Veterinary Parasitology 271 (2019) 80–86

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Isolation, genotyping and subtyping of single *Cryptosporidium* oocysts from calves with special reference to zoonotic significance

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ARTICLE INFO

Keywords:
Cryptosporidium
Single oocyst
PCR-RFLP
*gp*60
Zoonoses

ABSTRACT

The ability of the small-subunit ribosomal RNA (*SSU* rRNA) based nested PCR and Restriction Fragment Length Polymorphism (PCR-RFLP) to identify and genotype a single *Cryptosporidium* oocyst isolated from bovine faecal samples was evaluated in this study. In addition, subtyping was carried out by sequencing the 60 kDa glycoprotein (*gp*60) gene from the same single oocyst. Faecal samples were collected from 40 pre-weaned calves (5–20 days old) from 7 dairy farms located in 3 different counties within the Finger Lakes region of Upstate New York. All the samples were microscopically positive for *Cryptosporidium* spp. A total of 400 *Cryptosporidium* oocysts (10 single oocysts from each calf sample) were individually isolated and analyzed using a nested PCR targeting the *SSU* rRNA gene. The *SSU* rRNA gene was amplified in 324 (81%) individual oocysts. All *SSU* rRNA amplified individual oocysts DNA was genotyped using PCR-RFLP. *C. parvum* was the only identified species; 107 single oocysts generated PCR products from the A gene, 18 generated PCR products from the B gene and 199 generated PCR products from both. Sequence analysis of the *gp*60 gene in 99 individual oocysts revealed the presence of only subtype IIaA15G2R1 with 99.4–100% and 99.1–100% identity of nucleotides and amino acids, respectively. These sequences were identical (100%) in oocysts from 35 calves and exhibited mutations in the non-repeat region of the *gp*60 gene in those of 5 other calves. The examination of DNA from individual oocysts with genotyping and subtyping tools provides methodology to more clearly define the genetic characteristics of *Cryptosporidium* spp. on farms and within individual animals.

1. Introduction

Cryptosporidium is an important gastrointestinal protozoan parasite infecting a wide range of animals as well as humans worldwide. Among the approximately 100 *Cryptosporidium* species and genotypes described in various animals, *C. parvum* constitutes the most predominant zoonotic species that is of importance as an economic and welfare burden on livestock farming and public health (Feltus et al., 2006; Shirley et al., 2012; Feng et al., 2018). It is also responsible for about 85% of *Cryptosporidium* infections in pre-weaned dairy calves and about 1% in post-weaned ones. Therefore, pre-weaned calves are important reservoirs and sources for zoonotic cryptosporidiosis in humans (Chalmers and Katzer, 2013). In newborn calves, the disease is characterized by mild to severe watery diarrhea. *Cryptosporidium* infection in immunocompetent persons can result in acute self-limiting symptoms including diarrhea, nausea, vomiting and weight loss (Ryan et al., 2016). Moreover, infants and immunocompromised persons can manifest severe diarrhea, malnutrition, and chronic malabsorption, sometimes resulting in death (Shrivastava et al., 2017). Contact with infected calves has been implicated in transmission of zoonotic *C. parvum* to humans in the United States, United Kingdom, Ireland and Australia (Hunter et al., 2004; Roy et al., 2004). The development of molecular diagnostic tools for genotyping and subtyping of *Cryptosporidium* spp. has provided useful means that have aided in understanding host specificity of the parasite, tracking sources of infections, characterization of transmission dynamics of cryptosporidiosis and investigating outbreaks in animal and human communities (Xiao and Ryan, 2008). The nested PCR targeting the 18S small-subunit ribosomal RNA (*SSU* rRNA) gene is a sensitive assay used for detecting *Cryptosporidium* spp. PCR–restriction fragment length polymorphism (PCR-RFLP) of the *SSU* rRNA gene has been widely used in molecular epidemiology as a discriminatory tool to detect the presence of mixed *Cryptosporidium* species in animals and humans, and the semi-conserved and hyper-variable regions in the *SSU* rRNA gene provides the basis of genus-specific

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https://doi.org/10.1016/j.vetpar.2019.05.003
Received 4 March 2019; Received in revised form 6 May 2019; Accepted 8 May 2019

primers that are used in *Cryptosporidium* genotyping (Xiao et al., 1999, 2001; Jiang and Xiao, 2003; Xiao, 2010). Minor sequence differences exist among the five copies of the *SSU* rRNA gene in *C. parvum*, with four copies having the A type of sequence and one copy having the B type sequence (Le Blancq et al., 1997). They can be differentiated from each other in RFLP analysis of the PCR products using *Ssp*I (Xiao et al., 1999).

