Cryptosporidium parvum GP60 subtypes present in diarrheic dairy calves of two biogeographical regions of Chile

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Abstract

Background Cryptosporidium is an apicomplexan zoonotic pathogen primary causing diarrhea in vertebrate hosts notably bovines and humans. Here, we characterized Cryptosporidium isolates by using the GP60 gene fragment of C. parvum to observe the dynamics of cryptosporidiosis transmission in dairy calves from two distant biogeographical regions of Chile (Metropolitan and Los Rios Regions). We collected 72 fecal samples from diarrheic calves screening the parasite carried out microscopy of an acid-fast staining smear and molecular characterization employing PCR to directly detect the Sanger GP60 C. parvum subtype and simultaneously in one selected sample the NGS profile of the GP60 same gene fragment to determine same and/or others Cryptosporidium subtypes.

Results The IIaA15G2R1 subtype was present in the 100% of the bovine fecal samples studied from Los Rios Region. Along with this same subtype, another two were observed in the Metropolitan Region, IIaA17G2R1 and IIaA17G4R1. The NGS analysis of a single selected GP60 PCR amplicon of one selected sample of our study showed similarly the Sanger sequencing determined subtype, the IIaA17G4R1 in 90% of readable sequences observed. By using this approach another multiple low frequency IIa subtypes of C. parvum were observed confirming that in an infected host multiple subtypes of the parasite can be present.

Conclusions Cryptosporidiosis in these dairy farms calves in Chile is produced by C. parvum limited number of subtypes, being IIaA15G2R1 the most frequent. The IIa subtype family is considered prevalent in calves in South America. Subtypes IIaA17G2R1 and IIaA17G4R1 had been worldwide distribution. As all C. parvum subtypes observed in calves in Chile were isolated from diarrheic animals, so, it can be possible to relate its presence with the pathogenic role in the bovine host and with a potential digestive disease risk for humans.

Background

Cryptosporidium parvum (Protozoan, Apicomplexa) is the most important eukaryotic unicellular pathogen causing diarrhea in calves worldwide [1] and is one of the two leading causes of human cryptosporidiosis [2]. Acute diarrheic calves present lethargy, anorexia, fever accompanied by dehydration, collapse and death [3]. Furthermore, infection of dairy heifers results in less milk production due to nutrition complications such as nutrient malabsorption [4]. Bovine meat production is also impacted as cryptosporidiosis in pre-weaned calves results in lower average daily gain weight [5]. Cryptosporidium oocysts excreted by infected calves can contaminate the environment, facilitating transmission of the disease by fecal-oral route not only between animals but also to humans [6]. Indeed, cattle is the most important source of zoonotic Cryptosporidium [7]. Contaminated watersheds are an important source of Cryptosporidium infection to other animals [8] as well as to humans [9], and especially in developing countries where irrigation systems include rivers with scarce infrastructure for preventing fecal contamination [10]. Molecular identification of C. parvum isolates throughout GP60 based approach has been used widely to study the structure of the parasite populations and its dynamics of transmission in
calves [11]. The GP60 gene has nucleotide variation greater than the average in the genome of Cryptosporidium and its alleles are used to define groups (subtype families) among the different isolates [12]. Calves are frequently infected by the C. parvum IIa subtype family. A subtype, IlaA15G2R1 is considered highly pathogenic and is the most common infecting calves worldwide [13], meanwhile in Europe, Asia and Egypt the IId subtype family is mostly observed infecting these animals [14]. The main objective of the present work was to molecularly study the epidemiology of bovine cryptosporidiosis in Chile, by characterizing the GP60 subtypes of C. parvum infecting diarrheic dairy calves from two geographically distinct dairy zones.

