Short communication

Zoonotic Cryptosporidium parvum in Romanian newborn lambs (Ovis aries)

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A B S T R A C T

This study was undertaken to investigate the occurrence and public health significance of Cryptosporidium species/genotypes and subtypes in a newborn lambs. A total of 175 diarrheic fecal samples from lambs (younger than 21 days) were collected in seven sheep flocks located in western Romania, and were microscopically examined for the presence of Cryptosporidium oocysts after staining with modified Ziehl–Neelsen technique. Twenty-four (13.7%) fecal samples were tested Cryptosporidium positive by microscopy and were subjected for molecular characterization. All positive samples were successfully amplified through a nested polymerase chain reaction (PCR) of the small subunit (SSU) rRNA gene (18S). Cryptosporidium species were determined by restriction fragment length polymorphism (RFLP) analysis of the secondary PCR products using the conventional SspI and VspI restriction enzymes. The identified species were: Cryptosporidium parvum (20/24), C. ubiquitum (2/24) and C. xiaoii (2/24), respectively. PCR-RFLP results for C. ubiquitum and C. xiaoii isolates were confirmed by DNA sequencing. Subsequently, subtyping of seven randomly selected C. parvum isolates, based on sequence analysis of the GP60 gene, revealed the presence of five different subtypes (Ila17G1R1, Ila16G1R1, IldA20G1, IldA24G1 and IldA22G2R1) belonging in two zoonotic subtype families (Ila and Ild). These findings may suggest the potential role of the newborn lambs as a source for human cryptosporidiosis. This is the first published report about the presence of C. ubiquitum and C. xiaoii in lambs from Romania.

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1. Introduction

Species of the genus Cryptosporidium are widely distributed coccidian parasites, recognized as important zoonotic pathogens involved in enteric and gastric infections of a wide range of vertebrate hosts, worldwide (Xiao, 2010).

Results of several recent surveys, based on molecular tools, have shown that in ovine Cryptosporidium infections three species, namely C. parvum, C. xiaoii (previously recognized as the C. bosis-like genotype) and C. ubiquitum (formerly known as Cryptosporidium cervine genotype), appear to be more prevalent (Santin et al., 2007; Geurden et al., 2008; Mueller-Doblies et al., 2008; Quilez et al., 2008;
Fayer and Santín, 2009; Wang et al., 2010). The presence of other species and genotypes, such as C. andersoni, C. suis, C. hominis, C. pig genotype II and marsupial genotype, reported in a modest number of specimens, have been considered as oocysts passing through the intestinal tract (Fayer and Santín, 2009), and not sheep-adapted parasites.

Subtyping of sheep origin zoonotic C. parvum employing GP60 sequence analysis has been done in only few studies (Chalmers et al., 2005; Geurden et al., 2008; Quilez et al., 2008; Díaz et al., 2010; Sweeny et al., 2011). Results of these investigations have revealed that two major subtype families, Ila and Ild, with variable predominance according to geographic region, were responsible for C. parvum infections in sheep. Also, the presence of these zoonotic subtypes, especially in diarrheic lambs, highlighted the potential role of these livestock ruminants as a source for human cryptosporidiosis (Quilez et al., 2008; Díaz et al., 2010).

In Romania, a previous survey of ovine cryptosporidiosis was limited to one small study in which only two isolates were tested molecularly (Imre et al., 2011). Based on these considerations, additional studies to differentiate ovine Cryptosporidium isolates at the species/genotype and subtype levels are still required. Therefore, the study was undertaken to investigate the occurrence and public health significance of Cryptosporidium spp. in diarrheic newborn lambs from western Romania.

2. Materials and methods

During the period of December 2011–April 2012, a total of one hundred and seventy five fresh diarrheic fecal samples from lambs (Ovis aries), younger than 21 days, were collected in seven sheep flocks from western Romania. In survey area the ovine breeding is an important economic activity and is estimated at about 1.3 million in 2011 (unofficial data). Samples were collected directly from the rectum and transferred to sterile plastic jars.

Fecal samples were initially screened under light microscopy (magn. 400×) for the presence of Cryptosporidium oocysts using the modified Ziehl–Neelsen staining method (Henrickson and Pohlenz, 1981). The positive isolates were selected for molecular examination and were stored at 4°C in 2.5% potassium dichromate prior to processing.

Genomic DNA was isolated from Cryptosporidium-positive stool specimens using the QIAamp® DNA stool mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions, except the final step wherein the DNA was eluted with 100 μl AE (elution) buffer instead of 200 μl. The extracted DNA was stored at −20°C for further use.