The 60-kDa glycoprotein (*gp*60) gene, also known gp40/15, is highly polymorphic, and is used as a discriminatory marker for identifying human, animal and zoonotic *C. parvum* subtypes in DNA sequence analysis. To date, over 20 *C. parvum* subtype families have been identified with several subtypes within each family (Feng et al., 2018). Some of these families appear adapted to humans (IIc), while others are found in both ruminants and humans (IIa and IId). In industrialized countries, subtype family IIa, especially the IIaA15G2R1 subtype, is more prevalent in calves and humans and is considered the primary cause of zoonotic *C. parvum* infections in humans in these countries (Feng et al., 2013, 2018). Conversely, IIa subtypes are rarely seen in humans in developing countries, where most human infections are caused by anthroponotic IIc subtypes (Feng et al., 2018).

Controversies exist on within-isolate genetic diversity of *Cryptosporidium* spp. Although traditional Sanger sequencing of *gp*60 PCR products has revealed mostly a single *C. parvum* subtype in individual specimens from calves and humans, the analysis of PCR products using next-generation sequencing has shown a common occurrence of mixed subtypes in *C. parvum* specimens (Grinberg et al., 2013; Zahedi et al., 2017). This, however, does not appear to be the case for *C. hominis* isolates either by sequencing of *gp*60 PCR products or mapping of sequence reads to the reference *gp*60 gene sequence using the next-generation sequencing technology (Guo et al., 2015; Zahedi et al., 2017). This difference in observation of intra-isolate subtype diversity could be due to difference in transmission intensity between *C. parvum* and *C. hominis*.

This is the first report to genetically characterize *Cryptosporidium* oocysts from individual animals on farms using DNA isolated from a single *Cryptosporidium* oocyst using nested PCR and PCR-RFLP molecular assays specifically targeting the *SSU* rRNA and *gp*60 gene. This study aimed to address the ability of the nested PCR to amplify the *SSU* rRNA and *gp*60 genes from single *Cryptosporidium* oocysts isolated from bovine faecal samples. In addition, genotyping and subtyping of these oocysts were performed.

2. Material and methods

This study was approved by the Animal Care and Use Committee (IACUC), College of Veterinary Medicine, Cornell University. The faecal samples were collected from pre-weaned calves on dairy farms with informed consent from farm managers. Animal handling was performed in accordance with the Institutional protocol provisions and guidelines.

2.1. Samples collection and processing

A total of 40 pre-weaned Holstein dairy calves (5–20 days old) from seven different dairy farms located in three rural counties within the Finger Lake region of Upstate New York were enrolled in this study. These calves had neonatal diarrhea and were suspected to have cryptosporidiosis. Faecal samples were collected directly from the rectum of each animal by a clinician in sterile disposable plastic cups and transported in an insulated ice box to the laboratory for further processing and examination. All samples were concentrated and purified using a centrifugal Sheather's sugar flotation technique where approximately 1 g of feces was mixed with water, poured through gauze into a disposable 16 x 100 mm round-bottom glass centrifuge tube and centrifuged at 800 x *g* for 1 min. The tube was decanted, and the pellet was mixed and refilled with Sheather's sugar solution [specific gravity = 1.27] and a new coverslip [size = 18 mm] was placed on the top of the reverse meniscus with this tube being centrifuged for 8 min at 800 x *g*. The coverslip was then removed, placed on a new microscope slide and examined under the microscope. The resulting microscopic slide and cover glass containing the partially purified oocysts were washed with phosphate buffered saline (PBS) into a 1.5 ml microfuge tube. The tube was centrifuged, and the pellet was resuspended in 100–200 µl PBS.

