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Original

Detection and molecular characterization of *Cryptosporidium* spp. in dairy calves from the Valdivia province, Chile

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ABSTRACT

Objective. Detect acid-fast oocysts of Cryptosporidium spp. using the Ziehl-Neelsen stain (ZN) and apply molecular tools with the 18S rARN (18S rARN) and 60 kDa glycoprotein (gp60) genes to characterize the species and subtypes present in Valdivia province. **Material and methods**. 275 samples of calves from 15 dairy farms from the Valdivia province were analyzed by ZN stain. Samples that showed positive results to ZN were then submitted to PCR processing aiming as target the 18S rARN and gp60 genes, with subsequent sequencing of the latter. **Results**. 30 samples were positive to ZN, of which 46.6% (14/30) were confirmed by PCR amplification for 18S rARN and from those, 42.8% (6/14) also amplified the gp60 target using PCR. Posterior sequencing of this gene showed the presence of C. parvum in 100% of the isolates and the subtypes IIaA15G2R1 (3/6), IIaA14G1R1 (2/6) and IIaA17G1R1 (1/6). **Conclusions.** Subtype IIaA15G2R1 was predominant, being found within two communes of the province. The subtype IIaA14G1R1 was found in two farms belonging to the same commune and to our knowledge represents the first time this subtype has ever been reported in cattle in Chile and the continent. Finally, subtype IIaA17G2R1 has been found sporadically in calves just like in the present study. Furthermore, all three variants have already been reported infecting humans, however during the development of this study, we found no other records of studies that aimed the characterization of the subtypes of C. parvum from calves raising systems and workers, to have ever been conducted in Chile. Therefore, it is important to promote further investigation on this subject.

Keywords: Apicomplexa; calves; *Cryptosporidium parvum*; polymerase chain reaction (*Source: MeSH, USDA*).

RESUMEN

Objetivo. Detectar ooquistes ácido-alcohol resistentes de *Cryptosporidium* spp. en terneros mediante tinción Ziehl-Neelsen (ZN) y caracterizar molecularmente las especies y subtipos presentes en la

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provincia de Valdivia, a través del uso de herramientas moleculares con los genes 18*S rARN* y *gp*60 que codifica una glicoproteína de 60 kDa. **Materiales y métodos**. 275 muestras de heces de terneros pertenecientes a 15 predios lecheros de la provincia de Valdivia, fueron analizadas mediante tinción de ZN. Las muestras positivas a ZN, se analizaron por PCR mediante la amplificación de una región de los genes 18*S rARN* y *gp*60, con posterior secuenciación. **Resultados**. 30 muestras resultaron positivas a ZN, de las cuales el 46.6% (14/30) logró ser confirmado mediante PCR para 18*S rARN* y de estas, el 42.8% (6/14) fueron positivas a la PCR para *gp*60. La secuenciación arrojó la presencia de *C. parvum* en el 100% de las muestras y los subtipos IIaA15G2R1 (3/6), IIaA14G1R1 (2/6) y IIaA17G1R1 (1/6). **Conclusiones**. El subtipo IIaA15G2R1 predominó, encontrándose en dos comunas de la provincia. El subtipo IIaA14G1R1 fue encontrado en dos predios de una misma comuna y su hallazgo es el primero en bovinos en Chile y el continente. Finalmente, el subtipo IIaA17G2R1 ha sido encontrado esporádicamente en terneros, al igual que en esta investigación. Las tres variantes han sido reportadas en humanos, sin embargo, no hay registros de estudios que caractericen los subtipos de *C. parvum* en sistemas de crianza de terneros y trabajadores simultáneamente en Chile, por consiguiente, se debe indagar en el tema.

Palabras clave: Apicomplexa; terneros; *Cryptosporidium parvum*; reacción en cadena de la polimerasa (*Fuentes: MeSH, USDA*).

INTRODUCTION

Since the discovery of the genus *Cryptosporidium* by Ernest Tyzzer in 1910, this parasite has been considered a protozoan agent of importance for veterinary medicine, over human medicine (1). The genus *Cryptosporidium* has a worldwide distribution, affecting a wide range of animals including mammals, notably bovines, which are commonly affected by four species, *C. andersoni, C. ryanae, C. bovis* and *C. parvum*. The last three are considered potentially zoonotic, with *C. parvum* being a highly prevalent species in dairy farms (2,3).

Infection and cryptoporidiosis caused by *C. parvum* in dairy herds mainly affect younger animals (0 - 21 days of age), causing high morbidity and occasional mortality, but they also carry a number of productive, economic, and sanitary problems for the affected livestock (4). The infective forms (sporulated oocysts) of this protozoan agent are usually found in soil, food, and drinking water that has been contaminated with fecal matter from infected animals, which are then subsequently consumed by a susceptible host. On the other hand, cryptosporidiosis is also considered a public health problem, where people related to the livestock sector (farmers, veterinarians and workers) could also get infected through direct contact with parasitized animals or their fecal matter during their normal job routines if biosecurity and hygiene measures are neglected (4).

