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Short Communication

# Molecular characterization of *Cryptosporidium* isolates from calves in Argentina

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ABSTRACT

Cryptosporidiosis is responsible for significant fatalities of neonatal calves, resulting in substantial economic loss in dairy farming in several countries. Additionally, the high shedding of environmentally resistant oocysts by calves promotes contamination of drinking water and facilitates outbreaks of cryptosporidiosis in humans. Here we report on the *Cryptosporidium* species and GP60 subtypes of 45 calves originating from the Humid Pampa, the main productive dairy farming area of Argentina. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of the 18S rRNA gene was done to determine the infecting *Cryptosporidium* species and only *Cryptosporidium parvum* was detected. Subtyping by sequence analysis of the GP60 gene revealed 6 different alleles all pertaining to the zoonotic IIa family. Of these, IIaA23G1R1 represents a novel IIa subtype. Other identified subtypes, IIa18G1R1, IIaA20G1R1, IIaA21G1R1, and IIaA22G1R1 have been recognized in very few studies and/or with low frequencies. Interestingly, different alleles prevailed in the provinces of Buenos Aires (IIaA17G1R1 and IIaA21G1R1), Santa Fe (IIaA23G1R1), and Cordoba (IIaA20G1R1 and IIaA21G1R1), and different allele distribution patterns were observed. Subtypes IIaA18G1R1 and IIaA17G1R1, the latter often found in this study, are strongly implicated in zoonotic transmission, suggesting that calves may represent a potential source for human cryptosporidiosis in this region. This is the first published report of a molecular analysis of *Cryptosporidium* infection in dairy and beef calves from Argentina.



## 1. Introduction

*Cryptosporidium* spp. are ubiquitous enteric parasitic protozoa of vertebrates. Their monoxenic life cycle involves the formation of thick-wall oocysts which are released with host feces. Infection occurs through the oral–fecal route, by host-to-host contact, or through the ingestion of contaminated water or food (Bouzid et al., 2013).

Mainly four *Cryptosporidium* species have been reported in cattle: *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium ryanae*, and *Cryptosporidium andersoni*, but others have also been identified (e.g. *Cryptosporidium hominis*, *Cryptosporidium suis*, *Cryptosporidium meleagridis*, and others). While oocysts of *C. andersoni* can be distinguished from those of *C. parvum*, *C. bovis*, and *C. ryanae*, the latter

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three are very similar and molecular techniques are indispensable for their classification and detection (Chalmers and Katzer, 2013). In the case of the most pathogenic of these species, *C. parvum*, sporozoites invade the intestinal epithelium after oocyst excystation, producing villus shortening and destruction, which leads to reduced absorptive capacity (Klein et al., 2008). Clinical cases, which appear in 7–30 day-old calves, range from watery diarrhea, depression, anorexia, and abdominal pain, to death, due to dehydration and cardiovascular failure (Olson et al., 2004). The economic burden imposed by *C. parvum* infection of neonatal calves is mostly related to the special treatments needed to overcome diarrhea crisis with concomitant dehydration, resulting in growth retardation and mortality (Sanford and Josephson, 1982). Spread of cryptosporidiosis is facilitated by the highly efficient parasite dissemination strategy through environmentally resistant, long-lasting, and highly infective oocysts, and the absence of effective vaccines and/or chemotherapeutic agents (Wyatt et al., 2010). Importantly, *C. parvum* is a zoonotic and anthroponotic agent, and has been responsible for serious human diarrhea outbreaks both in industrialized and developing countries, affecting especially immunocompromised individuals and children (Jex and Gasser, 2010; Xiao and Feng, 2008).

Molecular characterization of circulating *Cryptosporidium* parasites can allow the evaluation of the distribution and zoonotic potential of species and subtypes as well as their transmission routes in humans and animals under different epidemiological situations (Xiao, 2010). The most common subtyping method described for *C. parvum* is the analysis of nucleotide repeats in the gene encoding a surface glycoprotein of 60 kDa (GP60) (Strong et al., 2000). Several *C. parvum* GP60 subgenotypes have been described in different parts of the world and, interestingly, association of some subtypes with zoonotic potential or pathogenicity has been demonstrated (Cama et al., 2007; Plutzer and Karanis, 2009).

