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Multilocus fragment analysis of *Cryptosporidium parvum* from pre-weaned calves in Colombia

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ABSTRACT

The intra-species genetic diversity of *Cryptosporidium parvum* in dairy cattle farms in the central area of Colombia was investigated using a multilocus fragment typing approach with nine variable-number tandem-repeat (VNTR) loci and the *gp60* gene. Genomic DNA of 70 *C. parvum* isolates from pre-weaned calves in 32 farms was analysed. Most markers showed two (ML1, MSB, CP47, and MSC6-7) or three alleles (5B12, Cgd2_3850, and Cgd6_5400), although they exhibited a major allele accounting for more than 69% of specimens, which explains their low discriminatory index. The TP14 microsatellite was monomorphic while a total of six alleles were found at the ML2 microsatellite. The two novel allelic variants (219bp, 245bp) exhibited by more than 36% of specimens at the latter locus were a remarkable finding. The 10-markers typing tool provided a Hunter-Gaston discriminatory value of 0.940 (95% CI, 0.918 – 0.961) and differentiated 22 multilocus subtypes (MLTs). Nevertheless, the combination of the three most informative markers (ML2, *gp60*, and Cgd2_3850) differentiated 68% of MLTs and hardly impaired the discriminatory index. The fact that many MLTs (13/22) were distinctive for individual farms provides evidence for the endemic nature of the infection and the major role played by transmission within farms. The eBURST algorithm suggested a low degree of genetic divergence. All but three MLTs were clustered in a clonal complex with a star-like topology typical of clonal expansion, however linkage analysis did not find evidence of linkage disequilibrium. Bayesian analysis also identified a genetic structure with $K = 3$ being the best estimation of ancestral clusters, although a large proportion of isolates (35%) could not be allocated to a single population, which indicates their mixed origin. The results confirm the genetic distinctiveness of *C. parvum* in cattle farms in this geographical area. This is the first multilocus analysis on the intra-specific variability of *Cryptosporidium* from calves in South America.

1. Introduction

Molecular tools are essential in unravelling the identity and transmission dynamics of *Cryptosporidium* isolates circulating in human and animal populations. This protozoan is a major contributor in diarrhoeal disease in humans and livestock worldwide, particularly cattle (Khan et al., 2018). Thirty-one species have been reported so far, based on sequencing of the small-subunit (SSU) *rRNA* gene which is considered the most reliable locus for identification of *Cryptosporidium* spp. Two species are responsible for the majority of human infections, including the anthroponotic *C. hominis* and the zoonotic *C. parvum* (Ryan et al., 2016). The latter is also one of the most prevalent enteric pathogens associated with neonatal calf diarrhoea, which highlights the economic significance and public health impact of cryptosporidiosis in cattle farms (Thomson et al., 2017).

Subtyping studies have showed a remarkable genetic diversity within *C. parvum* isolates, with the presence of human-specific, animal-specific and zoonotic subtypes. Fourteen allelic families (IIa → IIo) have been identified by phylogenetic analysis of the 60-KDa glycoprotein (*gp60*) gene, as well as several subtypes within each family. Some families, notably IIc, have only been identified in human cases, but others such as IId and especially IIa, are usually identified in both human and animal infections; remarkably, there is a high prevalence of subtype IIaA15G2R1 in humans and cattle in Europe, North America and

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Table 1
Primers and conditions of the PCR reactions for the amplification of diverse microsatellites and minisatellites.