2.2. Isolation of single oocyst

Single oocysts were isolated following similar protocols as described previously for isolation of single *Eimeria* oocysts (Shirley and Harvey, 1996; Wang et al., 2014), *Isospora* oocysts (Matsubar et al., 2017), and *Cryptosporidium andersoni* oocysts(Ikarashi et al., 2013) with minor modifications. Briefly, the purified *Cryptosporidium* oocyst suspension of each positive calf sample was serially diluted in a double volume of PBS to obtain a final concentration of one oocyst per microliter. One microliter of the final dilution was then transferred to a new glass slide with 1 µl of Sheather's sugar solution and examined under Differential Interference Contrast microscope (Olympus, BX51) to confirm the presence of single oocyst per microscopic slide. The individual oocyst from each slide was washed with PBS into a 1.5 ml microfuge tube, then centrifuged, and the 100 µl pellet was stored at -20°C for further molecular identification. These processes were repeated ten times for each calf sample to obtain ten individual slides each containing a single oocyst. To remove the possibility of cross-study DNA contamination new supplies were used throughout the entire oocyst collection procedure, including collection sample cups, flotation tubes, slides, coverslips, micropipette tips, and microfuge tubes.

2.3. Molecular identification of Cryptosporidium spp

DNA was extracted from a total of 400 individual *Cryptosporidium* oocysts (ten single oocysts from each calf) using the QIA-amp DNA mini kit (Qiagen, Germantown, Maryland, USA) proceeded by 5 cycles of freezing at −80 °C (10 min) and thawing at 56 °C (10 min). The *SSU* rRNA gene (˜840 bp) was amplified from 2 µl extracted DNA for each individual oocyst by nested PCR protocol using primers manufactured by Invitrogen, USA with the following sequences for the primary PCR: SSU F1: 5´- TTCTAGAGCTAATACATGCG-3´ (Xiao et al., 1999), SSU R1: 5´- CCCATTTCCTTCGAAACAGGA-3´ (Xiao et al., 2001). For the secondary PCR, the primers were as follows; SSU F2: 5´- GGAAGGGTTG TATTTATTAGATAAAG-3´ **(**Xiao et al., 1999**)**, SSU R2: 5´- CTCATAAG GTGCTGAAGGAGTA-3´ (Jiang et al., 2005). The primary and secondary PCR reactions and conditions were carried out according to Xiao and Ryan (2008). The secondary PCR products were examined by electrophoresis using 2% agarose gels stained with ethidium bromide and visualized on an ultraviolet trans-illuminator.

2.4. PCR-RFLP genotyping of Cryptosporidium spp

The secondary PCR product of each individual oocyst positive for the *SSU* rRNA gene was digested with each of the following enzymes; 20 U of *Ssp*I (New England Biolabs, USA) and 20 U of *Vsp*I (New England Biolabs, USA) according to the methods of Xiao et al. (1999); Xiao and Ryan (2008). The digested products and positive control were separated on 2.5% agarose gels stained with ethidium bromide and visualized on an ultraviolet trans-illuminator.

2.5. Subtyping of C. parvum

A nested PCR protocol was performed to amplify the *gp*60 gene (850 bp) from the same extracted DNA of each individual oocyst positive for the *SSU* rRNA gene and confirmed as *C. parvum* by PCR-RFLP. The primary PCR primers with the following sequences; AL3531 F1: 5´-ATAGTCTCCGCTGTATTC-3´ and AL3535 R1: 5´-GGAAGGAACGATGT ATCT-3´ were used (Peng et al., 2001). The secondary PCR primers

