

Intraspecific characterization of some *Cryptosporidium parvum* isolates from calves and lambs in Western Romania using molecular techniques

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Abstract. The paper shows the results of intraspecific characterization of 16 isolates of *Cryptosporidium parvum* from calves and lambs. The molecular approach included a two phases PCR, using gp60 as target gene. The PCR final product was sequenced and compared with referential gene sequence from Gene Bank. The analysis of 15 bovine *C. parvum* isolates from Western Romania revealed that they belong to two family subtypes: Ila (86.7%) and IId (13.3%). Two subtypes of Ila family were found: IlaA15G2R1 (53.4%) and IlaA16G1R1 (33.3%). The single ovine isolate of *C. parvum* belong to subtype IIdA 22G2R1. The intraspecific characterization of these isolates show the presence of zoonotic subtypes in calves and lambs from western Romania.

Keywords: *Cryptosporidium parvum*, subtype, Ila, IId, gp60

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Introduction

Protozoans of genus *Cryptosporidium* are parasites with a large host specificity, infecting many vertebrate species, including humans (Alves et al., 2005; Santín and Trout, 2007; Kvác et al., 2009). There are 19 valid species and 60 genotypes of *Cryptosporidium* known, and ten of them are found in bovine hosts (Feng et al., 2007; Plutzer and Karanis, 2007; Fayer, 2009). The most common mammalian species, *C. parvum*, infects mainly calves and lambs in the first month of life (Santín and Trout, 2007; Quílez et al., 2008). The interspecific differentiation between the two

major zoonotic species, *C. parvum* and *C. hominis*, is made by molecular analysis of subunit SSU-rRNA (18S) gene (Alves et al., 2001; Wu et al., 2003; Chalmers et al., 2005).

Intraspecific differentiation is also important for the proper understanding of the epidemiology of cryptosporidiosis. This is achievable by subtyping methods, the most popular being the sequential analysis of DNA glycoprotein 60 kDa (gp60) (Strong et al., 2000; Alves et al., 2003; Abe et al., 2006; Misić and Abe, 2007; Plutzer and Karanis, 2007). The gp60 gene represents the most polymorphic marker of *Cryptosporidium* genome and it is

similar to a microsatellite sequence repeated in tandem at the end of 5' and is encoded by TCA, TCG or TCT trinucleotides. Each subtype family of the two major zoonotic species differs from each other, especially by the number of trinucleotides repeated (TCA, TCG or TCT). The nomenclature of the family subtypes begins with the name of family subtype (Ia, Ib, Id, Ie, If etc. for *C. hominis* and Ila, Ilb, Ilc, Ild etc. for *C. parvum*) followed by the number of repetitive trinucleotides: TCA (coded by letter A), TCG (letter G) and/or TCT (letter T) where this is applicable (Xiao, 2010). Family subtypes Ila and Ild of *C. parvum* are found in both, humans and ruminants, and are considered the major zoonotic subtypes. Worldwide, the use of these second generation molecular tools has clarified certain aspects in the distribution of zoonotic *Cryptosporidium* species and subtypes, the pathways of transmission as well as population structure and host-parasite interactions (Fayer, 2009; Plutzer and Karanis, 2009; Xiao, 2010).

The aim of the present paper was to characterize the distribution of *Cryptosporidium* subtypes in calves and lambs from western Romania.

Materials and methods

Sixteen fecal samples (15 from calves and 1 from lambs) were collected from five farms from Arad, Bihor and Timiș counties in western Romania. DNA was extracted from fecal samples, after concentration with Mini-Bead-Beater/Silica technique, as described by Alves et al. (2001). Species determination was made by PCR-RFLP characterization of SSU-rRNA gene (18S) as described by Alves et al. (2003). Intraspecific characterization was performed using a two phase PCR, using gp60 as target gene, as described by Abe et al. (2006).

Final PCR products were sequenced with ABI 3100 autosequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were analyzed and compared with sequences from referential Gene Bank (Gene Bank®) using the ClustalX (<ftp://www.ftp-igbmc.u-strasbg.fr/pub/ClustalX>) and BLASTIN (www.ncbi.nlm.nih.gov) softwares.

Results

All 16 samples were identified as *C. parvum*. Intraspecific characterization of *C. parvum* isolates from cattle, as determined by secondary PCR sequenced product for the amplified gp60 gene, showed that the 15 isolates belong to two family subtypes: Ila (86.7%) and Ild (13.3%). In the Ila family subtype, two subtypes were found: IlaA15G2R1 (53.4%) and IlaA16G1R1 (33.3%). The ovine isolate of *C. parvum* belongs to family subtype Ild, subtype IldA22G2R1. These subtypes of *C. parvum* contain a number of copies of ATT and GTC trinucleotides from the repetitive area of the gp60 gene. This means that the family subtype IlaA15G2R1 contains 15 copies of ATT triplets (symbolized A15) and 2 copies of GTC trinucleotides (symbolized G2) accompanied by a repeated sequence of 13-15 bp (symbolized R1).

Discussions

Molecular epidemiology investigations carried out in neighboring countries (Serbia, Montenegro, Hungary) and other geographical areas showed that most *C. parvum* isolates from cattle belong to family subtype Ila, also identified in humans (Stantic-Pavlinic et al., 2003; Trotz-Williams et al., 2006; Mistic and Abe, 2007; Plutzer and Karanis, 2007; Santín and Trout, 2007; Thompson et al., 2007; Xiao et al., 2007; Xiao, 2010).

Worldwide, the most common subtype is IlaA15G2R1, which was identified also in the majority of our bovine samples (53.4%) from western Romania. In USA, 135 of 175 isolates belonged to this subtype (Trotz-Williams et al., 2006; Xiao et al., 2007), in Portugal 61/72 (Alves et al., 2005) and in India 5/9 (Feng et al., 2007). In Northern Ireland, a greater genetic diversity has been observed in allele II. The most common subtype was IlaA18G3R1 (120/214) followed by subtypes IlaA15G2R1 (28/214) and IlaA17G2R1 (19/214) (Thompson et al., 2007). Subtype IlaA15G2R1 was frequently identified in people from Australia, Japan, Kuwait, Northern Ireland, Portugal, Slovenia and the United States (Alves et al., 2005; Abe et al., 2006; Trotz-Williams et al., 2006; Thompson et al., 2007; Xiao, 2010).

This fact suggests that the family subtype IIa can spread easily within cattle populations and can be transmitted to humans too. In regions where both subtypes are found, (i.e. Spain), family subtype IIa infects preferentially calves, while family subtype IIc has a tropism for lambs and kids (Quílez et al., 2008). Subtype IIc was reported in this study in a lamb, suggesting that these animals can be reservoirs of family subtypes with zoonotic importance.

Therefore, the subtyping of *C. parvum* becomes a valuable tool in the analysis of infection sources and understanding of the dynamics of transmission of cryptosporidiosis. However, up to date, these investigations were performed only in 20 countries (Plutzer and Karanis, 2009). Moreover, regardless of the PCR technique used for genotyping or subtyping of *Cryptosporidium* isolates, all large specificity methods have problems in characterizing the dominant genotype. Almost all specific genotyping and subtyping tools detects only *C. hominis* and *C. parvum* species and their related genotypes. Further studies are required for a deeper understanding of the epidemiology of cryptosporidiosis in men and animals.

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