Currently, routine clinical diagnostic techniques for the detection of *C. parvum* are insufficient to clarify the possible route of transmission used by this agent in a disease outbreak (5). Therefore, molecular epidemiology of *C. parvum* can play an important role in the study of bovine cryptosporidiosis, developing genomics and molecular biology tools over time that allow us to unveil more information about this parasitic agent (6). The incorporation of research techniques such as the use of the Polymerase Chain Reaction (PCR) and variations, and the subsequent sequencing of the PCR products obtained from the amplification of the 18S rRNA and the gp60 gene that encodes the 60 kDa glycoprotein (gp60), allowed the genotyping and subtyping of different members of the genus *Cryptosporidium*, elucidating important epidemiological data such as the source of origin and transmission routes involved (6,7).

In Chile, few studies have managed to characterize -using molecular techniques- the species and subtypes of the genus *Cryptosporidium* present in dairy herds in the southern regions of the country, with the aim of improving knowledge on the epidemiology of bovine cryptosporidiosis at the local level. Therefore, the objective of the present research was to detect acid-fast oocysts belonging to the genus *Cryptosporidium* in dairy cattle from farms located in the Valdivia province, also determining the species and subtypes involved.

MATERIAL AND METHODS

Samples recollection. Fifteen dairy farms located in the Valdivia province (Los Ríos region, southern Chile) were visited between May 2015 and April 2017. Fecal samples were obtained by direct rectal palpation of calves under 2 months of age showing signs of diarrhea. The feces were then preserved in 70% ethanol and kept at refrigeration temperature (4–6°C) (8). A code corresponding to the borough of origin was assigned to each sampled farm (Eg: Mr: Mariquina; Mf: Máfil; Pl: Paillaco; LL: Los Lagos; V: Valdivia)

Oocyst recovery, Ziehl-Neelsen (ZN) staining and qualitative determination of the intensity of infection due to *Cryptosporidium* sp. Five g of fecal matter per sampled animal were weighed and then sieved with the aid of a fine metal mesh with an approximate pore of 36 µm, in order to separate the coarse material from the rest of the stool. The resulting screened samples were then introduced into 250 mL beakers and allowed to settle for 15 minutes. Once the sample settled, the supernatant was discarded, and the obtained sediment was transferred into a glass tube where 1 mL of petroleum ether was added to the sample. After homogenization, the samples were centrifuged at 216 x g for 5 minutes to recover and concentrate the oocysts from the sediment.

The resulting purified and concentrated samples were then subjected to ZN acid-fast staining as described by Muñoz et al (9, 10). Subsequently, the intensity of infection was then determined through the observation of 10 fields of 1000X chosen at random based on the following criteria proposed by Muñoz et al (10): Very low (+): 1-3 oocysts per field, Low (++): 4-10 oocysts per field, Intermediate (+++): 11-20 oocysts per field, High (++++): more than 20 oocysts per field. This classification was assigned after calculating the average number of *Cryptosporidium* sp. oocysts observed in the 10 fields of the sample. All positive samples were stored in 1.5 mL microcentrifuge tubes with 70% ethanol at a freezing temperature of –80° C, as suggested by Lalonde and Gajadhar (8).

DNA Extraction. The frozen microcentrifuge tubes containing the preserved positive samples were thawed at room temperature and 150 µL of purified sample per tube were obtained for the DNA extraction process. A specific kit for fecal matter (Quick-DNATM Fecal / Soil Microbe Microprep, Zymo Research®, Irvine, California) was used for this process following the manufacturer's guidelines. The obtained DNA was then quantified by spectrophotometry and stored at -80°C until subsequent molecular analysis.

PCR amplification of the 18S rRNA gene and endogenous bovine Cytochrome b gene (Cytb) (housekeeping). For the amplification of the 18S rRNA gene using conventional PCR, 49 µL of master mix (1 µL of each primer at 20 ng/µL; 5 µL of 250 mM dNTP mix; 6 µL of 25 mM MgCl; 1 Go Taq G2 Flexi DNA Polymerase unit; 10 µL of Go Taq Flexi Green Buffer; and sufficient nuclease-free water to fill the remaining volume) and 1 µL of genomic DNA from the sample were used. The primers utilized were genus-specific for *Cryptosporidium* spp. and corresponded to: Foward 5'-GTTAAGTATAAACCCCTTTACAAGTATC-3' and reverse 5'-CCTCCAATCTCTAGTTGGC-3' with a resulting 522 bp PCR product (9). The temperature cycle consisted of an initial denaturation performed at 95°C for 5 minutes, then 40 cycles at 95°C for 30 seconds, 52°C for 30 seconds, and 68°C for 30 seconds. After the thermal cycle completion, the mixture was then cooled to 4°C, loading 15 µL of PCR product on 2% agarose gel for electrophoresis (9).