were AL3532 F2: 5´- TCCGCTGTATTCTCAGCC-3´ and AL3534 R2: 5´-GCAGAGGAACCAGCATC-3´ (Peng et al., 2001). The primary and the secondary PCR reaction mixtures were each performed in a total volume of 100 µl consisted of Gene Amp 10X PCR buffer I (Applied Biosystem, USA), 200 µM for each of deoxynucleotide triphosphate (Invitrogen, Thermo Fisher Scientific, USA), 100 nM for each of forward and reverse primer (Invitrogen, Thermo Fisher Scientific, USA), 3 mM $MgCl_2$ (Invitrogen, Thermo Fisher Scientific, USA), 5 units Taq polymerase (Invitrogen, Thermo Fisher Scientific, USA) and 2 µl of DNA template. A 400 ng/µl of non-acetylated bovine serum albumin (Sigma, USA) were only added to the primary PCR reaction to reduce PCR inhibitors (Xiao et al., 2007). The reaction conditions for both primary and secondary reactions were the same and consisted of an initial denaturation (95 °C/3 min), 35 cycles of denaturation (94 °C/45 s), annealing (50 °C/ 45 s) and extension (72 °C/ 1 min), followed by a final extension (72 °C/ 10 min). The amplified PCR products were run on 2% agarose gel stained with ethidium bromide and visualized on ultraviolet trans-illuminator.

2.6. Sequence analysis

The amplified *gp*60 PCR products of 99 genotyped individual *C. parvum* oocysts were sequenced (Table 1). The sequencing was done using an ABI Automated 3730xl DNA Analyzer with forward and reverse primers used with the secondary PCR product. An additional sequencing primer; 5´-GAGATATATCTTGGTGCG-3´ was also used (Glaberman et al., 2002). The sequences for each individual oocyst were first evaluated using SeqMan Pro & MegAlign Pro (DNASTAR Lasergene 15) to generate a consensus sequence. The consensus nucleotide sequences of *gp*60 gene were analyzed using BLAST search in NCBI databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned with reference *gp*60 gene sequences available in the GenBank using the Clustal W method implemented in the MegAlign software (DNASTAR Lasergene 15). The nucleotide, amino acid identities and the deduced amino acid sequences of the obtained *gp*60 gene and reference sequences retrieved from the GenBank were compared using MegAlign program software. *C. parvum* subtypes generated in this study were

Table 1
Molecular genotyping and subtyping profiles of *Cryptosporidium* single oocysts isolated from dairy cow calves.