Results

Fifty percent (50%) of the samples presented microscopically Cryptosporidium oocysts, 18 samples from MR and 18 samples from LRR. From these samples, the genus specific SSU-rDNA PCR for Cryptosporidium was positive in 29 isolates and only 15 (51.7%) were GP60 positive PCR, of which 5 were from MR and 10 from LLR. Three C. parvum subtypes belonging to Ila subtype family were observed in the MR: IlaA15G2R1, IlaA17G2R1 and IlaA17G4R1. In the LRR, the subtype IlaA15G2R1 was observed in the 100% of the bovines parasite samples (Table 1).

NGS analysis of a single selected DNA sample of our study showed similarly the predominant Sanger IlaA17G4R1 GP60 subtype in 90% of the readable sequences along with others less frequent subtypes (Table 2)

Discussion

Of the 29 SSU-rDNA PCR Cryptosporidium positive samples only 51.7% were positive to GP60. The GP60 gene has a unique copy in the Cryptosporidium genome [15] instead of SSU-rDNA gene that possess five copies [16] making it a less sensitive in a PCR assay. Pre-weaning cattle are the most susceptible to infection especially by C. parvum [17], but it has been observed other parasites species such as C. bovis, C. ryanae and C. andersoni that could explain the lower number of positive samples by PCR in relation to the microscopy morphological tests.

The GP60 amplicons were sequenced all belonging to Ila subtype family (Table 1). Interestingly, in the LRR, the subtype IlaA15G2R1 was observed in the 100% of the samples. C. parvum subtype Ila predominates in calves in South America, in countries such as Argentina, Colombia and Brazil [18–20]. In Chile, IlaA15G2R1 predominates in the 86.6% of the samples which agrees with data from other countries studies. Feng et al. (2013) [21] described that the IlaA15G2R1 subtype has a high rate of transmissibility as an adaptive characteristic. IlaA17G2R1 has also been described in cattle in Europe and USA. The subtype IlaA17G4R1 has also been observed in Colombia, from diarrheic calves [20].

Although subtype diversity was observed in the samples, the predominant subtype was IlaA15G2R1 in both geographical regions of Chile, suggesting its highly infective characteristic. Most of the infections in
neonatal diarrheic calves in LRR can be consequence of the biogeographic characteristics of the region, with large number of surface watercourses [22,23].

Interestingly, the NGS analysis of a single selected DNA sample of our study showed similarly the predominant I1aA17G4R1 GP60 subtype in 90% of the readable sequences along with others less frequent subtypes. This result is presented confirming by using the NGS approach that multiple subtypes of C. parvum are present naturally in an infected host as reported before [24].

Conclusions

A general conclusion is that in two different biogeographical regions of Chile, cryptosporidiosis in neonatal calves is caused by C. parvum of limited number of subtypes. The main parasite subtype is I1aA15G2R1, which is the subtype in cattle mostly reported worldwide. The presence of C. parvum in Chile is a potential risk of infection for humans, especially for dairy farm workers and veterinarians, who are in most contact with infected animals. This study contributes to a better understanding of the dynamics of cryptosporidiosis transmission in Chile also in South America and globally.

Methods

Thirty-six (36) diarrheic calves, less than 30 days old, from two dairy farms located in Melipilla and El Monte counties in the Metropolitan Region (MR) 33°27′S 70°40′W, were selected for fecal sample collection. Another similar set of 36 calves were studied from dairy farms located in Mariquina, Rio Bueno and Valdivia counties in the Los Rios Region (LRR) 39°48′50″S 73°14′45″W. Sampling was performed directly from the rectum of the animals using a 50 ml conical centrifuge tubes (Thermo Fisher Inc., Pittsburgh, PA, USA) and preserved in 70% ethanol until processing. Fecal samples were centrifuged at 1,500 x g for 5 min, aliquots of 1 ml of sedimented slurry transferred to 1,5 ml microcentrifuge tubes and stored at 4 °C. Samples were smeared on glass slides, stained with modified Ziehl-Neelsen (mZN) and examined under optic microscope at 100X magnification. DNA was extracted from the Cryptosporidium positive samples with a commercial kit (ZR Fecal DNA MiniPrep ®, Zymo Research, CA, USA) following the manufacturer's protocols.