Locus	GenBank Acces no.	Primer sequence (5' → 3')	Annealing temp (°C)	Fragment size range (bp)	Reference
ML1	G35348	F1: CATGAGCTAAAAATGGTGG	55	218-242	(Cacciò et al., 2000; Chalmers et al., 2005)
		F2: CTAAAAATGGTGGAGAATATTC			
		R: HEX-CAACAAAATCTATATCCTC	50		
ML2	AF344880	F: CAATGTAAGTTTACTTATGATTAT	50	180-237	(Cacciò et al., 2001)
		R: FAM-CGACTATAAAGATGAGAGAAG			
TP14	XM627041	F1: TAATGCCCACCCATCTTCTT	61	279-333	(Mallon et al., 2003; Quílez et al., 2011)
		R1: TCCATCTGGGTCCATTTAGC			
		F2: CTAACGTTCACAGCCAACAGTACC	62		
		R2: FAM-GTACAGCTCCTGTTCCTGTTG			
5B12	AQ449854	F: TGACGATGAAGATGAGGGAAC	60	134-155	(Quílez et al., 2011)
		R: HEX-CAGGACAGATTTAGGAGGAGGA			
CP47	AF384127	F1: GCTTAGATTCTGATATGGATCTAT	43	417-479	(Gatei et al., 2007)
		R1: AGCTTACTGGTCCTGTATCAGTT	55		
		F2: ACCCCAGAAGGCGGACCAAGGTT			
		R2: FAM-GTATCGTGGCGTTCTGAATTATCAA			
MSB	XM627997	F: GGGAGGCATAGGGATGA	59	246-324	(Tanriverdi and Widmer, 2006)
		R: TAMRA-CTTTTGATCGCTTCTTTTCCA			
MSC6-7	BX538350	F1: ATTGAACAAACGCCGCAAATGTACA	55	517-570	(Gatei et al., 2007)
		R1:CGATTATCTCAATATTGGCTGTTATTGC	55		
		F2: GCTATTTGCTATCGTCTCACATAACT			
		R2: TAMRA-CTACTGAATCTGATCTTGCATCAAGT			
cgd2_3850	XM626569	F1: ATTGAAGATTGCGGATGATGGGGTT	70	151-205	(Ramo et al., 2016a).
		R1: TGGAGCGCCAAGTGCTGAAGA			
		F2: ATTTGCTGTTGCAACTGGTG	61		
		R2: TAMRA-GCCAAGTGCTGAAGAAGAGG			
cgd6_5400	XM627858	F: TAATCTTTGCGTGGGACCTC	60	251-312	(Ramo et al., 2016a).
		R1: GTGACTTGAATGACCCAGGA			
		R2: HEX-TGGAGTTTCTGAGACACAAAGA	59		

Australia (Ryan et al., 2014). Another class of highly polymorphic genetic markers characterized by allelic variability in repeat length are microsatellites and minisatellites, also known as short variable-number tandem-repeat (VNTR) loci. These markers are being increasingly used in multilocus schemes to investigate the intra-species diversity of *Cryptosporidium* spp. Multilocus methods improve resolution over analysis using a single locus, which underestimates genetic diversity where sexual reproduction occurs (Robinson and Chalmers, 2012). Nevertheless, a standardised multilocus scheme is not currently available (Chalmers et al., 2018).

Cryptosporidium has been recognised as a cause of diarrhoea in suckling calves in some South American countries (Del Coco et al., 2008; Meireles, 2010; Díaz-Lee et al., 2011; Pulido-Medellín et al., 2014; Holsback et al., 2018). However, the impact of bovine cryptosporidiosis in this continent is not well documented and data on the genetic diversity of *C. parvum* are limited. Modest numbers of specimens from calves have been characterised by *gp60* sequencing in Brazil, Argentina or Chile (Meireles et al., 2011; Paz e Silva et al., 2013; Tomazic et al., 2013; Del Coco et al., 2014; do Couto et al., 2014; Heckler et al., 2015; Mercado et al., 2015; Toledo et al., 2017) and no previous work with VNTR markers has so far been conducted. In Colombia, the single genotyping study reporting the distribution of *Cryptosporidium* species and *gp60* subtypes among pre-weaned calves highlighted this protozoan as a common and widespread pathogen in the central area of the country (Avendaño et al., 2018). Moreover, the latter study revealed the genetic distinctiveness of *C. parvum* in this geographical area, with the presence of eight subtypes within the IIa family, but the overwhelming predominance of an unusual subtype (IIaA18G5R1) previously unreported in natural infections in human or animal hosts. The genetic polymorphisms of *C. parvum* isolates from the latter contribution have been further characterised in the current study. For this purpose, a panel of nine VNTR markers was characterised by fragment length analysis, and the results were combined with the *gp60* subtype. Multilocus subtypes were also used to explore the population structure of *C. parvum* in cattle farms in this area of Colombia.

