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SHORT COMMUNICATION

Multiple *Cryptosporidium parvum* subtypes detected in a unique isolate of a Chilean neonatal calf with diarrhea

Ruben Mercado · Sebastian Peña · Luiz Shozo Ozaki · Fernando Fredes · Juan Godoy

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Abstract To further understand the composition of population of parasite in a single host, we analyzed the GP60 gene of *Cryptosporidium parvum* amplified from DNA of a randomly selected isolate found in the feces of a diarrheic calf from a dairy farm in Central Chile. Direct sequencing of the amplicon yield the IIaA17G4R1 *C. parvum* subtype. The same amplicon was cloned in *Escherichia coli* (22 clones) and sequenced, yielding three different GP60 subtypes, IIaA17G4R1 (16/22), IIaA16G4R1 (1/22), and IIaA15G4R1 (1/22), and four sequences with nucleotide substitutions in the serine repeats, which subtype would be otherwise IIaA17G4R1. It is thus possible to determine allelic polymorphism using Sanger sequencing with an additional step of bacterial cloning. The results also indicate the necessity to further characterize parasite populations in a single host to better understand the dynamics of *Cryptosporidium* epidemiology.

Keywords Diarrhea · Epidemiology · Polymorphism

R. Mercado (✉) · S. Peña
Facultad de Medicina, Universidad de Chile, Santiago, Chile
e-mail: rmercado@med.uchile.cl

L. S. Ozaki
Center for the Study of Biological Complexity and Department of Microbiology and Immunology Virginia Commonwealth University USA, Richmond, Virginia, USA

F. Fredes
Departamento de Medicina Preventiva Animal Facultad de Ciencias Veterinarias y Pecuarias Universidad de Chile, Santiago, Chile

J. Godoy
CARE Universidad Católica de Chile, Santiago, Chile

Introduction

Cryptosporidium parvum is a protozoan parasite that belongs to the phylum Apicomplexa (Xiao 2010). Intestinal infection caused by *C. parvum* in neotanal calves result in intense diarrhea or even death (Santin 2013; Lendner et al. 2015). In addition to direct economic losses due to lower growth of calves infected with *Cryptosporidium*, environmental contamination with parasite oocyst eliminated with the animal feces may occur (Nydam and Mohammed 2005). The zoonotic nature of this *Cryptosporidium* species makes its oocysts of great importance to human health especially to immunocompromised people (Caccio et al. 2013). *C. parvum* affects mostly calves less than 1 month of age. Oocyst shedding is mainly from these animals, and infection is dose-dependent of ingested oocyst and the frequency of calf to calf contact (Zambriski et al. 2013). Infection is characterized by different routes like animal-to-animal direct contact or contaminated water and food (Xiao 2010). On-farm transmission can be from reservoirs like rodents and birds. *C. parvum* oocysts survive well in farm waste products and soil, which are therefore also a potential source of infection (Smith et al. 2014). Molecular characterization has helped to better understand the epidemiology and epizootiology of *Cryptosporidium* infections (Xiao 2010). Molecular markers like the 60-kDa glycoprotein gene (GP60) (Strong et al. 2000) have been used to determine the genetic diversity of *C. parvum* population (Widmer 2009; Jex and Gasser 2010; Rieux et al. 2013a). The molecular classification is performed through GP60 allelic families and subtypes (Sulaiman et al. 2005). By directly sequencing a GP60 amplicon using Sanger sequencing method, it is only possible to detect the most frequent allele present in a *Cryptosporidium* isolate, preventing assessment of any allelic diversity and consequently the presence of mixed infections (Grinberg et al. 2013; Quinones-Mateu et al. 2014). The *C. parvum* subtype most frequently found around the

world using Sanger sequencing is the IIaA15G2R1 subtype (Jex and Gasser 2010; Xiao 2010). Nevertheless, mixed *Cryptosporidium* genotypes infecting the same host have been recently described, reinforcing the notion that parasites in a host are genetically heterogeneous populations (Rieux et al. 2013b; Shrestha et al. 2014). The diversity of *C. parvum* parasites in an individual opens the question on the accuracy of their genetic characterization. To advance the understanding of the genetic diversity of *C. parvum* in an isolate, we cloned the GP60 amplicon from one parasite isolate. By Sanger sequencing, only 22 cloned fragments we detected in addition to a major subtype (IIaA17G4R1), two minor (IIaA16G4R1 and IIaA15G4R1), and four sequences with base substitutions in the serine track resulting in S to P amino acid changes.