As a PCR inhibition control reaction, a segment of the Bovine Cytb gene was used. Master mix and DNA samples were used under the same proportion of each component as previously described for the amplification of the 18S rRNA gene. However, the primers used were the following: Citbf 5'-ATCATTTTGAGGTGCAACCG-3 'and Citbr 5'-TCCTAACAGGTCAGGTGAGA-3', with an expected PCR product of ~ 140 bp approximately.

Amplification of the gp60 gene encoding the 60 kDa glycoprotein (gp60). Subsequently, conventional PCR amplification of the gp60 gene was carried out on the samples that previously amplified the 18S rRNA gene, using 47.5 µL of master mix (1 µL of each primer at 20 ng/µL; 5 µL of 250 mM dNTP mixture; 6 µL of 25 mM MgCl; 1 unit of Go Taq G2 Flexi DNA Polymerase; 10 µL of Go Taq Flexi Green Buffer and the remainder in nuclease-free water) and 2.5 µL of warm sample DNA making up a 50 µL total reaction. This was done to identify the corresponding subtype present (5, 11), using the following primers: forward gp15-ATG 5'-ATGAGATTGTCGCTCATTATC-3 'and reverse gp15-STOP 5'-TTACAACACGAATAAGGCTGC-3. As

a result, 1000 bp PCR products were obtained. The temperature cycling protocol for this amplification step included 35 cycles at 95°C for 30 seconds, 50°C for 30 seconds, and 68°C for 1 minute, ending the cycle with a holding temperature of 14°C (11). The PCR products (15 µL) obtained were separated using a 2% agarose gel electrophoresis.

Purification of PCR products, sequencing and phylogenetic analysis. The amplicons were selected and extracted directly from 2% agarose gel to be subjected to purification using a kit for that sample matrix (Promega®, Madison, Wisconsin). As a result, approximately 50 µL of purified eluted DNA were obtained, from which 2 µL were extracted for quantification by spectral scanning UV-VIS spectrophotometry.

The sequencing was performed using the di-deoxyterminal method. The analysis of the obtained sequences and phylogeny was performed using Chromas Pro2.1.5 software. (Technelysium®, South Brisbane, Australia) and MEGA7. Subtyping was done as described by Sulaiman et al (12).

The Neighbor-Joining and Maximum Likelihood methods were used, applying 1000 replications for each phylogenetic construction. Twenty-two sequences of the gp60 gene described in bovines by different authors around the world, 2 outgroups (*C. meleagridis* and *C. hominis*) and the sequences obtained in this study were the basis for this construction.

RESULTS

From the total of 275 analyzed fecal samples obtained, 11% (30/275) showed at least one *Cryptosporidium* spp. oocyst, from which 50% (15/30) presented a very low (+) infection intensity, 36.6% (11/30) low (++), 6.7% (2/30) intermediate (+++), and finally 6.7% (2/30) showed high infection intensity (+ +++) (Figure 1).

The borough of Paillaco showed the highest number of samples with a very low intensity of oocysts. On the other hand, the results for the borough of Valdivia showed no evidence for this agent presence. Samples with intermediate intensity were only observed in the borough of Paillaco, while samples with high intensity were detected in the Mariquina and Máfil boroughs. In the case of the farms that belonged to the boroughs of Paillaco and Los Lagos, oocysts of *Cryptosporidium* spp. were observed only in a single farm per borough, meanwhile, in Mariquina and Máfil, 66.6% (2/3) and 50% (4/8) of the farms in each borough were affected by this parasite, respectively (Table 1 and Figure 2).

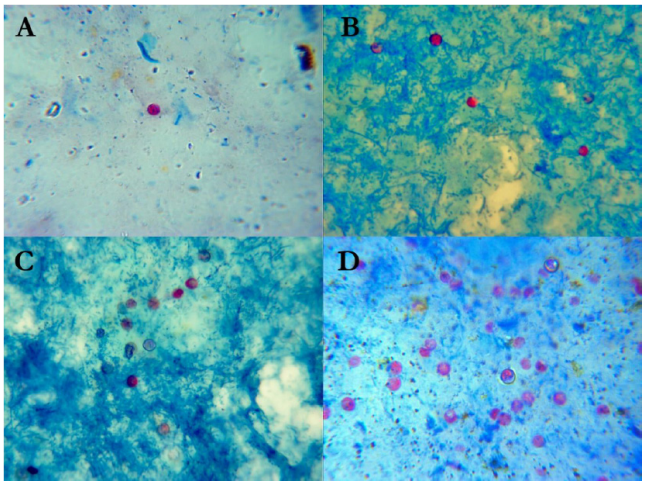

Figure 1. 1000x observation fields, where different intensities of infection are observed according to the amount of *Cryptosporidium* spp. acid-fast oocysts. (A = very low intensity, B = low intensity, C = intermediate intensity and D = high intensity).