2. Materials and methods

2.1. Cryptosporidium isolates

Genomic DNA of 70 *C. parvum* isolates from a previous study was used in this molecular analysis (Avendaño et al., 2018). These isolates were collected between 2010 and 2012 from naturally infected diarrhoeic (n: 25) and non-diarrhoeic (n: 45) calves younger than 35 days. The calves were from 32 dairy cattle farms located in four Departments in the central area of Colombia: Antioquia (n: 1), Boyacá (n: 4), Cundinamarca (n: 26) and Meta (n: 1). One isolate from each of 16 farms and 2 to 6 isolates from each of the remaining 16 farms were used. Calves were maintained under semi-extensive feeding conditions. *Cryptosporidium* species and *C. parvum gp60* subtypes in the previous study were determined based on a PCR-restriction fragment length polymorphism (RFLP) and sequence analyses of the *SSU-rRNA* and *gp60* genes, respectively (Xiao et al., 2001; Alves et al., 2003; Feng et al., 2007).

2.2. Multilocus fragment typing

An automated capillary electrophoresis (CE)-based DNA fragment analysis tool was used to categorize each isolate at nine VNTR markers, including five microsatellite (ML1, ML2, TP14, 5B12, CP47) and four minisatellite (MSB, MSC6-7, cgd2_3850, cgd6_5400) loci. The fragments were amplified using single (ML2, 5B12, MSB), heminested (ML1, cgd6_5400) and nested (TP14, CP47, MSC6-7, cgd2_3850) PCRs, with primers and conditions previously described (Gatei et al., 2007; Quílez et al., 2011; Ramo et al., 2016a). Reverse primers labelled with HEX (4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein), FAM (6-carboxyfluorescein), or TAMRA (6-carboxytetra-methylrhodamine) were used to allocate alleles with overlapping peaks to a specific locus. The primers used for PCR analysis of all gene targets, the annealing temperatures used, and the sizes of the expected PCR products are listed in Table 1.

According to the amplicon intensity, 0.5-to-2 µl samples of the micro- and minisatellite-labelled PCR products for each *C. parvum* isolate were mixed and subjected to CE on a 3500xL Genetic Analyzer and sized automatically using the GeneScan 600 Liz Size Standard (Applied Biosystems, Life Technologies). Data were stored and analysed with the aid of Gene Mapper software (version 4.1) to determine fragment sizes. At least two representative isolates for each allele were amplified using unlabelled primers and the above-mentioned PCR conditions, and they were subsequently analysed by bidirectional sequencing for length confirmation. Allele nomenclature was based on the fragment size (in base pairs) adjusted after comparison with sequence analysis of these representative isolates. Alleles were translated into numbers for multilocus analyses.

2.3. Multilocus subtype identification

The alleles at all nine VNTR loci were combined with the *gp60* subtype to provide the multilocus subtype (MLT) for each isolate. Each MLT was then assigned a number. The identification of two alleles at a single locus was designated a mixed infection, and the two possible MLTs were considered. The Hunter-Gaston discriminatory index (HGDI) and 95% confidence intervals were calculated for each VNTR locus and the multilocus analysis. For this purpose, the VNTR diversity and confidence extractor software (V-DICE) available at the Health Protection agency bioinformatics tools website were used (http://www.hpa-bioinfotools.org.uk/cgi-bin/DICI/DICI.pl) (Hunter and Gaston, 1988).

2.4. Data analysis

The evolutionary relationships of the isolates was analysed by the eBURST algorithm (http://eburst.mlst.net/). Clonal complexes were defined as clusters of closely related MLTs that differ from one another at one locus [single locus variants (SLVs)]. The MLTs that were not clustered in any clonal complex were classified as singletons (Feil et al., 2004). Allelic linkage disequilibrium (LD) among different loci was assessed by measuring the standardised index of association (I_A^S) using the software LIAN v. 3.7 (http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl) (Haubold and Hudson, 2000). The population structure was determined using the software STRUCTURE v. 2.3 (http://pritchardlab.stanford.edu/structure.html). This program utilises a Bayesian algorithm in order to identify distinct sub-populations and determine fractions of the MLT for each isolate belonging to each subpopulation (Pritchard et al., 2000). The most probable number of ancestral populations was defined by the *K* value in accordance with previous reports (Evanno et al., 2005).

