Short communication

Genotype and subtype analyses of *Cryptosporidium* isolates from cattle in Hungary

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Abstract

Seventy nine faecal samples from calves with diarrhoea were collected on 52 farms from different counties in Hungary to investigate the species, genotypes and subtypes of *Cryptosporidium* in calves. Oocysts from faecal sample collected from each animal were concentrated using sucrose gradient centrifugation and examined by Immuno Fluorescence Assay (IFA). Genomic DNA was extracted from microscopically positive samples and nested PCR was performed to amplify the partial SSU rRNA and GP60 genes of *Cryptosporidium* that were subsequently digested by SspI, VspI and MboII restriction enzymes to determine the *Cryptosporidium* species and genotype present. *Cryptosporidium parvum* was detected in 21 samples while the *Cryptosporidium* deer-like genotype was found in another sample. The sequence and phylogenetic analysis of 21 isolates of the GP60 PCR products showed that the most common *C. parvum* subtype is IIaA16G1R1. Interestingly, two isolates were found to contain the *C. parvum* allele IId in addition to identifying another containing a new *C. parvum* IIa A18G1R1 subgenotype firstly described in the *C. parvum* complex. These findings suggest that cattle can be a source of cryptosporidial infections for humans and animals in Hungary. This is the first published description of *Cryptosporidium* subgenotyping in Hungary.

Keywords: *Cryptosporidium parvum*, cattle, genotype, subgenotype, Hungary.

1. Introduction

Currently 16 *Cryptosporidium* species are considered to be valid, however *C. hominis* and *C. parvum* are detected most commonly from various water samples and human and domestic ruminant faeces (Hunter and Thompson, 2005, Slapeta 2006) with *C. hominis* responsible for human to human transmission and *C. parvum* responsible for animal to human transmission (Becher et al., 2004, Santin et al., 2004, Fayer et al., 2006). Over the past 20 years, cattle have been identified as being a reservoir host for taxa transmitted from animals to humans, however, a remarkable assemblage of species affects cattle, including both cattle-specific, in addition to a zoonotic species. Cattle have been described as major hosts for *Cryptosporidium parvum*, *C. bovis*, *Cryptosporidium* deer-like genotype and *C. andersoni* which show a host age related susceptibility: *C. parvum* predominates in pre-weaned calves, *C. bovis* and *C. deer*-like genotype in post-weaned calves and *C. andersoni* in older calves and adult cattle (Santin et al., 2004, Fayer et al., 2006, Robinson et al., 2006, Slapeta 2006), although *C. bovis* and *C.* deer-like genotype have been reported in all age groups (Feng et al., 2006). Their findings clearly demonstrate that neonatal calves are an important source of zoonotic cryptosporidiosis in humans, although little is known about its transmission dynamics. Recently, researchers have used highly discriminatory sub-typing techniques (sequence analysis of the GP60 gene), useful for tracking infection sources and examining the transmission dynamics of *C. parvum* (Xiao et al., 2006, Abe et al., 2006). The next advance in our understanding of the epidemiology of cryptosporidiosis is likely to come from more detailed characterization of *Cryptosporidium* strains within the same species and genotype (Hunter and Thompson, 2005). Identification of the isolates at the subgenotype level will be useful for control of the cryptosporidial infections and for understanding of the population structure of *C. parvum* genotypes.

79 The aim of the present work was to use sequences of GP60 to identify subgenotypes of *C.*
80 *parvum* from cattle farms in Hungary.
81
82 **2. Materials and methods**
83
84 *2.1. Material collection and microscopic examination*
85 Seventy nine cattle faecal samples were collected from rectums of pre-weaned calves
86 with diarrhoea from 52 farms in nine counties in Hungary (Fig 1) in the period between
87 May-June 2006. Microscopic examinations using immunofluorescence (CRYPTO CEL,
88 Cellabs Pty Ltd, Brookvale, Australia) were performed on all samples after discontinuous
89 sucrose gradient purification and diethyl-ether/PBS 2:1 v/v biphasic concentration as
90 described (Karanis et al., 2006, Gomez-Couso et al., 2006). The faecal samples were
91 preserved in 2.5 % potassium dichromate and kept at 4 °C. One fifth of the sample
92 suspension in 2.5 % potassium dichromate was processed for genotypic analysis.
93
94 *2.2. DNA extraction and genetic analysis*
95
96 The microscopically positive samples were selected for PCR-RFLP analyses of the SSU
97 rRNA gene (Xiao et al., 2001, Feng et al., 2006), and sequencing of the GP60 gene Alves
98 et al., 2003, Sulaiman et al., 2005).
99 DNA was extracted from faecal samples using the Quiamp DNA Stool Mini Kit (Qiagen
100 GmbH, Hilden, Germany), according to the manufacturer's description, with the addition
101 of three 10-minute freeze-thaw cycles after resuspension in lysis solution in order to
102 rupture the *Cryptosporidium* oocysts. Liquid nitrogen was used for freezing, and thawing
103 was carried out at 70 °C in a Dry Thermo unit (DTU-2B, Taitec, Japan). DNA was eluted
104 in 100 µl buffer and stored at –20 °C until use.