Cryptosporidium species were determined by a nested polymerase chain reaction (PCR) of the small subunit (SSU) rRNA gene (18S), followed by restriction fragment length polymorphism (RFLP) analysis of the secondary PCR products using the conventional SspI (New England BioLabs®, Beverly, MA, USA) and VspI (Promega®, Madison, WI, USA) restriction enzymes. Primers and amplification conditions used in the nested PCR protocol, and the RFLP procedure were performed as described by Xiao and Ryan (2008), except the PCR reaction mixture volume which was prepared in 25 μl instead of 100 μl.

The presence of diagnosed Cryptosporidium species, through the nested PCR–RFLP method, was confirmed by DNA sequencing of 11 samples from the five positive sheep flocks. Subtyping of 7 randomly selected C. parvum isolates was based on sequence analysis of the secondary nested PCR products of the 60 kDa glycoprotein (GP60) gene. In this case, PCRs were performed as described previously (Alves et al., 2003). For two isolates of C. xiaoi and C. ubiquitum, sequencing procedure targeted the secondary PCR product of the SSU rRNA gene. Amplification products were direct sequenced on an ABI Prism 3130 automated sequencer (Applied Biosystems, Foster City, CA) using the forward and reverse primers of the secondary PCR. Single-strand DNA sequencing reactions were performed using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The nucleotide sequences obtained were aligned and analysed, together with the reference sequences downloaded from the GenBank database, using online versions of Clustalw2 (available http://www.ebi.ac.uk/Tools/msa/clustalw2/) and BLAST® (Basic Local Alignment Search Tool; available http://blast.ncbi.nlm.nih.gov) software. The C. parvum subtypes were named according to the proposed nomenclature system by Sulaiman et al. (2005) and the representative DNA sequences were deposited in GenBank under accession numbers JX258863–JX258866.

To compare the prevalences between age groups, the Statistic Calculator (StatPac® Inc.) was used, and the differences were considered significant when p values were less than 0.05.

3. Results and discussion

Overall, 24 (13.7%) out of 175 diarrheic fecal samples were diagnosed as Cryptosporidium positive by microscopic examination. Five out of seven sheep flocks were found to be Cryptosporidium positive and the percent of infected newborn lambs varied between 11.1% and 35.0% within the flocks (Table 1). The percentage of animals shedding oocysts according to selected age groups was 13.8% (1–7 days), 16.2% (8–14 days), and 10.2% (15–21 days), respectively. These differences were not statistically significant p > 0.05.

Molecular characterization of Cryptosporidium isolates through the nested PCR–RFLP procedure targeting the SSU rRNA gene was successfully done in all (24) microscopic positive samples. Thus, RFLP analysis of the secondary PCR amplicons with SspI and VspI endonucleases demonstrated the presence of three Cryptosporidium species (Xiao and Ryan, 2008), including C. parvum (20/24; 83.3%) in four flocks, C. ubiquitum (2/24; 8.3%) in one flock, and C. xiaoi/C. bovis (2/24; 8.3%) in two flocks, respectively (Table 1). DNA sequencing of the SSU rRNA PCR products from two C. ubiquitum RFLP positive samples confirmed the identification of this species based on the homology of 99.9% with the reference sequences retrieved from GenBank accession numbers JN247402 and HQ822139, respectively. Similarly, the nucleotide sequence obtained from two PCR–RFLP indistinguishable C. xiaoi/C. bovis isolates showed 99.8%
homology compared to the C. xiao GenBank accession numbers GU553016 and EF514234, respectively. Subsequently, subtyping of seven C. parvum isolates, based on sequence analysis of the GP60 gene, revealed the presence of five different subtypes belonging to the zoonotic subtype families Ila and IId, respectively. So, the identified subtypes revealed high sequence homology (99.9–100%) to Gen-Bank deposited isolates and were defined as follows: IlaA17G1R1 (n = 2; GenBank reference sequence: AM988863), IlaA16G1R1 (n = 1; AM937009), IIdA20G1 (n = 2; FJ917375), IIdA24G1 (n = 1; HQ005751), and IIdA22G2R1 (n = 1; GU214368), respectively. Interestingly, each isolate was found only in one flock (Table 1).

This is the first published report about the presence of C. ubiquitum and C. xiao in lambs from Romania. C. parvum was the major species involved in the neonatal cryptosporidiosis outbreaks in sheep flocks from western Romania. In accordance with our results, dominance or exclusive presence of zoonotic C. parvum has been confirmed in other recent molecular surveys conducted in the United Kingdom (28 lambs out of 33, Mueller-Doblies et al., 2008) and Spain (154/154, Quilez et al., 2008; 14/23, Diaz et al., 2010). C. ubiquitum, found only in two samples out of 24 in this study, has been detected to be most prevalent in lambs from Belgium (9/10, Geurden et al., 2008), China (74/82, Wang et al., 2010) and the United States (48/57, Santin et al., 2007). Likewise, the sheep adapted C. xiao, the other species diagnosed in two cases in the current survey, was genetically confirmed as predominant in lambs from Australia (285/387, Sweeney et al., 2011) or as single species in Spain (1/1, Navarro-i-Martinez et al., 2007).