2.5. Nucleotide sequence accession numbers

Representative nucleotide sequences generated in the current study were deposited in the GenBank database under accession numbers MG924432 to MG924435, MG924439 to MG924446 and MG924450.

3. Results

3.1. Allelic diversity

The numbers and sizes of alleles and the HGDI values identified at each VNTR locus are summarised in Table 2. The analysis of allelic variation revealed that most loci exhibited two (ML1, MSB, CP47, and MSC6-7) or three (5B12, Cgd2_3850, and Cgd6_5400) alleles and the TP14 microsatellite was monomorphic. A major allele was exhibited by more than 69% of isolates at all these markers, with a single locus (Cgd2_3850) displaying a HDGI value higher than 0.450. In contrast, a total of six alleles more evenly distributed were identified at the ML2 microsatellite, which explains the highest HGDI value (0.822) exhibited by this marker. This discriminatory index was higher than that reported for *gp60* sequencing, which differentiated a total of eight subtypes in a previous study with the same stock of samples. Sequencing of representative isolates for each allele revealed novel allelic variants at the ML2 (219 and 245bp), 5B12 (173bp) and Cgd2_3850 (169bp) loci.

Table 2
Microsatellites and minisatellites alleles identified using capillary electrophoresis in *C. parvum* isolates from calves in Colombia. The adjusted size after sequencing (base pairs) and number assigned to each allele for multilocus analysis is indicated.

Locus Alelle (bp) (assigned number)	Nº of isolates (%) (n: 70)[a]	Nº of farms (n: 32)
ML1 [HGDI = 0.089 (0.000 – 0.182)][b]		
226 (2)	3 (4.6)	3
238 (1)	62 (95.4)	24
ML2 [HGDI = 0.822 (0.802 – 0.842)]		
219 (2) [d]	11 (18)	5
231 (1)	15 (24.6)	5
233 (3)	11 (18)	6
235 (6)	2 (3.3)	2
237 (4)	11 (18)	8
245 (5) [d]	11 (18)	4
TP14 [HGDI = 0.000 (0.000 – 0.101)]		
324 (1)	67 (100)	30
MSB [HGDI = 0.031 (0.000 – 0.089)]		
316 (2)	1 (1.5)	1
322 (1)	64 (98.5)	28
CP47 [HGDI = 0.239 (0.119 – 0.360)][c]		
417 (1)	55 (85.9)	25
420 (2)	7 (10.9)	5
417 + 420	2 (3.1)	2
MSC6-7 [HGDI = 0.058 (0.000 – 0.134)]		
516 (2)	1 (1.5)	1
549 (1)	64 (97)	29
516 + 549	1 (1.5)	1
5B12 [HGDI = 0.223 (0.098 – 0.347)]		
167 (2)	7 (10.9)	6
169 (1)	56 (87.5)	24
173 (3) [d]	1 (1.6)	1
Cgd2_3850 [HGDI = 0.459 (0.352 – 0.566)]		
169 (3) [d]	4 (6.1)	1
193 (1)	46 (69.7)	21
199 (2)	15 (22.7)	10
193 + 199	1 (1.5)	1
Cgd6_5400 [HGDI = 0.190 (0.072 – 0.309)]		
271 (3)	1 (1.6)	1
277 (1)	56 (88.9)	24
283 (2)	1 (1.6)	1
277 + 283	5 (7.9)	1
GP60 [HGDI = 0.506 (0.363 – 0.650)]		
IIaA15G2R1 (1)	5 (7.6)	3
IIaA16G2R1 (2)	3 (4.5)	1
IIaA17G4R1 (3)	2 (3)	1
IIaA18G5R1 (4)	46 (69.7)	22
IIaA19G6R1 (5)	2 (3)	2
IIaA20G5R1 (6)	3 (4.5)	3
IIaA20G6R1 (7)	4 (6.1)	1
IIaA20G7R1 (8)	1 (1.5)	1

[a] Not all 70 *C. parvum* isolates could be typed at every loci.
[b] Hunter-Gaston Discriminatory Index (index [confidence interval 95%]).
[c] CP47 alleles were identified as IA29G10 (417bp) and IIA30G10 (420bp) by sequencing, in accordance with the nomenclature proposed by Gatei et al. (2007).
[d] Alleles not described previously.

