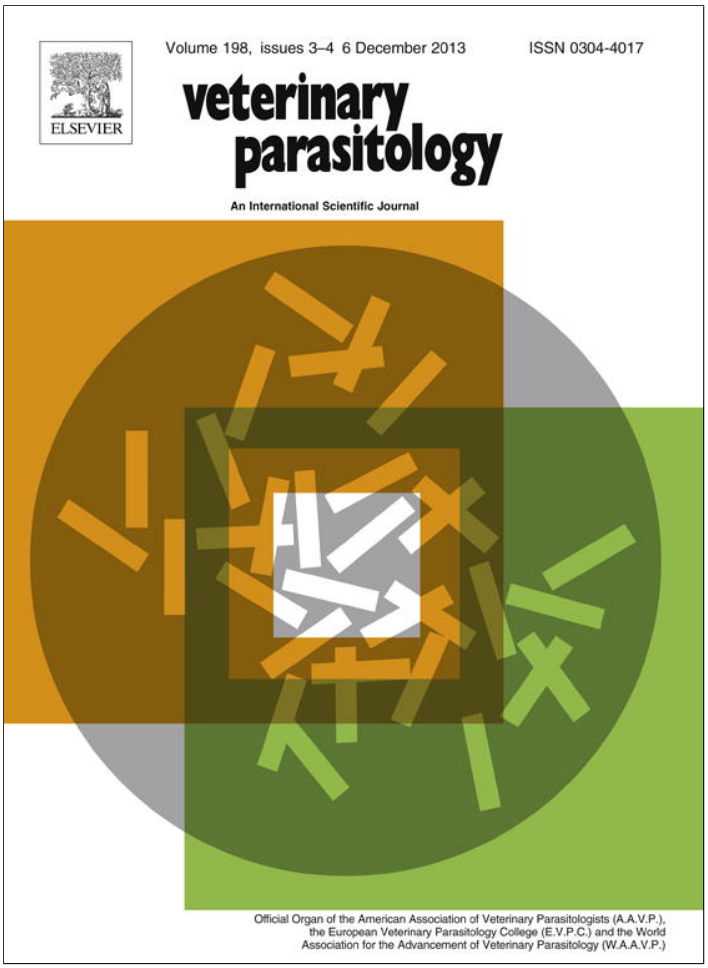

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Short Communication

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

Table 1
Cryptosporidium parvum GP60 subtypes found in calves originating from three different provinces in Argentina.

Subtype	Samples			Total	Geographic region[b]	References
	Buenos Aires	Santa Fe	Cordoba			
IIaA17G1R1	10	0	0	10	England (7 of 51) North Ireland (1 of 215) Hungary (3 of 21) The Netherlands (14 of 129)	Brook et al. (2009) Thompson et al. (2007) Plutzer and Karanis (2007) Wielinga et al. (2008)
IIaA18G1R1	0	0	1	1	England (2 of 51) Hungary (1 of 21) Serbia, Montenegro (2 of 18) The Netherlands (2 of 129)	Brook et al. (2009) Plutzer and Karanis (2007) Misic and Abe (2007) Wielinga et al. (2008)
IIaA20G1R1	4	1	3	8	Serbia, Montenegro (2 of 18)	Misic and Abe (2007)
IIaA21G1R1	9	3	3	15	Sweden (2 of 110) Argentina (1 of 1)	Silverlås et al. (2013) Del Coco et al. (2012)
IIaA22G1R1	2	3	0	5	Czech Republic (12 of 243) Germany (1 of 53) Sweden (7 of 198)	Kváč et al. (2011) Broglia et al. (2008) Silverlås et al. (2013)
IIaA23G1R1	1	6	0	7	None	None
	26	13	7	46[a]		

[a] 46 subtypes were identified in 45 samples as in one sample both subtypes, IIaA18G1R1 and IIaA20G1R1, were detected.
[b] Country in which the respective subtype has been reported in cattle. Also the number of positive samples and the examined study group is given.

States, and Zambia (Feng et al., 2007; Geurden et al., 2006; Maikai et al., 2011; Ng et al., 2011; Silverlås et al., 2010). Often an age-related variation of *Cryptosporidium* species has been observed, where depending on the region studied, either *C. bovis* or *C. parvum* were reported to dominate in calves, while other *Cryptosporidium* sp. prevailed in cattle of older age (Fayer et al., 2007, 2006; Kvác et al., 2006; Silverlås et al., 2010; Khan et al., 2010; Santín et al., 2008).