105

106 *2.3. SSU rRNA PCR*

107 SSU rRNA nested PCR was performed as described by Xiao et al., (2001). Briefly, firstly
108 the 1325 bp PCR product and secondly the 826-864 bp PCR product was amplified in
109 standard mixtures of 50 µl containing 800 nmoles of each SSU rRNA specific primer, 200
110 µM dNTP, 1.5 mM $MgCl_2$ and 2.5 U HotStarTaq DNA polymerase (Qiagen GmbH,
111 Hilden, Germany). The templates were subjected to 35 amplification cycles (94 °C for 45
112 s, 55 °C at primary PCR and 58 °C at secondary PCR for 45 s, 72 °C for 60 s) followed by
113 one cycle of 10 min at 72 °C. The PCR products were analysed by 1.5 % agarose gel
114 electrophoresis and visualised after ethidium bromide staining.

115

116 *2.4. RFLP analysis*

117 Restriction fragment length polymorphism analysis of secondary PCR products was
118 performed initially using SspI and VspI before also including MboII in order to
119 differentiate *C. parvum, C. bovis* and *Cryptosporidium* deer-like genotype (Xiao et al.,
120 2001, Feng et al., 2006). The master mix used for restriction digestion contained 2 µl of
121 reaction buffer, 7.8 µl of nuclease free water and 5 units of VspI /SspI/MboII (Promega,
122 Wisconsin, USA) per reaction according to the description of the manufacturer. Each
123 reaction mixture contained 10 µl of master mix and 10 µl of secondary PCR product.
124 Restriction digestion was carried out at 37 °C for 2 h.

125

126 *2.5. Sequence of the GP60 gene*

127 A fragment of the GP60 gene (800-850 bp) was amplified with primers AL3531 (5' ATA
128 GTC TCC GCT GTA TTC 3'), AL3535 (5' GGA AGG AAC GAT GTA TCT 3') and
129 AL3532 (5' TCC GCT GTA TTC TCA GCC 3'), AL3534 (5' GCA GAG GAA CCA GCA
130 TC 3') in standard mixtures of 50 µl containing 800 nmoles of each GP60 specific primer,

200 µM dNTP, 1.5 mM $MgCl_2$ and 2.5 U HotStarTaq DNA polymerase (Qiagen GmbH, Hilden, Germany). The templates were subjected to 35 amplification cycles (94 °C for 45s, 50 °C for 45s, 72 °C for 60s) followed by one cycle of 10 min. at 72 °C (Alves et al., 2003). The PCR product was analysed by agarose gel electrophoresis and visualised with ethidium bromide staining. In some cases the primer set AL3532 (5' TCC GCT GTA TTC TCA GCC 3') and LX0029 (5' CGA ACC ACA TTA CAA ATG AAG T 3') was used for secondary PCR and 400 bp of PCR product was amplified (Sulaiman et al., 2005).

The secondary PCR products were sequenced on an ABI Prism 3100 (Applied Biosystems, Japan) Genetic Analyser using a Big Dye Terminator V.3.1 cycle sequencing kit (Applied Biosystems, Japan). The accuracy of the data was confirmed with bidirectional sequencing with the forward and reverse primers used in secondary PCR and an intermediary sequencing primer (5'-GAG ATA TAT CTT GGT GCG-3'). The recently proposed nomenclature was used in naming *C. parvum* subtypes (Sulaiman et al., 2005). Nucleotide sequences of the GP60 gene reported in this paper were aligned using ClustalW. The phylogenetic tree was constructed using the neighbour joining algorithm based on evolutionary distances calculated by the Kimura two-parameter model with 1,000 bootstrap sampling and was drawn using the Treecon program. The GP60 sequence of *C. meleagridis* (AF401497) was used to root the tree. Data reported in this paper of the GP60 nucleotide sequences are available in the GenBank database under the accession numbers EF073047-EF073051.

3. Results and discussion

According to the IFA results, 39 samples (~50%) were positive, 17 of which contained only one or two oocysts while 22 samples contained more than 100 oocysts (from 10 µl out of the 1.2 ml concentrated sample). Of the 22 samples containing more than 100