3.2. Multilocus subtypes

The multilocus subtype (MLT) of each isolate was determined based on the combination of alleles at the nine mini- and microsatellite loci and the *gp60* subtype. A total of 61 isolates from 26 farms had complete allele data for all ten loci and 22 MLTs were identified (Table 3). The two most prevalent MLTs (13 and 15) were identified in ten specimens and four farms. Nine isolates showing a biallelic profile at a single locus

Table 3
Multilocus subtypes identified in *C. parvum* isolates from calves in Colombia, based on the combination of nine microsatellites/minisatellites and the *gp60* locus.

MLT	Alleles identified at each locus[a]										Nº of isolates[b] (n: 61)	Nº of farms (n: 26)
	TP14	MSB	MSC 6-7	ML1	cgd6_5400	5B12	CP47	cgd2_3850	ML2	GP60		
1	1	1	1	1	1	2	1	2	4	6	3	3
2	1	1	1	1	1	2	1	2	4	8	1	1
3	1	1	1	1	1	2	2	1	2	4	3	2
4	1	1	1	1	1	1	1	1	1	2	3	1
5	1	1	1	1	1	1	1	1	1	4	3	1
6	1	1	1	1	1	1	1	1	3	1	3	1
7	1	1	1	1	1	1	1	1	3	3	2	1
8	1	1	1	1	1	1	1	1	3	4	4	3
9	1	1	1	1	1	1	1	1	6	4	1	1
10	1	1	1	1	1	1	1	1	4	4	5	3
11	1	1	1	1	1	1	1	1	4	5	2	2
12	1	1	1	1	1	1	1	1	5	1	1	1
13	1	1	1	1	1	1	1	1	5	4	10	4
14	1	1	1	1	1	1	1	1	2	4	2	2
15	1	1	1	1	1	1	1	2	1	4	10	4
16	1	1	1	1	1	1	1	3	2	7	4	1
17	1	1	1	1	1	1	2	1	3	4	1	1
18	1	1	1	1	1	1	2	1	6	4	1	1
19	1	1	1	1	1	1	2	1	2	4	4	3
20	1	1	1	1	2	1	1	1	5	4	5	1
21	1	1	1	1	3	1	1	1	3	1	1	1
22	1	1	2	1	1	1	1	1	5	1	1	1

[a] The number assigned to each allele is shown in Table 2.
[b] Only alleles that amplified at all ten markers were used for the multilocus analysis. Samples revealing mixed infection at a specific marker were unfolded and assigned to the corresponding MLT.

(CP47, cgd6_5400, cgd2_3850 and MSC6-7) were scored as having two potential MLTs; five of these isolates belonged to the same farm and showed a biallelic profile at locus Cgd6_5400. Numerous MLTs (13/22) were distinctive for individual farms. The remaining nine MLTs were simultaneously identified in two or more farms. A comparison between MLTs and the previously reported subtypes determined by *gp60* sequencing showed that each subtype corresponded to a single MLT, with the exception of subtypes IIaA15G2R1 and IIaA18G5R1. The multilocus analyses differentiated additional allelic variants in specimens subtyped as IIaA15G2R1 (4 MLTs) and IIaA18G5R1 (12 MLTs). The HGDI value of the ten-satellite tool was 0.940 (95% CI, 0.918 – 0.961). The inclusion of the three most discriminatory loci (ML2, *gp60* and Cgd2_3850) in the multilocus analysis differentiated a total of 15 MLTs and provided a similar value (HGDI: 0.904; 95% CI, 0.873–0.936).