Materials and methods

A fecal sample was collected on September 2011 from a 1-month-old calf showing watery diarrhea, anorexia, and depression. The calf was from one of the many dairy farms located in Central Chile. The sample was collected direct from the animal rectum in a 50-ml conical tube, stored in 70 % ethanol and maintained at 4 °C until processing. Presence of parasite was checked using the modified Zielh-Neelsen (mZN) staining method (Henriksen and Pohlenz 1981). The stained smear was microscopically analyzed under ×1000 magnification.

Total DNA was extracted using ZR Fecal DNA MiniPrep ® (Zymo Research, California, USA). A 150-μl aliquot of sedimented fecal material was transferred to one ZR BashingBead ™ lysis tube and filled with 750 μl of lysis solution. The tube was vortexed at maximum speed for 5 min followed by centrifugation at 13,500*g* for 1 min. Four hundred microliters of the supernatant was transferred to the kit column according to the manufacturer's protocol. The DNA was eluted in 100 μl of Elution Buffer and kept frozen at −20 °C until molecular analyses.

The *Cryptosporidium* GP60 gene was amplified by PCR using the following primers (Strong et al. 2000): forward gp15-ATG 5′-ATG AGA TTG TCG CTC ATT ATC-3′) and reverse gp15-STOP 5′-TTA CAA CAC GAA TAA GGC TGC-3′. An amplicon of about 1000 base pairs (bp) was obtained in a total volume of 25 μl PCR mix containing 250 μM of premixed dNTPs, 2 ng/μl of each primer, 10X HotMaster® 10X Reaction Buffer (5 Prime, Maryland, USA), 2.5 units of HotMaster® Taq Polimerase. The DNA template (2.5 μl) was added and PCR performed as follows: initial denaturation at 95 °C for 5 min, 30 cycles at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 68 °C at 60 s. Amplification of DNA was checked by agarose (1 %) gel electrophoresis, and DNA bands visualized by staining with Gel-Red® (Biotium Inc., Hayward, USA). An aliquot of the amplified DNA fragment was purified and directly sequenced (Sanger method) using the forward gp15-ATG and reverse gp15-STOP primers in external facilities (Macrogen Inc, Korea).

One microliter of the GP60 amplicon was ligated into TOPO® TA cloning vector (Invitrogen, California, USA) by adding 1 μl of cloning kit salt solution and 3 μl of water, according to the manufacturer's protocol. One microliter of the vector was then added and carefully homogenized. After incubation at room temperature for 5 min, ligation mixture was added to 100 μl of Z-Competent™ (*E. coli* strain DH5-α, Zymo Research, California, USA) bacterial cells in a 1.5-ml microcentrifuge tube on ice. The cell suspension (10, 20, and 75 μl) was plated on LB nutrient agar plates containing 50 μg/ml ampicillin and 40 μg/ml X-gal. The agar plates were incubated at 37 °C overnight until formation of bacterial cell colonies. Selected colonies (36) were submitted to colony PCR using the following primers: forward M13 Forward (−20) 5′-GTA AAA CGA CGG CCA G-3′ and reverse M13 reverse 5′-CAG GAA ACA GCT ATG AC-3′, in a total volume of 10 μl containing 250 μM of premixed dNTPs, 2 ng/μl of each primer, 1X HotMaster® buffer, 5 units of HotMaster® Taq Polimerase (5 Prime, Maryland, USA). PCR was performed under the following conditions: initial denaturation at 95 °C for 5 min, 30 cycles at 95 °C for 30 s, annealing at 50 °C for 45 s and extension at 68 °C at 60 s. One microliter of each PCR mixture was checked by agarose gel (1 %) electrophoresis and the gel visualized by staining with Gel-Red® (Biotium Inc., Hayward, USA). PCR mixtures showing an amplicon of about 1400 bp were further processed with ExoSAP-It (USB-Affimetrix, California, USA) and sequenced with the forward and reverse M13 primers in external facilities (Macrogen Inc., Korea).

Raw sequences were edited and analyzed with Sequencher® 5.1 (Gene Codes Corporation, MI, USA). The GP60 serine track coding was analyzed and subtypes obtained according to Sulaiman et al. (2005).

Results

Twenty-two sequences were obtained out of the 36 processed colonies from the cloning of the GP60 amplicon obtained from a single *C. parvum* isolate. The sequences revealed three GP60 subtypes belonging to the IIa subtype family of *C. parvum*. The subtype IIaA17G4R1 appears in 16 (16/22, 73 %) of the analyzed sequences. Two additional subtypes were detected, IIaA15G4R1 (1/22, 4.5 %) and IIaA16G4R1 (1/22, 4.5 %) (Table 1). The remaining four sequences (4/22, 18 %) showed T to C nucleotide substitutions inside the serine

Table 1 Sequence of the serine track of the three GP60 subtypes and their frequency

Serine track	Subtype	Frequency
TCATCATCGTCATCGTCATCGTCATCGTCATCATCATCATCATCATCATCATCATCATCATCAACATCA	IIaA17G4R1	16
TCATCATCGTCATCGTCATCGTCATCGTCATCATCATCATCATCATCATCATCATCATCA□□□ACATCA	IIaA16G4R1	1
TCATCATCGTCATCGTCATCGTCATCGTCATCATCATCATCATCATCATCATCATCATCA□□□□□□ACATCA	IIaA15G4R1	1

track changing, the codons TCG and TCA (Ser) to CCG and CCA (Pro), respectively (Table 2).