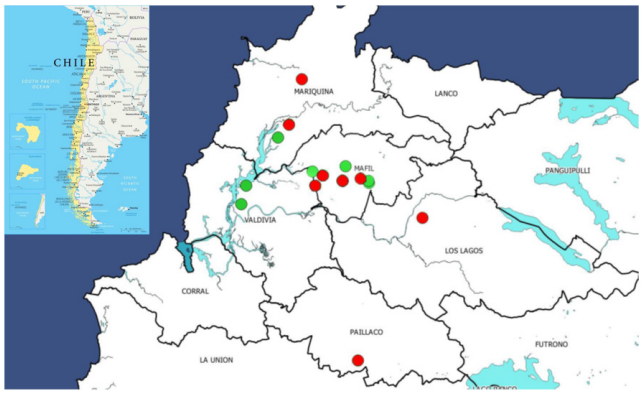

Figure 2. Map indicating the sampled farms (n = 15) and the presence of *Cryptosporidium* spp. in each one of them. The "green" colored circles indicate the absence of the parasite in the corresponding farm and the "red" colored circles represent the presence of it (Elaborated in QGIS 2.18).

Table 1. Frequency of samples according to the borough, farm, and intensity of infection by *Cryptosporidium* spp. in fecal matter samples from dairy calves in the Valdivia Province between the years 2015 – 2017.

Cod	Absent	Intensity of infection			
		+	++	+++	++++
Mariquina	**40**	**2**	**0**	**0**	**1**
Mr1 (19)	17	2	0	0	0
Mr2 (7)	7	0	0	0	0
Mr3 (17)	16	0	0	0	
Máfil	**132**	**3**	**5**	**0**	**1**
Mf1 (22)	22	0	0	0	0
Mf2 (6)	6	0	0	0	0
Mf3 (51)	51	0	0	0	0
Mf4 (8)	8	0	0	0	0
Mf5 (25)	24	1	0	0	0
Mf6 (7)	4	2	1	0	0
Mf7 (8)	7	0	1	0	0
Mf8 (14)	10	0	3	0	1
Paillaco	**25**	**7**	**5**	**2**	**0**
Pl1 (39)	25	7	5	2	**0**
Los Lagos	**24**	**3**	**1**	**0**	**0**
Ll1 (28)	24	3	1	0	0
Valdivia	**24**	**0**	**0**	**0**	**0**
V1 (7)	7	**0**	**0**	**0**	**0**
V2 (17)	17	**0**	**0**	**0**	**0**
Total	**245**	**15**	**11**	**2**	**2**

Cod: Borough Cod. (number of farms)

The result of the DNA extraction process was quantified, showing the presence of DNA in all samples. PCR was performed on the ZN staining positive samples, of which 46.6% (14/30) amplified the expected PCR product for the 18S rRNA gene (Figure 3).

The samples that did not show the expected PCR product to the amplification of the 18S rRNA gene were subjected to an inhibition control, subjecting the samples to a conventional PCR for the bovine endogenous Cytb gene, where it was elucidated that 75% (12/16) of these were inhibited at the time of performing the technique for the aforementioned gene (Figure 4).

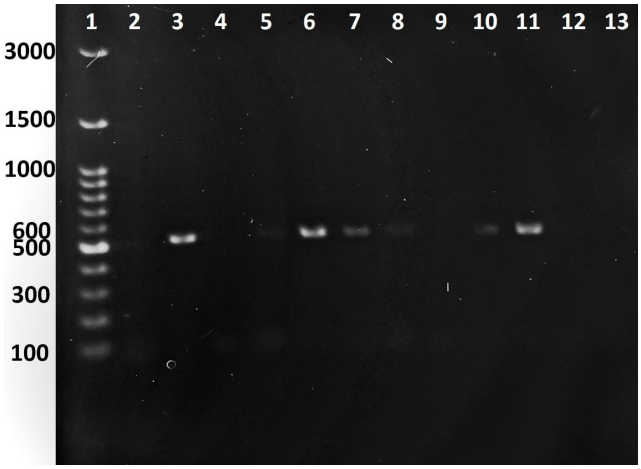

Figure 3. 2% agarose gel electrophoresis. PCR for 18S rRNA gene from samples positive for *Cryptosporidium* spp. by ZN. Lane 1. 100 bp molecular weight marker; Lane 2. Negative control corresponding to "mastermix" without DNA; Lane 3. Positive control corresponding to *C. parvum* sample confirmed by previous sequencing; Lanes 4-12. ZN positive samples; Lane 13. Negative control corresponding to "mastermix" without DNA. Lanes 5 to 8, 10 and 11 corresponds to positive samples.

