) and bovines in Sweden (GenBank Acc. No. FJ917374). The fact that this type of sequence was found only in humans in the present study indicates the possibility of anthroponotic transmission. In contrast, the second type of sequences was seen in a human (RH8) and two bovine isolates (RB26 and RB2). It should be noted that subtypes IIdA21G1a and IIdA26G1 were also already found in human isolates (Alves et al. 2006, Nazemalhosseini-Mojarad et al. 2011). The subtype IIdA27G1 was the most frequent subtype in calves in this study, and represents a new subtype.

In summary, our epidemiological analysis suggests that in Timis County, Romania, there is a high prevalence of *C. parvum* subtype family IId characterised in this study, in contrast to

the high prevalence of the family Ila in previous studies also in this region (Imre et al. 2011). Human cryptosporidiosis appears to occur primarily through zoonotic transmission and cattle appear to be a major zoonotic reservoir. Results of this and previous studies, however, need to be confirmed by analyses of more

samples. These studies should also be performed in other regions of Romania and should include better collection of epidemiological data, with the purpose of more conclusive identification of infection sources, reservoir hosts and transmission dynamics of *Cryptosporidium* spp. in humans in this country.

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