3.3. Population analysis

The eBURST analysis revealed two clonal complexes and one singleton, with the main cluster containing all but three MLTs. The second clonal complex was formed only by two MLTs differing in the *gp60* subtype (Fig. 1). The linkage analyses of all specimens as a single population suggested a predominantly panmictic structure within the *C. parvum* population in this geographical area. Evidence of linkage disequilibrium (LE) was not found since the pairwise variance (V_D: 1.3196) was lower than the 95% critical value (L: 1.3221), although the P value almost reached statistical significance (I_As: 0.0186; P: 0.056). Values remained not significant when counting only once all repeated MLTs (I_As: 0.0051; V_D: 1.2365; L: 1.6017; P: 0.378). The results of analysis with STRUCTURE indicated that the most likely number of clusters was three ($K = 3$). Two thirds of isolates (65.7%) were considered to belong to any of the three clusters since the probability of

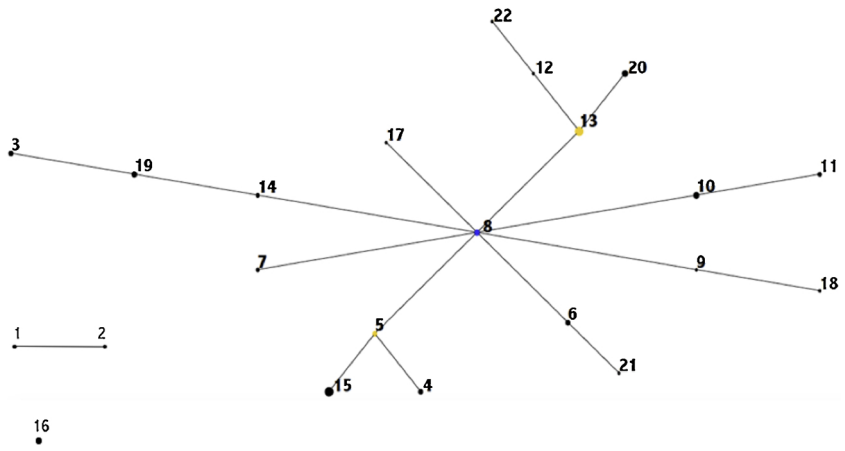

Fig. 1. Relationships among 22 multilocus subtypes (MLTs) of *Cryptosporidium parvum* identified in calves using the eBURST algorithm. Each circle represents a MLT; the size is proportional to the number of isolates. MLTs related by single-locus variants are linked by lines. The allelic profile of each MLT is indicated in Table 3.

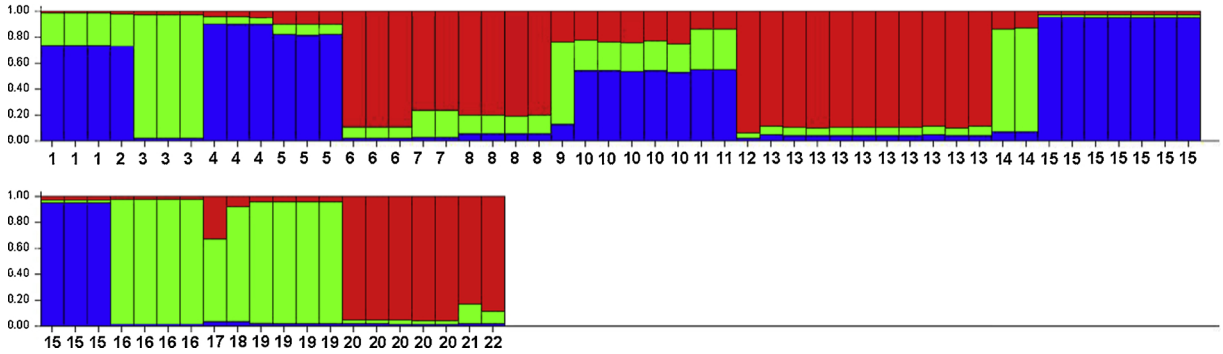

Fig. 2. Bayesian analysis of 61 *C. parvum* isolates from calves in Colombia as inferred by STRUCTURE. The bar plot shows the most probable number of ancestral clusters ($K = 3$) represented by different colours. Each bar represents an isolate. The colours within the bar reflect the isolate's estimated proportion of membership (shown on the y-axis) in that cluster. The MLT number for each isolate is shown in the x-axis. The allelic profile of each MLT is indicated in Table 3. Analyses were conducted on allelic data at VNTR loci and the GP60 subtype.

belonging to them was higher than 0.8. The remaining isolates could not be allocated to a single population and were considered to have a mixed ancestry (Fig. 2).