157 oocysts all were found to be PCR positive and subsequent RFLP and GP60 sequencing
158 results identified *C. parvum* in 21 samples and *Cryptosporidium* deer-like genotype in the
159 other. In terms of geographical distribution we were able to detect *C. parvum* in all
160 counties except county Somogy whereas only county Szolnok was positive for the
161 *Cryptosporidium* deer-like genotype (Fig 1). Our results support the earlier
162 epidemiological findings, that *Cryptosporidium* is a frequent pathogen of calves with
163 diarrhoea in Hungary (detected in 70% of the herds) (Nagy, 1995).
164 Alignment of GP60 sequences obtained with reference sequences downloaded from the
165 GenBank indicated, positives obtained during this study belonged to the *C. parvum*
166 subtype family IIa and IId (Fig 2). Within the IIa family all sequences were identical in
167 the non-repeat region (i.e. had one copy of sequence ACATCA immediately after the
168 trinucleotide repeats) while the trinucleotide repeat region all contained one copy of the
169 TCG repeat and 16, 17 or 18 copies of TCA. In the case of the IId family, the sequences
170 had one copy of the TCG repeat and 19 or 22 copies of the TCA repeat. Altogether, three
171 *C. parvum* IIa subtypes and two *C. parvum* IId subtypes were found with the
172 *Cryptosporidium* subgenotype IIaA16G1R1 being the most common, detected in 15
173 (71.4%) calves out of 21. The subgenotype IIaA17G1R1 was found in three cases while
174 the IId subgenotype family (IIdA22G1 and IIdA19G1) was detected in two.
175 Furthermore we found a novel *C. parvum* subgenotype (IIa A18G1R1) in the *C. parvum*
176 complex.
177 Until now the IIaA16G1R1 subtype was described in the US and Canada totalling three
178 calves in Michigan and four in Ontario however this subtype is not frequent in North
179 America and has not yet been detected in human patients (Peng et al., 2003,
180 Trotz-Williams et al., 2006, Xiao et al., 2006).
181 The IIaA17G1R1 played an important role in the three *Cryptosporidium* outbreaks in the
182 UK as it was detected in nine human patients and also in water samples. This subgenotype

183 has been detected in four samples from calves and humans in Slovenia in addition to one
184 calf in Ireland and is known as the Moredun isolate found in calves in the USA (Chalmers
185 et al., 2005, Peng et al., 2001, Thompson et al., 2006, Xiao et al., 2006).
186 Within the subtype family IId, the IIdA17G1 and IIdA21G1 isolates have previously been
187 found in calves and sheep in Portugal. The IIdA22G1 and IIdA19G1, which were
188 detected in Hungarian calves in this report, were detected in human patients in Portugal
189 (Alves et al., 2006).
190 Interestingly, subtypes such as IIaA16G2R1 and IIaA15G2R2, which have been found
191 frequently in Europe (Portugal, Slovenia, Ireland) and appear to be the major subtypes
192 responsible for zoonotic cryptosporidiosis (Alves et al., 2003, 2006, Stantic-Pavlinic et
193 al., 2003, Glaberman et al., 2002), were not detected in this study.
194 The potential for human infection of subtype IIaA16G1R1 (found in 71.4% of the
195 Hungarian samples) and of the new subtype IIaA18G1R1 (found in one Hungarian
196 sample) is yet to be determined. Since the IIdA22G1 and IIdA19G1 genotypes found in
197 this study and the IIaA17G1R1 genotype detected in Europe have already been detected
198 in human patients a clear public health risk in terms for the potential of zoonotic
199 transmission exists from subtype family IId in Hungary (Chalmers et al., 2005, Alves et
200 al., 2003, 2006).
201 Several species of *Cryptosporidium* are known to be transmissible between humans and
202 animals with *C. parvum* being the most common zoonotic species identified in domestic
203 ruminants (Alves et al., 2003, 2006, Nagy, 1995), causing diarrhoea and impairing the
204 gain of body weight. Direct contact with infected cattle is a major transmission pathway
205 for human infection along with indirect transmission through drinking water (Hunter and
206 Thomson, 2005). A recent study confirmed *Cryptosporidium* oocyst contamination in
207 various types of Hungarian surface water (Plutzer et al., in press). Environmental
208 pollution with human and domestic-animal faecal material is recognized as a potential

pathogenic pathway for wildlife infections with the zooanthropomorphic protozoan parasite. In order to prevent these pathways potential control measures and the role of veterinary and medical professionals in the prevention of cryptosporidiosis need to be defined. A benefit of this subgenotyping approach is its ability to differentiate various effects such as geographic variation and relationships to demographic and epidemiological data thereby giving the possibility to determine infection sources accurately (Peng et al., 2001). This is the first investigation of *Cryptosporidium* (sub) genotyping in Hungary and will complement any further investigation into species differences regarding risk factors for different (sub) genotypes, especially for human adapted *Cryptosporidium* and zoonotic (sub) genotypes.

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Fig 1.

The map of Hungary indicates the nine counties (grey colored), where cattle faecal samples were collected (all together 79 samples from 52 farms).

Fig 2.

Phylogenetic tree of GP60 sequences. The numbers on the branches are bootstrap values greater, than 50%. The name of the isolates, the original hosts, the localities and the

assession numbers in the Gene Bank (in case of the retrieved sequences) are shown in parentheses.