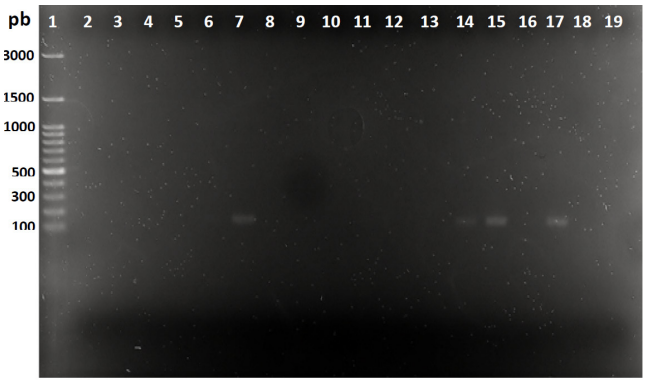

Figure 4. 2% agarose gel electrophoresis, PCR for the Cytb gene ("housekeeping") in samples negative to the 18S rRNA gene. Lane 1 contains a 100 bp molecular weight marker; Lane 2 Negative control corresponding to "mastermix" without DNA; Lanes 3-18 contain DNA samples that showed no amplification to the PCR round for the 18S rRNA gene; Lane 19 has a negative control corresponding to "mastermix" without DNA. Lanes 7, 14, 15 and 17 contain samples that amplified the Cytb gene based PCR.

The samples that generated a PCR product for the 18S rRNA gene were subjected to a second amplification, but this time targeting the gp60 gene. 42.8% (6/14) of the samples generated the expected amplicon (Figure 5).

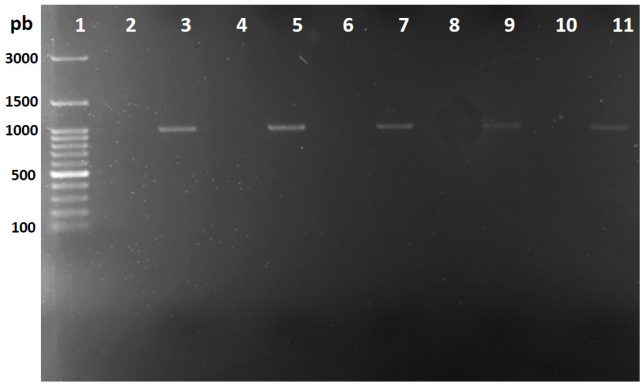

Figure 5. 2% agarose gel electrophoresis, PCR round for gp60 gene of the samples that previously amplified the 18S rRNA gene of *Cryptosporidium* spp. Lane 1 contains a molecular weight marker of 100 base pairs (bp), lane 2 contains negative control corresponding to "mastermix" without DNA, lane 3, 5, 7, 9 and 11 contain positive samples to the amplification of the gp60 gene and, finally, lanes 4, 6, 8 and 10 are empty.

All the samples that evidenced the presence of parasite DNA in the PCR for both target genes were purified and the genetic material present was quantified. In this process, three samples of the PCR product of the 18S rRNA gene did not yield conclusive results at the time of sequencing, therefore, only 78.5% (11/14) successfully completed the process.

The aligned sequences showed 99–100% identity with other *Cryptosporidium* sequences, specifically with the *C. parvum* species, with an "e-value" equal to 0.00. On the other hand, as a result of subtyping using the gp60 gene segment, the subtypes IIa A15G2R1 (3/6), IIa A14G1R1 (2/6) and IIa A17G2R1 (1/6) were obtained (Figure 6, Table 2).

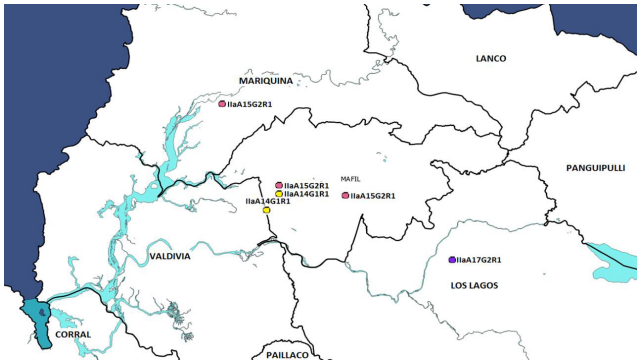

Figure 6. Valdivia province map, illustrating the geographic distribution of *Cryptosporidium parvum* subtypes found in the sampled dairy farms. Pink ovals (n=3) correspond to subtype IIaA15G2R1, yellow ovals (n=2) to IIaA14G1R1 and purple (n=1) ovals are indicative of the presence of subtype IIaA17G2R1 (Elaborated in QGIS 2.18).

The sequences obtained in the current study were grouped with the rest of the sequences of the allelic family IIa of bovine origin and yak (*Bos grunniens*), an animal member of the Bovidae family that inhabits the Himalayas. The clade that groups the parasites obtained in the Valdivia province was supported by a bootstrap value of 84% in the Neighbor-Joining method and 99% in the Maximum Likelihood method. In both trees generated, the sequences obtained are far from the subtypes belonging to the allelic family IId (Figure 7 and 8). The analysis carried out using the Maximum Likelihood method made it possible to divide the *C. parvum* IIa sequences obtained into two clades (bootstrap 99%), where those from Argentina and Sweden are grouped into a clade supported by a 52% bootstrap. Similarly, both phylogenetic analyzes are topographically similar.