All DNA samples were tested by PCR with SSU-rDNA Cryptosporidium specific primers [25] and COX1 bovine specific primers [26] to rule out PCR inhibitory activity. The DNA samples positive in both tests were then submitted to PCR for amplification of the GP60 gene, using 2.5 μl of extracted DNA and the primers gp15-ATG (5’ ATG AGA TTG TCG CTC ATT ATC 3’) and gp15-STOP (5’ TTA CAA CAC GAA TAA GG C TGC 3’) [15], resulting in an expected amplicon of about 1,000 bp. For determining the species and subtype family of each isolate, each consensus sequences were aligned using BLAST (Basic Local Alignment Search Tool) to sequences deposited in Genbank (NCBI). Sequences from each sample were subtyping by using the methodology proposed by Sulaiman et al. (2005) [27]. Next Generation Sequence (NGS) analysis of a single selected DNA sample were conducted in the Ion Torrent PGM platform using Ion 314™ Chip (Thermo Fisher, CA, US), using a third-party sequencing service. After filtering and quality
trimming, the resulting FASTA formatted sequences were analyzed with the FASTX toolkit integrated into the online data analysis platform Galaxy [28] for determining the number of TCA/TCG repeats determined using the collapse sequences option for parasite subtyping [27].

Declarations

Ethics approval and consent to participate

This protocol was approved by the Bioethics Advisory Committee of the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT), Santiago, Chile (N°018/FONDECYT/Medicina G2-G3/0499). Verbal consent was obtained from farms owners in previously studies for obtaining fecal samples used in this work.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interest.

Availability of data and materials

The datasets used and analyzed for this study are available from the corresponding author on reasonable request.

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Authors’ contributions

SP carried out the DNA isolation, performed PCR, bioinformatics analysis and drafted the manuscript. PM, ER and FF contributed to recollect part of the samples and revised the manuscript. LSO performed PCR, bioinformatics analysis and revised the manuscript. RM conceive the study and design, perform microscopy examination, carried out bioinformatics analysis and drafted the manuscript. All authors read and approved the final manuscript.
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Abbreviations

$GP60$: 60 kDa glycoprotein

$SSU-rDNA$: Small Subunit Ribosomal DNA

$PCR$: Polymerase Chain Reaction

$MR$: Metropolitan Region

$LRR$: Los Rios Region

$NGS$: Next Generation Sequencing

$mZN$: Modified Ziehl-Neelsen

$COX1$: Cyclooxygenase 1

$BLAST$: Basic Local Alignment Search Tool

$NCBI$: National Center for Biotechnology Information

References

1. Tsukano K, Fukuda T, Otsuka M, Nishi Y, Inoue H, Sarashina S, Suzuki K. Advantage of parenteral nutrition for diarrheic calves. J Vet Med Sci. 2018;80:1808–12.

2. Feng Y, Ryan UM, Xiao L. Genetic Diversity and Population Structure of Cryptosporidium. Trends Parasitol. 2018;34:997–1011.

3. Blanchard PC. Diagnostics of Dairy and Beef Cattle Diarrhea. Vet Clin North Am - Food Anim Pract. 2012;28:443–64.

4. Lorenz I, Fagan J, More SJ. Calf health from birth to weaning. II. Management of diarrhoea in preweaned calves. Ir Vet J. 2011;64:

5. Shivley CB, Lombard JE, Urie NJ, Koprul CA, Santin M, Earleywine TJ, Olson JD, Garry FB. Preweaned heifer management on US dairy operations: Part VI. Factors associated with average daily gain in preweaned dairy heifer calves. J Dairy Sci. 2018;101:9245–58.