4. Discussion

Multilocus genotyping is more informative than the analysis of a single locus in exploring the intra-species diversity of *Cryptosporidium*, given the occurrence of genetic recombination in the sexual phase of the protozoan life cycle. Combinations with more than 55 VNTR loci have been used to date and recommendations on the validation of markers for a multilocus scheme have been proposed, although no standardised panel has been universally adopted so far (Robinson and Chalmers, 2012; Hotchkiss et al., 2015; Pérez-Cordón et al., 2016; Chalmers et al., 2017, 2018). A review of multilocus studies with *C. hominis* and *C. parvum* revealed that different sets of markers are required for typing each species (Robinson and Chalmers, 2012). Studies with *C. parvum* from domestic ruminants indicated that differences also apply to host factors, with some loci being much more useful for analysing isolates from either calves or lambs (Ramo et al., 2016a,b).

The genetic diversity or *C. parvum* in cattle is not well documented in South America. Previous studies based on sequence analysis of the *gp60* gene, which is the most popular marker for *Cryptosporidium* subtyping, have shown the genetic richness of the protozoan in this area. A wide range of subtypes belonging to a single *gp60* allelic family have been reported in Brazil (IIaA14G2R1, IIaA14G2R2, IIaA15G2R1, IIaA16G3R2, IIaA17G2R1, IIaA18G1R1, IIaA18G2R2, IIaA19G2R1, IIaA19G2R2, IIaA20G1R1; IIaA20G2R1, IIaA20G2R2) (Meireles et al., 2011; Paz e Silva et al., 2013; do Couto et al., 2014; Heckler et al., 2015; Toledo et al., 2017), Argentina (IIaA16G1R1, IIaA17G1R1, IIaA18G1R1, IIaA19G1R1, IIaA20G1R1, IIaA21G1R1, IIaA22G1R1, IIaA23G1R1) (Tomazic et al., 2013; Del Coco et al., 2014) and Chile (IIaA15G4R1, IIaA16G4R1, IIaA17G4R1) (Mercado et al., 2015). The fact that many of these subtypes were novel allelic variants or were not shared among different countries has been related to the geographic isolation of the protozoan in cattle farms (Avendaño et al., 2018). The latter authors conducted the first large study of *Cryptosporidium* species and subtypes in Colombia, which revealed the distinct identity of *C. parvum* circulating in calves. A rare subtype, IIaA18G5R1, was by far the most prevalent and widely distributed in the study, and three novel subtypes were found (IIaA19G6R1, IIaA20G6R1 and IIaA20G7R1).

Isolates from the above-mentioned investigation have been further analysed in the present study using a panel of nine VNTR markers, which were selected based on their resolution ability for typing *C. parvum* from calves (Ramo et al., 2016a). None of the VNTR loci exceeded the genetic variability of the *gp60* locus (8 alleles). Seven markers were either biallelic (ML1, MSB, CP47, MSC6-7) or triallelic (5B12, cgd6_5400, cgd2_3850) but exhibited a major allele accounting for more than 69% of specimens, which explains their low discriminatory index. It is worth mentioning that no variation was found at the TP14 microsatellite, which is considered a relevant locus in multilocus studies in Europe and the United States (Quílez et al., 2011, 2013; Herges et al., 2012; Hotchkiss et al., 2015). This marker was required to achieve 95% of MLTs in previous studies with either *C. parvum* or *C. hominis* (Robinson and Chalmers, 2012). The 324bp fragment exhibited by all isolates in this study was also the predominant allele found in calves in northern Spain (Ramo et al., 2016a). Nevertheless, the latter authors reported two additional allelic variants (333bp and 342bp) that increased the HDGI value to 0.663, which indicates that geographical factors should also be considered when selecting the most informative loci.

The greatest number of alleles was detected at the ML2 locus (6 alleles), which provided a HGDI value even higher than *gp60* sequencing. This microsatellite has been reported to be among the most polymorphic markers for typing *C. parvum* from humans, pre-weaned livestock or waterborne outbreaks in Europe (Cacciò et al., 2001; Hunter et al., 2007, 2008; Quílez et al., 2011, 2013; Díaz et al., 2012). The most prevalent allele at this marker (ML2-231) was also the most common allelic variant in calves in Spain (Quílez et al., 2011; Díaz et al., 2012; Ramo et al., 2016a). Nevertheless, more than 36% of specimens exhibited two novel allelic variants 219bp and 245bp in length, which further supports the genetic uniqueness of *C. parvum* in cattle farms in this geographical area.