Table 2. Results obtained according to each analysis: Ziehl-Neelsen (ZN), PCR gene 18S rRNA, gp60, and amplified gp60 segment sequencing (N = 275).

N	ZN (+ Samples/Total Samples)	18*S rARN* (+ Samples/Total Samples)	*gp*60 (+ Samples/Total Samples)	*gp*60 sequences (number of samples)
275	30/275	14/30	6/14	*C. parvum* IIaA15G2R1 (3) *C. parvum* IIaA14G1R1 (2) *C. parvum* IIaA17G2R1 (1)

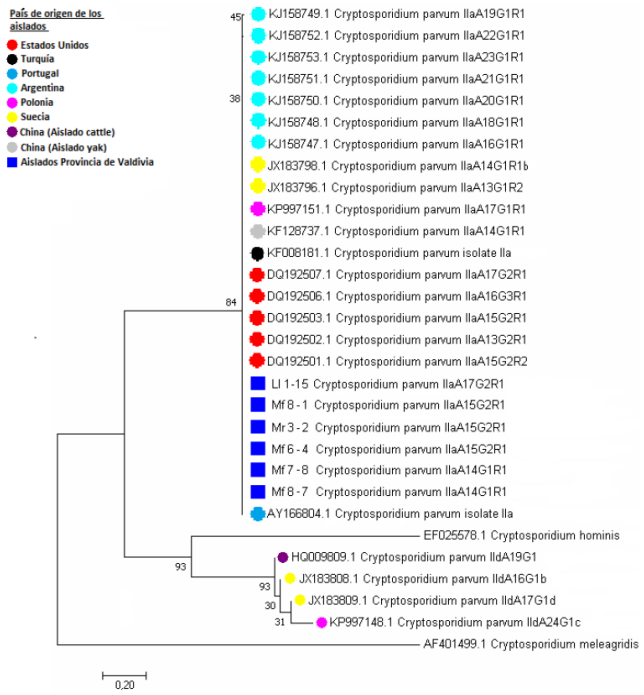

Figure 7. Phylogenetic analysis using the Neighbor-Joining method, between nucleotide sequences of the *Cryptosporidium parvum* gp60 gene from bovines inhabiting different parts of the world (GenBank) and from the present study.

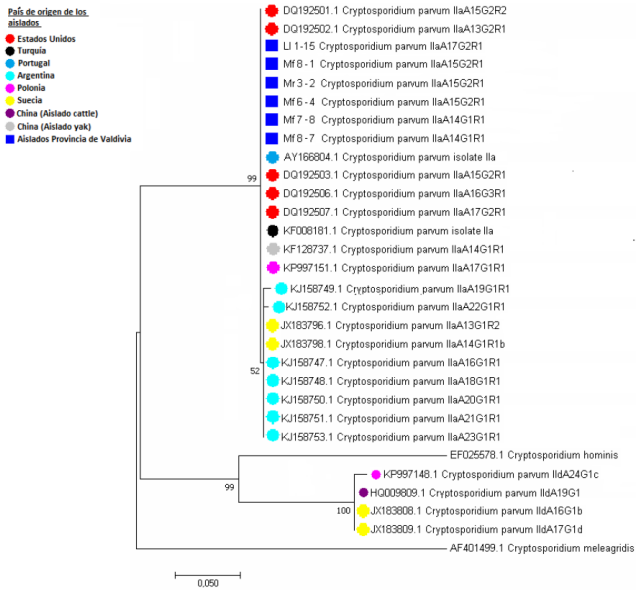

Figure 8. Phylogenetic analysis through the Maximum Likelihood method, based on the 2-parameter Kimura model, between nucleotide sequences of the *Cryptosporidium parvum* gp60 gene from bovines inhabiting different parts of the world (GenBank) and from the present study.

DISCUSSION

The percentage of samples from diarrheal animals positive for *Cryptosporidium* sp. using ZN staining (11%, 30/275) was lower than those reported by previous studies conducted in Chile (9, 10), where 49.8% to 57.9% of parasitized animals were obtained from a total of 205 and 221 dairy calves in the Metropolitan and Los Ríos regions, respectively. The reason behind these notorious differences might be that farmers and veterinarians from the farms that joined this study had already implemented control and prevention measures intending to control diarrhea in calves, such as early separation from the mother, supervised and ensured colostrum consumption by the newly born calves, individual pens for the newborn calves, the adoption of a maximum number of animals per collective pen avoiding overcrowding, and the availability of active pharmacological principles (halofuginone) indicated for the treatment of bovine cryptosporidiosis, all of which have direct and indirect implications to reduce the transmission of this protozoan between individuals from the same farm. On the other hand, neonatal diarrhea in calves can have multiple origins and may suffer from variations that could depend on the geographical location of the farm, the size of the herd, and its internal management which leads to differences within the prevalences of bacterial, viral and / or parasitic agents (e.g. *Eimeria* sp.) that could be more or less prevalent in one area than another (13). Therefore, it is presumed that animals with diarrheal samples that turned out negative to ZN processing could be affected by a different infectious agent rather than *Cryptosporidium* sp. or even be due to a non-infectious origin, a possibility that was not evaluated in the present investigation.