6. Al Mawly J, Grinberg A, Prattley D, Moffat J, Marshall J, French N. Risk factors for neonatal calf diarrhoea and enteropathogen shedding in New Zealand dairy farms. Vet J. 2015;203:155–60.
7. Ryan U, Fayer R, Xiao L. Cryptosporidium species in humans and animals: current understanding and research needs. Parasitology 2014;141:1667–85.

8. Toledo RDS, Martins FDC, Ferreira FP, De Almeida JC, Ogawa L, Dos Santos HLEPL, Dos Santos MM, Pinheiro FA, Navarro IT, Garcia JL, Freire RL. Cryptosporidium spp. And Giardia spp. In feces and water and the associated exposure factors on dairy farms. PLOS ONE 2017;12:e0175311.

9. Mahon M, Doyle S. Waterborne outbreak of cryptosporidiosis in the South East of Ireland: weighing up the evidence. Ir J Med Sci. 2017;186:989–94.

10. Verbyla ME, Symonds EM, Kafle RC, Cairns MR, Iriarte M, Mercado-Guzman A, Coronaro O, Breitbart M, Ledo C, Mihelcic JR. Managing Microbial Risks from Indirect Wastewater Reuse for Irrigation in Urbanizing Watersheds. Environ Sci Technol. 2016;50:6803–13.

11. Rieux A, Paraud C, Pors I, Chartier C. Molecular characterization of Cryptosporidium isolates from pre-weaned calves in western France in relation to age. Vet Parasitol. 2013;197:7–12.

12. Abal-Fabeiro JL, Maside X, Bello X, Llovo J, Bartolome C. Multilocus patterns of genetic variation across Cryptosporidium species suggest balancing selection at the gp60 locus. Mol Ecol. 2013;22:4723–32.

13. Xiao L. Molecular epidemiology of cryptosporidiosis: An update. Exp Parasitol. 2010;124:80–9.

14. Mi R, Wang X, Huang Y, Zhou P, Liu Y, Chen Y, Chen J, Zhu W, Chen, Z. Prevalence and molecular characterization of Cryptosporidium in goats across four provincial level areas in China. PLOS ONE 2014;9:e111164.

15. Strong WB, Gut J, Nelson RG. Cloning and sequence analysis of a highly polymorphic Cryptosporidium parvum gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. Infect Immun. 2000;68:4117–34.

16. Le Blancq SM, Khramtsov N V, Zamani F, Upton SJ, Wu TW. Ribosomal RNA gene organization in Cryptosporidium parvum. Mol Biochem Parasitol. 1997;90:463–78.

17. Qi M, Wang H, Jing B, Wang D, Wang R, Zhang L. Occurrence and molecular identification of Cryptosporidium spp. in dairy calves in Xinjiang, Northwestern China. Vet Parasitol. 2015;212:404–7.

18. Del Coco VF, Cordoba MA, Bilbao G, de Almeida Castro AP, Basualdo JA, Fayer R, Santin M. Cryptosporidium parvum GP60 subtypes in dairy cattle from Buenos Aires, Argentina. Res Vet Sci. 2014;96:311–4.

19. Heckler RP, Borges DGL, Bacha FB, Onizuka MKV, Teruya LES, Neves JPL, Leal CRB, de Lemos RAA, Meireles MV, Borges FDA. First genetic identification of Cryptosporidium parvum subtype IlaA14G2R1 in beef cattle in Brazil. Prev Vet Med. 2015;121:391–4.

20. Avendano C, Ramo A, Vergara-Castiblanco C, Sanchez-Acedo C, Quilez J. Genetic uniqueness of Cryptosporidium parvum from dairy calves in Colombia. Parasitol. Res. 2018;117:1317–23.