The combination of nine VNTR markers with the *gp60* subtype provided a more robust analysis than *gp60* alone, and identified 22 multilocus subtypes within 61 isolates. Most MLTs (16/22) were differentiated within the two most prevalent subtypes (IIaA15G2R1 and IIaA18G5R1), thereby supporting the usefulness of multilocus typing as compared to sequencing of the *gp60* gene. It is significant to note that the combination of only the three most informative markers (ML2, *gp60* and Cgd2_3850) differentiated 68% of MLTs and hardly impaired the discriminatory index, showing a marker redundancy that has been reported in most multilocus schemes (Robinson and Chalmers, 2012). Likewise, the exclusion of *gp60* and ML2 loci dramatically reduced the numbers of MLTs (n = 9), indicating that both markers contributed the most to the discriminatory power of the multilocus approach.

The genetic diversity found in our study is similar to that reported among 118 calves in the United Kingdom using a six-loci approach (23 MLTs) (Hotchkiss et al., 2015), but much lower to that detected in Ireland (78 MLTs) or Spain (70 MLTs) using panels of seven and twelve markers, respectively (de Waele et al., 2013; Ramo et al., 2016a). Many MLTs (13/22) were confined to individual farms, a finding that has been related to the endemic nature of infection and intensive farming practices with limited exchange of animals between herds (Tanriverdi and Widmer, 2006; Quílez et al., 2011; Drumo et al., 2012; Ramo et al., 2016a). In the current study, dairy calves in most farms (31/32) were

reared under a semi-extensive system, but the introduction of new animals occurred rarely. Most herds breed their own replacement females rather than buying young heifers, suggesting that *Cryptosporidium* infection is mainly transmitted within cattle farms.

Analyses of *C. parvum* populations have shown a significant diversity in terms of the role of genetic exchange, with differences at a local geographical level that could determine the degree of recombination (Tanriverdi et al., 2008). Evidence of these variations has been reported in Scotland, with panmictic, epidemic or clonal subpopulations among specimens from humans and livestock (Mallon et al., 2003; Morrison et al., 2008). The *C. parvum* population structure ranged from basic clonality (humans) to epidemic clonality (livestock) in France, and a predominant pattern of clonality was found in Italy (Ngouanesavanh et al., 2006; Drumo et al., 2012). In contrast, an overall panmitic structure was reported in cattle farms and/or humans in the Upper Midwest of the United States, Ireland and northern Spain (Herges et al., 2012; de Waele et al., 2013; Ramo et al., 2016a).

In the current study, the analysis with the eBURST algorithm showed that all but three MLTs were linked by SLVs, suggesting a low degree of genetic divergence. The distinctive identity of the remaining three MLTs was associated with the *gp60* gene, since all these specimens belonged to any of the three subtypes with 20 TCA repeats in the trinucleotide region. MLTs in the main clonal complex formed a network with a star-like structure typical of clonal expansion, but linkage analysis did not find evidence of linkage disequilibrium. Nevertheless, the *P*-value was close to the statistical significance at the 5% level, advising subsequent studies with more exhaustive sampling. This observation was supported by an analysis using STRUCTURE, which indicated that the population structure is best explicated by $K = 3$ ancestral types, but a large proportion of isolates (35%) could not be assigned to only one of these three populations as they showed mixed ancestry.

To the best of our knowledge, this is the first multilocus analysis on the intra-specific variability of *Cryptosporidium* from calves in South America. The results reveal that this fragment analysis approach based on the combination of ten markers is a useful tool for strain typing and epidemiological tracking, but should be optimized by selecting the most informative markers at a local level in order to improve both the cost effectiveness and the time involved in testing. Our findings also confirm the distinctiveness of *C. parvum* infection in cattle farms in this geographical area and indicate a moderate genetic diversity. Further investigations with a larger sample set of specimens from this and other areas should be conducted to better understand the genetic diversity of the protozoan in South America.

Declarations of interest

None.

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