Discussion

Different subtypes of *C. parvum* based on the GP60 gene within the same host were found in Argentina in a study with 73 dairy calves, of which two showed two different subtypes: IIaA21G1R1 and IIaA22G1R1 (Del Coco et al. 2014). Similarly, using high throughput sequencing, Grinberg et al. (2013) found 10 different GP60 subtypes of *C. parvum*. These studies indicate that mixed infection with multiple subtypes in one isolate is more of a rule than an exception. Intra-isolate diversity seems to be maintained through time. In a deep-sequencing study, repeated propagation of infection with *C. parvum* did not reduce the genetic diversity (Widmer et al. 2014).

Directly sequencing the GP60 amplicon, the IIaA17G4R1 was the only observed subtype. By sequencing, individual fragments present in the amplicon and separated by cloning showed that this subtype is majoritarian representing 73 % of the sequences analyzed. In this way, two more subtypes were observed, IIaA15G4R1 and IIaA16G4R1. Sanger sequencing can thus be used to resolve genetic diversity of the GP60 gene in intra-host population of *C. parvum*, by adding the step of bacterial cloning. Diversity was observed even with a relatively small amount of sequencing (22 cloned fragments) although variants at very small proportion might be missed, as shown in Grinberg et al. (2013).

The major GP60 subtype of the *C. parvum* sample here analyzed is one different from the most globally representative GP60 subtype, the IIaA15G2R1. Of the three subtypes found (IIaA15G4R1, IIaA16G4R1, and IIaA17G4R1), two are similar to the subtype observed by Waldron et al. (2011). The subtypes IIaA15G4R1 and IIaA17G4R1 they found were from sporadic cases of cryptosporidiosis in human samples. In calf samples, all from New South Wales, Australia, they found the subtype IIaA17G4R1. None of the calves had parasite with the major subtype IIaA15G4R1.

Four of the cloned sequences in our study showed nucleotide substitutions in the serine track, changing the codon of serine to that of proline, a change new to previously described GP60 polymorphisms. The DNA polymerase used in this study was a non-proofreading polymerase (HotMaster, 5 Prime Inc.), and the polymerase fidelity may be the cause of the observed polymorphism. This is unlikely, however, as the change was a constant substitution of a T for a C (Table 2). Central to these substitutions is the biological consequence of a change of the amino acid serine by a proline in GP60, as the latter amino acid is known to cause drastic structural changes inside a protein. These four clones, without the nucleotide substitutions would nevertheless be classified as IIaA17G4R1. The results obtained in regard to mixed infection were similar to those of Grinberg et al. (2013) and Rieux et al. (2013b).

In conclusion, it is clear that multiple subtypes of *Cryptosporidium* GP60 can be present in an infection by the parasite. The biological significance of mixed infection is still to be determined. Detection of the different subtypes is possible using a tool sufficiently sensitive to detect the different sequences in one isolate (Quilez et al. 2014), in this case

Table 2 Nucleotide substitutions in the serine track

Sequence	Serine track	aa change
IIaA17G4R1[a]	TCATCATCGTCATCGTCATCGTCATCGTCATCATCATCATCATCATCATCATCATCATCATCAACATCA	n/a
RARE_er12	TCATCATCGTCATCGTCATCGTCATCGTCACCATCATCATCATCATCATCATCATCATCATCAACATCA	Pro
RARE2_jl12	TCATCATCGTCATCGTCATCGTCACCGTCATCATCATCATCATCATCACCATCATCATCATCAACATCA	Pro-Pro
RARE3_cata	TCATCATCGTCATCGTCACCGTCATCGTCATCATCATCATCATCATCATCATCATCATCATCAACATCA	Pro
RARE4_mpaz	TCATCATCGTCACCGTCATCGTCATCGTCATCATCATCATCATCATCATCATCATCATCATCAACATCA	Pro

[a] Reference sequence

conventional Sanger sequencing of fragments individually separated by conventional bacterial cloning. We aim to study multiple samples from infected animals reared in the same or different locations to further understand the dynamics of parasite infection and transmission at population levels using informative markers such as the GP60 subtyping.

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