21. Feng Y, Torres E, Li N, Wang L, Bowman D, Xiao L. Population genetic characterisation of dominant Cryptosporidium parvum subtype IlaA15G2R1. Int J Parasitol. 2013;43:1141–7.
22. Muñoz P, Mercado R, Morales G, Bravo V, Raffo E. Cryptosporidium spp., comparative diagnosis and geospatial distribution in diarrheic calves from dairy farms, Valdivia, Chile. Rev. MVZ Cordoba 2014;19:3954–61.

23. Wells B, Shaw H, Hotchkiss E, Gilray J, Ayton R, Green J, Katzer F, Wells A, Innes E. Prevalence, species identification and genotyping Cryptosporidium from livestock and deer in a catchment in the Cairngorms with a history of a contaminated public water supply. Parasit Vectors 2015;8:66.

24. Mercado R, Peña S, Ozaki LS, Fredes F, Godoy J. Multiple Cryptosporidium parvum subtypes detected in a unique isolate of a Chilean neonatal calf with diarrhea. Parasitol Res. 2015;114:1985–8.

25. Muñoz P, Fredes F, Díaz-Lee A, Mercado R, Ozaki L. Detección de Cryptosporidium spp. en terneras de lecherías de la Región Metropolitana mediante Ziehl Neelsen y confirmada por inmunocromatografía y ensayo molecular. Arch Med Vet. 2011;43:111–6.

26. Estrada-Chávez C, Otero FD, Díaz CA, Villegas-Sepúlveda N, González RP, Salazar DG. Concordancia de la PCR y métodos rutinarios para el diagnóstico de tuberculosis bovina. Vet Mex. 2004;35:225–36.

27. Sulaiman IM, Hira PR, Zhou L, Al-Ali FM, Al-Shelahi FA, Shweiki HM, Iqbal J, Khalid N, Xiao L. Unique endemicity of cryptosporidiosis in children in Kuwait. J Clin Microbiol. 2005;43:2805–9.

28. Blankenberg D, Gordon A, Von Kuster G, Coraor N, Taylor J, Nekrutenko A. Manipulation of FASTQ data with Galaxy. Bioinformatics 2010;26:1783–5.

Tables

Table 1: Frequency of GP60 subtypes found in the two regions and respectively counties of Chile.

| Region | County    | N° | Subtype          |
|--------|-----------|----|------------------|
| MR     | El Monte  | 2  | IIaA15G2R1       |
|        | El Monte  | 1  | IIaA17G2R1       |
|        | El Monte  | 1  | IIaA17G4R1       |
|        | Melipilla | 1  | IIaA15G2R1       |
| LRR    | Rio Bueno | 6  | IIaA15G2R1       |
|        | Valdivia  | 2  | IIaA15G2R1       |
|        | Mariquina | 2  | IIaA15G2R1       |
| Total  |           | 15 |                  |
Table 2: Subtype and frequency (parentheses) of the NGS study of a single selected sample. The occurrence of each allele is shown in terms of percentage of the 100% of the readable sequences analyzed.

| Subtype       | Frequency (%) |
|---------------|--------------|
| IIaA17G4R1    | 90.47%       |
| IIaA16G4R1    | 4.91%        |
| IIaA18G4R1    | 1.38%        |
| IIaA15G4R1    | 0.72%        |
| IIaA18G3R1    | 0.72%        |
| IIaA16G5R1    | 0.54%        |
| IIaA19G4R1    | 0.30%        |
| IIaA17G3R1    | 0.18%        |
| IIaA15G4R2    | 0.12%        |
| IIaA11G4R1    | 0.06%        |
| IIaA13G4R1    | 0.06%        |
| IIaA14G4R1    | 0.06%        |
| IIaA15G2R1    | 0.06%        |
| IIaA16G3R1    | 0.06%        |
| IIaA17G4R2    | 0.06%        |
| IIaA17G5R1    | 0.06%        |
| IIaA18G5R1    | 0.06%        |
| IIaA19G5R1    | 0.06%        |
| IIaA20G4R1    | 0.06%        |
| IIaA20G5R1    | 0.06%        |