GP60 gene sequences of the 45 individuals of the study group could be amplified and 43 of these were successfully analyzed by direct sequencing. Of the two remaining individuals, the GP60 subtype could be determined after amplicon cloning and sequencing. As suspected by the direct sequencing chromatogram, in one of the samples two GP60 subgenotypes were identified using this approach. In order to assign GP60 alleles to subtype families, an alignment and phylogenetic analysis were carried out (Fig. 2, Supplementary material). Altogether six GP60 subtypes, all belonging to the IIa family, were identified in the study region (Table 1). Notably, at amino acid site 99, a non-synonymous nucleotide exchange in the coding triplet "GAC" to "GGC", resulting in an exchange of Asn to Gly, was exclusively observed in the 46 sequences of Argentine origin but not in any of the compared reference sequences of the Ic, Ie, If, Ib, IIa, IIc, IIb, and IId family (Strong et al., 2000). The subtype IIaA21G1R1 ($n = 15$) predominated in the study and was found to be frequent in the provinces of Buenos Aires (9 of 26), Cordoba (3 of 7), and Santa Fe (3 of 13). As to our knowledge, IIaA21G1R1 has so far only been identified in two calves from Sweden (Silverlås et al., 2010), and in a single calf from Argentina (Del Coco et al., 2012), and has not been reported in other regions of the world. The novel subtype IIaA23G1R1 ($n = 7$) was frequent in our study group predominating in Santa Fe (6 of 13) while it was identified only once in Buenos Aires (1 of 23). So far, it is the GP60 IIa subtype with the highest number of trinucleotide repeats observed. Two further subtypes, IIaA20G1R1 ($n = 8$) and IIaA22G1R1 ($n = 5$), were also rather frequently identified in this study and have been rarely reported by others: subtype IIaA20G1R1 has only once been reported before, from a region covering Serbia and Montenegro (Misic and Abe, 2007), while subtype IIaA22G1R1 has been reported in only three previous studies, from Germany, Sweden, and the Czech Republic (Broglia et al., 2008; Kváč et al., 2011; Silverlås et al., 2013). The subtype IIaA18G1R1 seems to be geographically widespread as it has been observed in Serbia and Montenegro; England, Hungary, and The Netherlands, yet, as in this study, always with a very low frequency (Misic and Abe, 2007; Plutzer and Karanis, 2007; Wielinga et al., 2008; Brook et al., 2009). In contrast, subtype IIaA17G1R1 ($n = 10$) has been frequently reported in this as well as in other studies and is thought to be globally distributed (Brook et al., 2009; Jex and Gasser, 2010; Plutzer and Karanis, 2007; Thompson et al., 2007; Wielinga et al., 2008).

As has been recently pointed out, the overwhelming majority of data on GP60 variation from cattle belongs to subfamily IIa and has been reported so far from relatively few industrialized countries (Australia, Northern Ireland, Portugal, The Netherlands, Spain, the USA, and others; Jex and Gasser, 2010). As yet no studies have been carried out in the investigated region, which may account for the observation that the majority of the identified subtypes (5 of 6) have only occasionally and with low frequency been reported.

Two of the subtypes identified in this study, IIaA17G1R1 and IIaA18G1R1, have also been reported in humans, which may suggest a potential risk of zoonotic transmission in the studied region (O'Brien et al., 2008; Wielinga et al., 2008; Zintl et al., 2009). Noteworthy, humans suffering from cryptosporidiosis who were found to be infected with subtype IIaA17G1R1 were particularly linked to zoonotic exposure (Chalmers et al., 2011). With respect to zoonotic transmission, the potential role of the other IIa

subgenotypes identified in this study has still to be defined (Jex and Gasser, 2010).

In conclusion, only *C. parvum* of the GP60 subtype family IIa was found in calves originating from a region of high dairy farm activity. Of the six identified GP60 alleles, IIaA23G1R1 represents a novel subtype of *C. parvum* while four other identified GP60 subtypes (IIaA18G1R1, IIaA20G1R1, IIaA21G1R1, and IIaA22G1R1) have been rarely reported, and if so, with low frequency. The frequent finding of subtype IIaA17G1R1, strongly implicated in zoonotic transmission, suggests that calves might constitute an important source for human cryptosporidiosis in Argentina. Additional studies involving more extensive sampling and including humans are underway to determine the prevalence of cryptosporidiosis, association of neonatal death of calves with *Cryptosporidium* species and genotypes, and the degree of zoonotic transmission.

Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2013.09.022.

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