From an analytical point of view, the sensitivity of ZN staining is variable since 8 and 22,813 oocysts / mL have been established as the lower detection limit in distilled water artificially inoculated with *Cryptosporidium* sp. oocysts and fecal matter from diarrheal calves, respectively (9, 14). Therefore, and considering the high proportion of individuals with very low (50%) and low (36.6%) oocysts burdens detected, it is possible to infer that we are missing a high number of false negative cases that were not detected by the initial screening.

As a result of the amplification of the 18S rRNA gene segment, it was possible to confirm the presence of genetic material corresponding to *Cryptosporidium* in 46.6% (14/30) of the ZN-positive samples, while the remaining 53.4% (16/30) did not generate the expected PCR product. A similar situation was observed in the study carried out by Ghaffari et al (15), where a very important proportion of 41.1% (14/34) of the samples initially submitted to the Auramine Phenol technique and later to 18S rRNA PCR showed inhibition. This could be explained by the degradation or low concentrations of the parasite DNA within the sample or to the inhibition of the PCR, which could be explained by the presence of high concentrations of organic (bile salts) or inorganic inhibitors such as calcium and phytic acid, the latter being one of the most important since it corresponds to a natural component of cereal and oilseed seeds commonly used for animal consumption, maintaining constant levels of this acid at the gastronintestinal level (16, 17). This clearly demonstrates the importance of the nature of the sample when extracting the DNA, where the presence of inhibitors can skew the results and cause the appearance of false negative results.

To evaluate the presence of PCR inhibitors, a segment of the bovine Cytb gene was amplified. This additional process evidenced that 75% (12/16) of the negative 18S rRNA gene PCR samples may have suffered inhibition of the reaction. In turn, it was observed that the samples that were not inhibited in the Cytb gene PCR (25%; 4/16) had very low or low infection intensities, therefore, it could be inferred that the amount of oocysts/mL of fecal matter contained within the original samples could be so low that their genetic material was possibly not sufficient to be detected in the first PCR reaction for the 18S rRNA gene. This was verified by Muñoz et al (9), who determined that the detection limit of this molecular technique is approximately 11,406 oocysts / mL of diarrheal calf feces. Consequently, it is presumed that the samples processed in the present study showed a lower amount of oocysts when compared to the described detection limit.

According to the results obtained by the 18S rRNA gene PCR, the amplification of the gene segment was possible on 46.6% (14/30) of the samples. Only 11 samples had a monospecific infection by *C. parvum* which was determined by sequencing and alignment of the amplicons obtained. This agrees with other studies on pre-weaning calves from around the world, which classify the species *C. parvum* as the predominant species of the genus at this age stage (3). In Estonia, a study by Santoro et al (18) determined prevalences of 95.5% from a total of 224 samples with positive results to the presence of *C. parvum*, where the remaining 3.6% and 0.9% corresponded to *C. bovis* and *C. ryanae*, respectively. A study by Kváč et al (19) in the Czech Republic also showed a high prevalence of 87.2% (159/182) of *C. parvum* in calves up to 2 months of age. It is worth mentioning that in both studies a segment of the 18S rRNA gene was used as the target gene, similar to the present study. On the other hand, the unique presence of *C. parvum* in the studied animals was probably influenced by the age of the selected animals, since the species *C. ryanae*, *C. bovis* and *C. andersoni* are common in animals belonging to older age groups (3.20).

Only six samples amplified the PCR product for gp60 which corresponds to 42.8% of the samples positive for the 18S rRNA gene (6/14), showing a discrepancy in the number of samples that generated a PCR product for both molecular markers. This difference has also been presented in other studies (21) and is related to the exchange of genes that can occur during the gametogonia phase (sexual reproduction) between different populations of *C. parvum* present within the same host, being able to generate genetic recombinations in their gene sequence (22).

With the subtyping of *C. parvum* through the gp60 gene, it was determined that all the subtypes found belonged to the allelic family IIa, distributed in three boroughs of the province of Valdivia. This family is widely identified worldwide, both in cattle and humans, due to its zoonotic character (23,24). The IIaA15G2R1 subtype was found in 3/6 samples from three farms located in two different boroughs (Mariquina and Mafil), while the IIaA14G1R1 (2/6) and IIaA17G2R1 (1/6) subtypes were found in a single borough, Máfil and Los Lagos, respectively. This variation in the regional or national distribution of the subtypes has been previously analyzed, this is the case of Ng et al (25) who described the distribution of the *C. parvum* subtypes in Australia where they studied six dairy farms located in two states (Western Australia and New South Wales), reporting that the IIaA17G2R1 variant was widely located in the only farm studied in the state of New South Wales in 16 individuals, whereas in Western Australian farms it was only found in one isolate. The IIaA15G2R1