Farm	No. of animals	Animal code (age in days)	No. of isolated single oocysts from each animal	No. of *SSU* rRNA positive oocysts	PCR-RFLP genotyping			No. oocysts amplified by *gp*60 gene	*C.parvum* subtype[c]	No. of sequenced *gp*60 gene amplicons	GenBank accession numbers
					C. Parvum						
					A gene	B gene	A&B				
I	2	LE1 (1)	10	9	9	–	–	9	IIaA15G2R1	9	MK099816
		LE40 (20)	10	10	–	–	10	10	IIaA15G2R1	2	MK099855
II	1	HE2 (5)	10	10	–	–	10	10	IIaA15G2R1	10	MK099817
III	4	S3 (8)	10	1	1	–	–	1	IIaA15G2R1	1	MK099818
		S37 (5)	10	10	–	10	–	10	IIaA15G2R1	2	MK099852
		S38 (20)	10	10	10	–	–	10	IIaA15G2R1d	2	MK099853
		S39 (20)	10	10	10	–	–	10	IIaA15G2R1e	2	MK099854
IV	10	T4 (7)	10	9	–	–	9	9	IIaA15G2R1	2	MK099819
		T5 (7)	10	8	–	–	8	8	IIaA15G2R1	2	MK099820
		T6 (7)	10	8	–	–	8	8	IIaA15G2R1	4	MK099821
		T7 (7)	10	7	–	–	7	7	IIaA15G2R1	2	MK099822
		T8 (14)	10	9	–	–	9	9	IIaA15G2R1	2	MK099823
		T9 (14)	10	10	–	–	10	10	IIaA15G2R1	2	MK099824
		T10 (14)	10	10	–	–	10	10	IIaA15G2R1	2	MK099825
		T11 (14)	10	10	–	–	10	10	IIaA15G2R1	2	MK099826
		T12 (14)	10	8	–	–	8	8	IIaA15G2R1	2	MK099827
		T13 (14)	10	10	–	–	10	10	IIaA15G2R1	2	MK099828
V	4	FL18 (7)	10	3	–	–	3	3	IIaA15G2R1	2	MK099833
		FL19 (10)	10	4	–	–	4	4	IIaA15G2R1	2	MK099834
		FL20 (14)	10	6	6	–	–	6	IIaA15G2R1	2	MK099835
		FL21 (7)	10	7	–	–	7	7	IIaA15G2R1	2	MK099836
VI	6	CS22 (14)	10	7	1	–	6	7	IIaA15G2R1	2	MK099837
		CS23 (14)	10	9	–	–	9	9	IIaA15G2R1	2	MK099838
		CS24 (14)	10	10	–	–	10	10	IIaA15G2R1	2	MK099839
		CS25 (14)	10	10	–	–	10	10	IIaA15G2R1	2	MK099840
		CS26 (14)	10	10	–	–	10	10	IIaA15G2R1	2	MK099841
		CS27 (14)	10	10	–	–	10	10	IIaA15G2R1	3	MK099842
VII	13	CTD14 (20)	10	4	4	–	–	4	IIaA15G2R1	2	MK099829
		CTD15 (20)	10	4	–	–	4	4	IIaA15G2R1	2	MK099830
		CTD16 (14)	10	7	7	–	–	7	IIaA15G2R1	2	MK099831
		CTD17 (6)	10	7	–	7	–	7	IIaA15G2R1	2	MK099832
		CTD28 (20)	10	10	10	–	–	10	IIaA15G2R1	4	MK099843
		CTD29 (20)	10	9	9	–	–	9	IIaA15G2R1	2	MK099844
		CTD30 (14)	10	10	10	–	–	10	IIaA15G2R1	2	MK099845
		CTD31 (10)	10	7	7	–	–	7	IIaA15G2R1	2	MK099846
		CTD32 (10)	10	7	7	–	–	7	IIaA15G2R1a	2	MK099847
		CTD33 (7)	10	7	7	–	–	7	IIaA15G2R1b	2	MK099848
		CTD34 (7)	10	7	7	–	–	7	IIaA15G2R1	2	MK099849
		CTD35 (5)	10	10	–	1	9	10	IIaA15G2R1c	2	MK099850
		CTD36 (5)	10	10	2	–	8	10	IIaA15G2R1	2	MK099851
Total	40		400	324 (81%)[a]	107	18	199	324 (100 %)[b]		99	

[a] The percentage was calculated in relation to the number of individual *Cryptosporidium* spp. oocysts from each animal.
[b] The percentage was calculated in relation to the number of oocysts amplified by *SSU*RNA gene.
[c] A consensus nucleotide sequence was generated for *gp*60 gene of 2 single oocysts from each calf except calf S3 only one oocyst.

named and designated according to Sulaiman et al. (2005) and submitted to the GenBank under accession Nos., **MK099816-MK099855** (Table 1).

3. Results

The results of this study revealed that all the enrolled calves (n = 40) were positive for *Cryptosporidium* oocysts upon microscopic examination and molecular analysis. The *SSU* rRNA gene was amplified from 324/400 individual oocysts (81%) (Table 1). The PCR-RFLP genotyping of all *SSU* rRNA amplified PCR products from the 324 single oocysts indicated that all oocysts were of the same species, *C. parvum*. Based on the restriction banding patterns of *Ssp*I and *Vsp*I restriction enzymes, the *SSU* rRNA PCR products of *C. parvum* were differentiated into type A and type B genes. A total of 107 *C. parvum* single oocysts produced type A gene product, 18 produced type B gene product and 199 had both gene products (Table 1). There was a marked difference in the banding profiles of the type A gene (108, 254 and 449 bp) and the type B gene (119, 254 and 449 bp) following digestion with the SspI enzyme (Fig. 1A). The VspI enzyme restriction banding profile for the type A gene (102, 104 and 628 bp) was nearly identical to the type B (102, 104 and 625 bp) (Fig. 1B). SspI differentiates both gene types better than the VspI enzyme (Fig. 1).The *gp*60 gene was amplified from all 324 single oocysts (100%) that were confirmed as *C. parvum* based on PCR-RFLP analysis of the *SSU* rRNA gene (Table 1).

