InnoVationinfo

ISSN: 2581-7566

Volume 4: 2

Journal of Applied Microbiological Research

J Appl Microb Res 2021

Genetic Diversity of *Cryptosporidium parvum* in Diarrheic Dairy Calves of Two Biogeographical Regions of Chile

Rubén Mercado[1] *
Sebastian Peña[1]
Pamela Muñoz[2]
Eduardo Raffo[3]
Fernando Fredes[4]
Luiz S Ozaki[5]

[1]Parasitology Unit, Faculty of Medicine, University of Chile, Santiago, Chile
[2]Institute of Animal Pathology, Austral University of Chile, Valdivia, Chile
[3]Department of Agriculture, Protection of Renewable Natural Resources Division, Agricultural and Livestock Service, Valdivia, Chile
[4]Department of Animal Preventive Medicine, Universidad of Chile, Santiago, Chile
[5]Department of Immunology and Microbiology, Centre for Biological Data Sciences, Life Sciences, Virginia Commonwealth University, Richmond, Virginia, United States

Abstract

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. 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. 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.

Keywords: *Cryptosporidium*, GP60; Dairy calves, Epidemiology, Diarrhea, Chile.

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: Cyclooxigenase 1; BLAST: Basic

Article Information

Article Type: Research
Article Number: JAMBR 151
Received Date: 08 February, 2021
Accepted Date: 20 December, 2021
Published Date: 27 December, 2021

***Corresponding author:** Dr. Rubén Mercado, Associate Professor, Parasitology Unit, Faculty of Medicine, University of Chile, Av. Las Palmeras #299, Interior Parque Quinta Normal, Comuna de Santiago, Santiago, Chile, Tel: +56 2 2977 0733, Email: rmercado@med.uchile.cl

Citation: Mercado R, Peña S, Muñoz P, Raffo E, et al. (2021) Genetic Diversity of *Cryptosporidium parvum* in Diarrheic Dairy Calves of Two Biogeographical Regions of Chile J Appl Microb Res. Vol: 5 Issu: 1 (27-30).

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Local Alignment Search Tool; NCBI: National Center for Biotechnology Information.

Introduction

Cryptosporidium parvum (Protozoan, Apicomplexa) is the most important cause of eukaryotic unicelular pathogen diarrhea in calves worldwide and is one of the two leading causes of human cryptosporidiosis [1,2]. 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 as well as to humans, and especially in developing countries where irrigation systems include rivers with scarce infrastructure for preventing fecal contamination [8-10]. 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, IIaA15G2R1 is considered highly pathogenic and is the most common infecting calves worldwide, meanwhile in Europe, Asia and Egypt the IId subtype family is mostly observed infecting these animals [13,14]. 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.

Materials and 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 4ºC. 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 and COX1 bovine specific primers to rule out PCR inhibitory activity [15,16]. 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 GGC TGC 3'), resulting in an expected amplicon of about 1,000 bp [15]. 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) [17]. 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 for determining the number of TCA/TCG repeats determined using the collapse sequences option for parasite subtyping [18].

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 IIa subtype family were observed in the MR: IIaA15G2R1, IIaA17G2R1 and IIaA17G4R1. In the LRR, the subtype IIaA15G2R1 was observed in the 100% of the bovine's parasite samples (Table 1). NGS analysis of a single selected DNA sample of our study showed similarly the predominant Sanger IIaA17G4R1 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 instead of SSU-rDNA gene that possess five copies making it a less

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

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%)

Table 2: Subtype and frequency (parentheses) of the NGS study of a single selected sample. The occurrence of each allele is show in terms of percentage of the 100% of the readable sequences analyzed.

sensitive in a PCR assay [19,20]. Pre-weaning cattle are the most susceptible to infection especially by *C. parvum*, 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 simples by PCR in relation to the microscopy morphological tests [21]. The GP60 amplicons were sequenced all belonging to IIa subtype family (Table 1). Interestingly, in the LRR, the subtype IIaA15G2R1 was observed in the 100% of the samples. *C. parvum* subtype IIa predominates in calves in South America, in countries such as Argentina, Colombia and Brazil [22-24]. In Chile, IIaA15G2R1 predominates in the 86.6% of the samples which agrees with data from other countries studies. Feng et al. described that the IIaA15G2R1 subtype has a high rate of transmissibility as an adaptive characteristic [25]. IIaA17G2R1 has also been described in cattle in Europe and USA. The subtype IIaA17G4R1 has also been observed in Colombia, from diarrheic calves [24]. Although subtype diversity was observed in the samples, the predominant subtype was IIaA15G2R1 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 [26,27]. Interestingly, the NGS analysis of a single selected DNA sample of our study showed similarly the predominant IIaA17G4R1 GP60 subtype in 90% of the readable sequences along with others les 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 [28].

Conclusion

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 IIaA15G2R1, 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.

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.

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.

Funding

No institutional funding resources to this research were employed.

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.

Acknowledgement

We want to thank Dr. Mario Duchens of the Department of Animal Production, Faculty of Veterinary and Animal Sciences, Universidad of Chile, Santiago, Chile for the important contribution in the recollection of part of the samples.

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Citation: Mercado R, Peña S, Muñoz P, Raffo E, et al. (2021) Genetic Diversity of *Cryptosporidium parvum* in Diarrheic Dairy Calves of Two Biogeographical Regions of Chile J Appl Microb Res. Vol: 5 Issu: 1 (27-30).