subtype found in the present study is the most prevalent around the world to be found in calves from dairy production systems (5,19,26,27), and was observed in three different farms from our region. The isolated IIaA17G2R1 subtype has been described sporadically (25), so it can be concluded that this subtype plays a minor role in the etiology of bovine cryptosporidiosis. Finally, the IIaA14G1R1 subtype observed twice (2/6) has not been previously reported in mammals from America, including domestic bovines. It has only been diagnosed in calves from dairy production systems in Sweden (28), in addition to another isolated report in China issued by Mi et al (29) who found this subtype in yak (Bos grunniens), a wild bovine species that inhabits the Himalayas that was domesticated for the production of milk, wool, meat, and bone.

Both variants IIaA15G2R1 and IIaA14G1R1 of *C. parvum* were located simultaneously in two farms belonging to the borough of Máfil (Figure 6). This result may be related to the transmission dynamics, which in turn is strongly rooted in the geographical conditions of the study area, where the present water bodies belong to the Valdivia river basin, originating smaller surface water sources (streams and estuaries and among others) that creates connections between the dairy farms present in the borough, forming a wider hydrographic network. This, combined with the mismanagement of the slurry produced by agricultural-livestock activities, allows microbiological communication due to the contamination of surface and even underground water bodies (30). Transport through surface water courses is useful for infectious forms of *Cryptosporidium*, which are resistant to freshwater conditions of rivers (1). Another form of transfer of microbiological agents such as *Cryptosporidium*, is the introduction of animals from infected farms to a susceptible one. This would imply that subtypes present in other local, national or foreign productive systems could expand to other herds free of this parasitic agent. However, the sampled farms had closed breeding systems, generating their own animal replacements without having to resort to buying animals elsewhere. Therefore, the detection of three different subtypes (IIaA15G2R1, IIaA14G1R1 and IIaA17G1R1) may be due to existing geographic segregation, and by reduced animal movement between farms in the area (31).

It is important to mention that the finding of two subtypes of *C. parvum* (IIaA15G2R1 and IIaA14G1R1) in the same farm could be due to the presence of a mixed infection within the sampled herd; however, massive cloning or sequencing techniques (NGS) (11, 32, 33) must be performed in order to identify the heterogeneity of the gp60 gene and the diversity of *C. parvum* subtypes present within the same host.

On the other hand, the three variants of *C. parvum* found have also been reported in humans, suggesting a possible zoonotic transmission. The IIaA15G2R1 subtype has been reported by Raehmouni et al (27) in stool samples of both human and farm cattle origin from a rural sector of Tunisia, highlighting a possible exchange of this subtype between the two species, which has also been supported by other authors with other subtypes (23). In the case of subtype IIaA17G2R1, its zoonotic transmission has been epidemiologically proven in an outbreak that occurred in a summer camp in North Carolina (United States), where individuals were infected by food intake and close contact with infected individuals, who also had contact with breeding calves in the place (34). The IIaA14G1R1 subtype has been diagnosed in three HIV-positive patients in Malaysia and one immunocompetent patient from Slovakia (35,36), however, its potential zoonotic (25,26) or anthropozoonotic (37) nature has not yet been determined due to the scarcity of studies indicating its simultaneous presence in humans and domestic animals in the same geographic area. In the present study, a lack of internal biosecurity standards was observed, such as the non-use of disposable gloves when handling animals, a practice that generates a high risk of worker exposure to the agent.

In conclusion, the ZN staining allowed to detect *Cryptosporidium* oocysts in 11% (30/275) of the samples from the georeferenced farms of the Valdivia province, collaborating with epidemiological studies of this parasite and its distribution within the province and also at the national level. Amplification of *Cryptospordium* 18S rRNA and gp60 genes was successful only in 46.6% and 42.8% from ZN-positive samples and from samples that generated a PCR product for the 18S rRNA gene, respectively, due to to the use of polymorphic genes as a target gene (such as the gp60 gene) and to the genetic recombination that these agents can carry out, among their different variants or populations (19, 32). On the other hand, through sequencing, the discovery of *C. parvum* and the subtypes IIaA15G2R1, IIaA17G2R1, and IIaA14G1R1 was

achieved. The description of a new subtype in South America (IIaA14G1R1) that affects dairy herds, sets a precedent for future studies of this nature that could be developed inside or outside the continent, in both domestic and wild species, and even in humans. This finding highlights the importance of elucidating the species and even more so, the subtypes present within a given area, since it allows to find the origin and possible transmission route that *Cryptosporidium* spp. (or *C. parvum* in this case) has taken, which helps to establish and create contingency plans during outbreaks of the disease in bovine rearing systems.

Conflict of interests

All authors declare that during the preparation and preparation of this work there was no conflict of interest.

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