The higher percent of infection was recorded in lambs aged between 8 and 14 days. This observation was in agreement with data published by Causapé et al. (2002) in Zaragoza (north–eastern Spain), but contrary with findings obtained by Santin et al. (2007) in Maryland (US), where the peak of Cryptosporidium infection in lambs was reported at 21 days of age.

Sequence analysis of the GP60 gene revealed a considerable genetic variability of 7 C. parvum isolates from lambs. It is worth noting that 4 out of 5 identified subtypes were found in different sheep flocks due, probably, to different management practices. To our knowledge, of the identified isolates only IIdA24G1 and IIdA20G1 subtypes were previously reported in lambs (Quilez et al., 2008; Sweeney et al., 2011). The overwhelming predominance of the IId subtype family was described in one large sample size study with extensive genetic diversity (11 identified subtypes) conducted in north–eastern Spain (Quilez et al., 2008), where most isolates (134 out of 137) belonged to this family subtype. In contrast, other surveys, in which a low number of ovine C. parvum isolates from United Kingdom, Belgium and north–west of Spain were subtyped, showed the more frequent appearance of subtypes belonging to the family Ila (Chalmers et al., 2005; Geurden et al., 2008; Diaz et al., 2010). Most of the GP60 genotypes found in this study have been reported previously in humans in different countries such as Slovenia (Soba and Logar, 2008), Kuwait (Sulaiman et al., 2005) and Australia (Waldron et al., 2011).

From an epidemiological perspective and in agreement with the previous survey (Imre et al., 2011), our results demonstrate that Cryptosporidium may be involved in the etiology of enteric infections of newborn lambs in sheep flocks from western Romania. Likewise, the predominance of C. parvum and the presence of C. ubiquitum with zoonotic potential (Xiao, 2010) in sampled diarrheic animals, emphasize the potential role of these small ruminants as a source of human cryptosporidiosis. Moreover, this matter is also highlighted by the occurrence of members of C. parvum family alleles Ila and IId, known to be transmissible between humans and animals. To obtain a complete epidemiologic picture of Cryptosporidium infection in this

### Table 1

Distribution of Cryptosporidium species and subtypes in newborn lambs at seven sites in western Romania.

<table>
<thead>
<tr>
<th>Collection site</th>
<th>No. of animals examined/Cryptosporidium positive</th>
<th>Prevalence (95% CI)</th>
<th>Occurrence of infection in age groups</th>
<th>Species identified (number)</th>
<th>Subtypes (number)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1–7 days (&lt;br&gt;(n = 58)</td>
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<tr>
<td>Flock A</td>
<td>26/5</td>
<td>19.2 (8.6–38.0)</td>
<td>2</td>
<td>C. parvum</td>
<td>IlaA17G1R1 (2)</td>
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<tr>
<td>Flock B</td>
<td>29/0</td>
<td>0.0 (0.0–11.5)</td>
<td>–</td>
<td>C. parvum</td>
<td>IIdA20G1 (2)</td>
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<tr>
<td>Flock C</td>
<td>34/7</td>
<td>20.5 (10.4–36.9)</td>
<td>2</td>
<td>C. xiaoii</td>
<td>IIdA24G1 (1)</td>
</tr>
<tr>
<td>Flock D</td>
<td>18/2</td>
<td>11.1 (3.3–33.1)</td>
<td>–</td>
<td>C. parvum</td>
<td>IIdA22G2R1 (1)</td>
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<td>Flock E</td>
<td>16/3</td>
<td>18.7 (6.8–43.4)</td>
<td>–</td>
<td>C. xiaoii</td>
<td>IlaA16G1R1 (1)</td>
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<tr>
<td>Flock F</td>
<td>32/0</td>
<td>0.0 (0.0–10.5)</td>
<td>–</td>
<td>C. parvum</td>
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<tr>
<td>Flock G</td>
<td>20/7</td>
<td>35.0 (18.1–56.9)</td>
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<td>Total</td>
<td>175/24</td>
<td>13.7 (9.4–19.6)</td>
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<td>8–14 days (&lt;br&gt;(n = 68)</td>
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<td>15–21 days (&lt;br&gt;(n = 49)</td>
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region, and to characterize the transmission dynamics and zoonotic potential of *C. parvum* isolates, further molecular studies focused on human cryptosporidiosis and other livestock are needed.

**Conflict of interest statement**

The authors have no financial or personal relationship with other people or organizations that could inappropriately influence or bias this paper.

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