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Cryptosporidium parvum GP60 subtypes in dairy cattle from Buenos Aires, Argentina

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ABSTRACT

Cryptosporidium parvum from 73 dairy calves less than two months old from Buenos Aires province (Argentina) were molecularly characterized using sequence analysis of the GP60 gene. Seventy-five sequences were obtained, and seven different subtypes were identified, all belonging to the IIa subtype family. The most common subtypes were IIaA20G1R1 (27/75), IIaA22G1R1 (16/75), and IIaA18G1R1 (13/75). Subtypes IIaA21G1R1, IIaA23G1R1, IIaA16G1R1 and IIaA19G1R1 were found sporadically. Two samples contained mixed infections with IIaA21G1R1 and IIaA22G1R1. A significant association was found between subtypes and geographic location, whereas there was no relation between subtypes and presence of diarrhea. Three of the subtypes found in this study (IIaA16G1R1, IIaA18G1R1, and IIaA19G1R1) were previously identified in humans. These findings suggest that cattle could play an important role in the transmission of cryptosporidiosis to humans in Buenos Aires province.

Cryptosporidiosis is a significant cause of diarrhea in humans and domestic animals (Santín and Trout, 2008). Cattle are mainly infected by four species of *Cryptosporidium*: *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium ryanae*, and *Cryptosporidium andersoni* (Fayer et al., 2010). Of these, only *C. parvum* is an important zoonotic pathogen and its presence in cattle is a public health concern because human cryptosporidiosis is more common where dairy farming is most intensive (Xiao and Feng, 2008). Zoonotic transmission of *Cryptosporidium* can occur through direct contact as well as through ingestion of food and water contaminated with oocysts (Del Coco et al., 2009).

Molecular tools are required to identify *Cryptosporidium* species, genotypes and subtypes; this information is critical to identify the cause of infection and to determine the source of contamination (Xiao and Ryan, 2008). Because of its zoonotic potential and intra-species diversity subtyping tools have been developed for *C. parvum*. The most widely used subtyping method is DNA analysis of the 60 kDa glycoprotein (GP60) gene (Xiao, 2010).

Beef and dairy cattle are among the most economically important agricultural production systems in Argentina. In 2005, there were ~56 million head of cattle in this country maintained on 213,706 farms. Argentina is divided into seven agricultural regions: Central, Cuyo, Border, Finishing, Mesopotamia, North West and Patagonia (Aznar et al., 2011). The central region territory, which includes Buenos Aires province, has 30 million head of cattle and the highest human population density in Argentina (INDEC, 2010).

Molecular characterizations of *C. parvum* at subtype level in cattle, important for establishing identification for epidemiological purposes, have been conducted in several countries (Xiao et al., 2007; Santín et al., 2008; Brook et al., 2009; Misic and Abe, 2007; Silverlås et al., 2010, 2013). But in Argentina data are scarce with only two studies reporting *C. parvum* subtypes in dairy cattle. The first study found subtype IIaA21G1R1 in a calf (Del Coco et al., 2012). The second study reported 6 subtypes (IIaA17G1R1, IIaA18G1R1, IIaA20G1R1, IIaA21G1R1, IIaA22G1R1 and IIaA23G1R1) from 45 calves (Tomazic et al., 2013). Because all other reports of cryptosporidiosis in cattle in Argentina are based on microscopy (Bellinzoni et al., 1990; Del Coco et al., 2008; Tiranti et al., 2011), understanding of the potential public health significance of zoonotic *C. parvum* infection in Argentina remains unclear and constitutes an impediment to developing appropriate interventions. In this study, we molecularly characterized *C. parvum*

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isolates obtained from young cattle located on dairy farms in the province of Buenos Aires, Argentina.

A total of 73 *C. parvum* isolates from pre-weaned calves (<2 months of age) from dairy farms in 12 municipalities in Buenos Aires were studied (Table 1). Fecal samples were examined to establish their consistency (McAllister et al., 2005), and screened by microscopy for *Cryptosporidium* oocysts using an acid-fast staining technique. Then, oocysts were concentrated using a sucrose flotation protocol (Smith, 2008). DNA was isolated using a DNeasy Tissue Kit (Qiagen, Valencia, California) with modifications (Fayer et al., 2010). Presence of *C. parvum* was determined by nested PCR and sequencing of a SSU rRNA gene fragment (Santín et al., 2008).

To subtype *C. parvum*, a two step nested PCR protocol was performed to amplify a 450-bp fragment of the GP60 gene (Sulaiman et al., 2005). For primary and secondary PCR, the PCR mixture contained 1x PCR buffer, 3 mM $MgCl_2$, 0.2 mM Dntp, 2.5 U taq (Qbiogene, Irvine, California), 2.5 μl BSA (0.1 g/10 ml) and 1 μM of each forward and reverse primer in a 50 μl reaction volume. Each of 35 cycles consisted of 94C for 45 s, 50C for 45 s, and 72C for 1 min after an initial hot start at 94C for 5 min and ending with 72C for 10 min. PCR products were subjected to electrophoresis in a 1% agarose gel and visualized by staining the gel with ethidium bromide. PCR products were purified using Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT™) (USB Corporation, Cleveland, Ohio), and sequenced in both directions using the same secondary PCR primers in 10 μl reactions using Big Dye™ chemistries and an ABI3130 sequencer analyzer (Applied Biosystems, Foster City, California). Sequence chromatograms of each strand were aligned and examined with Lasergene software (DNASTAR, Inc., Madison, Wisconsin). *C. parvum* subtypes were determined by sequence analysis of the GP60 gene and named using the protocol previously described (Sulaiman et al., 2005). This nomenclature is based on the number of TCA (designated by the letter A), TCG (designated by the letter G) and ACATCA (designated by the letter R) repeats in the microsatellite region. When a mixed infection within a specimen was suspected from the sequence traces, the PCR products of GP60 were cloned using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, California) and transformants were selected from each specimen and screened by PCR, and sequenced in both directions as described above using M13 forward and reverse primers. Up to eight clones from each specimen were sequenced. Sequences obtained in the present study were deposited in the GenBank database under accession numbers KJ158747-KJ158753.