Most studies on bovine cryptosporidiosis have been conducted in industrialized countries, while much less information is available for developing countries (Jex and Gasser, 2010). In Argentina, *Cryptosporidium* sp. has been reported by microscopic observation of oocysts in stained fecal smears, and reports have been so far limited to estimations of dairy calf herd prevalence and infection in dairy calves according to age (Del Coco et al., 2008; Tiranti et al., 2011). As molecular characterization of *Cryptosporidium* isolates has not been carried out, the involved *Cryptosporidium* species and zoonotic subtypes remain unknown making it difficult to draw conclusions to animal and/or human health. Therefore, the aim of the present work was to determine the *Cryptosporidium* species and genotypes infecting calves from the main productive dairy farming region of Argentina. In addition, GP60 allele typing was carried out to assess the zoonotic potential of the encountered *C. parvum*.

## 2. Materials and methods

Fecal samples ($n = 45$) were collected from calves of both sexes aged from 5 to 60 days, from 16 dairy and 2 beef farms located in the Argentine provinces of Buenos Aires (9 farms), Santa Fe (6 farms), and Cordoba (3 farms). All but three sampled animals displayed diarrhea. Of the non-diarrheic animals, two originated from beef farms and the remaining one, from a dairy farm. After verification of the presence of oocysts using the modified Ziehl–Neelsen method DNA was isolated from 0.2 g fecal samples as described by Peng et al. (2003). Purified DNA was quantified in a Nanodrop spectrophotometer and stored at −20 °C until further analysis.

DNA samples were subjected to PCR-RFLP as described in Xiao et al. (1999) using direct PCR. In addition, 10 samples were subjected to direct sequencing after amplification of the hypervariable region of the 18S rRNA gene as described previously (Coupe et al., 2005; Macrogen, Seoul, South Korea). Four of these 10 sequences were longer than the minimum sequence length of 200 bp as stipulated by the GenBank database and were deposited under the accession numbers KC995120–KC995123.

*C. parvum* isolates were subtyped by direct sequencing of an 878 bp PCR amplicon of the gene encoding the 60 kDa glycoprotein (GP60), as described by Alves et al. (2003). Amplicons of two samples were ligated into the pJET1.2/blunt vector (CloneJET$^{TM}$ PCR Cloning Kit, Fermentas, Lithuania), as recommended by the manufacturer. After transformation of *E. coli* TOP 10 cells (Invitrogen, Carlsbad, CA), three qualified clones were selected, purified, and subsequently sequenced using the forward and reverse amplification primers on an ABI 3500xL equipment (Applied Biosystems, Carlsbad, CA). Assignment of GP60 alleles to their respective family was done by phylogenetic analysis including previously defined reference alleles. GP60 subtypes were designated based on the number of trinucleotides TCA and TCG, and the hexanucleotide ACATCA in the polymorphic repeat region (Sulaiman et al., 2005). Nucleotide sequences were deposited in the GenBank database under accession numbers KC995124–KC995163, KF147536–KF147540, and KF289038.

## 3. Results and discussion

By PCR-RFLP, exclusively *C. parvum* and no other *Cryptosporidium* species could be identified in our study group, consisting of 45 samples from 18 different farms in the major dairy farming region of Argentina (Fig. 1, Supplementary material). In 10 of the 45 samples, the species *C. parvum* was additionally verified by sequencing of the hypervariable region of the 18S rRNA gene.

A predominance of *C. parvum* has been also reported in studies carried out in other countries that included young calves (less than 1 month of age) with and without diarrhea (Broglia et al., 2008; Brook et al., 2009; Feng et al., 2007; Kvác et al., 2006; Plutzer and Karanis, 2007; Thompson et al., 2007). It is noteworthy, however, that an exclusive identification of *C. parvum* has also been reported in some studies of randomly sampled cattle in Iran, Australia, North America and Europe (Nazemalhosseini-Mojarad et al., 2011; O'Brien et al., 2008). In contrast, a predominance of *C. bovis* has been reported in preweaned calves of Sweden, but also in some farms of Australia, China, India, Nigeria, United