Statistical analysis was performed using SPSS (Statistical Package for Social Science) for Windows, version 11.5, 2002. Differences among proportions were calculated by Chi-Square Test for values of $P < 0.05$.

Seventy-five sequences of the GP60 gene were identified from the 73 *C. parvum* specimens examined. Seven subtypes were identified (Table 1), all belonging to the zoonotic family IIa which is the most commonly reported in cattle and humans worldwide (Xiao, 2010). Subtypes within family IIa differ from each other by the number of trinucleotide repeats (TCA or TCG) coding for the amino acid serine, in addition subtypes have one or two copies of the sequence ACATCA immediately after the trinucleotide repeats. All subtypes found in this study have only one copy of the sequence ACATCA (R1) immediately after the trinucleotide repeats and a single copy of the trinucleotide TCG (G1) with a variable number of copies of the TCA trinucleotide repeat (A16, A18, A19, A20, A21, A22, and A23) (Table 1). Two calves from two different locations showed mixed infection with subtypes IIaA21G1R1 and IIaA22G1R1 (Table 1). Concurrent infections with multiples subtypes have already been described in cattle isolates (Xiao et al., 2007).

Five of the 7 subtypes identified in this study (IIaA18G1R1, IIaA20G1R1, IIaA21G1R1, IIaA22G1R1 and IIaA23G1R1) have been previously detected in cattle in Argentina (Del Coco et al., 2012; Tomazic et al., 2013). Identification of subtypes IIaA16G1R1 and IIaA19G1R1 represents the first time those subtypes have been found in Argentina. The results indicate a substantial genetic diversity in Buenos Aires, similar to reports from Sweden (Silverlås et al., 2010, 2013), Czech Republic (Kváč et al., 2011), Australia (Nolan et al., 2009), and Canada (Trotz-Williams et al., 2006). However, it is interesting that subtype IIA15G2R1, the overwhelmingly dominant subtype in cattle in most studies worldwide (Feng et al., 2013), has not been found in Argentina.

The most common subtype in this study, IIaA20G1R1, was previously reported in Serbia and Montenegro (Misic and Abe, 2007), Sweden (Silverlås et al., 2013), and Argentina (Tomazic et al., 2013). But in none of those studies was IIaA20G1R1 the most common subtype. The most prevalent subtypes identified in those studies were IIA21G1R1 in Argentina, and IIA16G1R1 in Serbia, Montenegro, and Sweden.

The second most commonly subtype (IIaA22G1R1) was indeed the most widely distributed (identified in 9 of 12 locations). Subtype IIaA22G1R1 has also been reported in Germany (Broglia et al., 2008), Czech Republic (Kváč et al., 2011), Sweden (Silverlås et al., 2013), and Argentina (Tomazic et al., 2013). But, in none of those studies was IIaA22G1R1 a common subtype.

Table 1
Cryptosporidium parvum GP60 subtypes identified in dairy cattle from Buenos Aires province, Argentina.

Municipalities	No. of *Cryptosporidium parvum* positive samples	No. of GP60 sequences obtained	*C. parvum* subtypes identified (Number found)
Tandil	20	20	IIaA18G1R1 (4) IIaA20G1R1 (6) IIaA21G1R1(4) IIaA22G1R1 (2) IIaA23G1R1 (4)
Tres Arroyos	3	3	IIaA18G1R1 (1) IIaA20G1R1 (1) IIaA22G1R1 (1)
Bolivar	2	2	IIaA22G1R1 (2)
General Alvarado	1	1	IIaA22G1R1 (1)
General Lamadrid	11	12	IIaA16G1R1 (4) IIaA18G1R1 (1) IIaA20G1R1 (5) IIaA21G1R1 (1) IIaA22G1R1 (1)
Trenque Launquen	4	5	IIaA21G1R1 (1) IIaA22G1R1 (4)
Chavez	3	3	IIaA18G1R1 (3)
Benito Juarez	3	3	IIaA20G1R1 (1) IIaA21G1R1 (2)
Azul	15	15	IIaA18G1R1 (3) IIaA19G1R1 (1) IIaA20G1R1 (7) IIaA21G1R1 (2) IIaA22G1R1 (2)
Lobería	1	1	IIaA18G1R1 (1)
Rauch	6	6	IIaA20G1R1 (4) IIaA22G1R1 (2)
Olavarría	4	4	IIaA20G1R1 (3) IIaA22G1R1 (1)
Total	73	75	IIaA16G1R1 (4) IIaA18G1R1 (13) IIaA19G1R1 (1) IIaA20G1R1 (27) IIaA21G1R1 (10) IIaA22G1R1 (16) IIaA23G1R1 (4)