Sequence analysis of the *gp*60 gene products from 99 representative individual *C. parvum* oocysts revealed that all sequences were of the same subtype IIaA15G2R1. This subtype has 15 copies of TCA (A) and 2 copies of TCG (G) trinucleotide repeats that encode for amino acid serine and one copy of the sequence ACATCA (R1) immediately after the trinucleotide repeat. The *gp*60 sequences of *C. parvum* subtype IIaA15G2R1 from this study exhibited 99.4–100% nucleotide and 99.1–100% amino acid identities. These sequences were identical (100%) in 35 calves (animal code: LE1-CTD31, CTD 34, CTD 36, S37 and LE40). Sequences with nucleotide substitutions in the non-repeat region of the *gp*60 gene were from 5 of the calves. The latter were designated as subtype IIaA15G2R1a (code: CTD32), IIaA15G2R1b (code: CTD33), IIaA15G2R1c (code: CTD35), IIaA15G2R1d (code: S38) and IIaA15G2R1e (code: S39). Subtype IIaA15G2R1a exhibited a non-synonymous mutation at position 12 (TTC phenylalanine →leucine TTG) and synonymous mutation at codon 606 that did not result in an amino acid substitution (AGA→AGG; both are arginine). Moreover, non-synonymous mutation was observed in subtype IIaA15G2R1b and resulted in an amino acid substitution at position 604 (arginine AGA→ glycine GGA) and 625 (AGT serine → GGT glycine). G to A transition at codon 667 of subtype IIaA15G2R1c resulted in aspartic acid to asparagine substitution (Table 2 & Fig. 2). The *gp*60 gene sequences of subtype IIaA15G2R1d showed synonymous mutation at codon 66 (GGC→GGG, both are glycine) and 465 (GTT→GTC, both are valine). Furthermore, amino acid replacement was observed at codon 302 of *gp*60 sequences of subtype IIaA15G2R1e (glutamic acid GAA → valine GTA). There was 99.7–100% nucleotide and 99.1–100% amino acid identity between 60 gene sequences of *C. parvum* subtype IIaA15G2R1 in this study and the same subtype of bovine and human origin retrieved from GenBank.

4. Discussion

The ability of the nested PCR and PCR-RFLP to identify and genotype a single *Cryptosporidium* oocyst was evaluated in this study. Subtyping was done by sequencing the *gp*60 gene PCR products. The *SSU* rRNA gene was amplified from 324/400 individual oocysts purified from 40 pre-weaned calves (5–20 days old). The lack of amplification of the 76 remaining oocysts may have been due to the loss of oocysts during washing and transfer or insufficient DNA from the single oocyst leading to failure of *SSU* rRNA gene amplification. The presence of infection in all investigated calves indicated that the animals were infected immediately after birth, and previous work in this region has revealed that calf infections are relatively ubiquitous (Fayer et al., 2006; Santin et al., 2004; Xiao et al., 2007; Szonyi et al., 2010). Others have also detected *Cryptosporidium* spp. commonly in 3 days old calves (Avendaño et al., 2018) and reported the establishment of the infection during the first 2 weeks of life (Trotz-Williams et al., 2007; Santin et al., 2008; Avendaño et al., 2018).

There is evidence of mixed species and intra-species genetic diversity within *Cryptosporidium* spp. populations. Genotyping of individual oocysts or sporozoites can identify these mixed species/genotypes but this work is technically challenging and rarely reported (Tanrıverdi et al., 2002; Ikarashi et al., 2013). Based on the results of PCR-RFLP, all *SSU* rRNA positive single oocysts were genotyped as *C. parvum*. This species was shown to have A and B copies of the *SSU* rRNA gene based on *Ssp*I restriction patterns, as indicated previously by PCR-RFLP of the gene using the *Ssp*I restriction enzyme (Xiao et al., 1999).

C. parvum has been reported as the most dominant species identified in pre-weaned calves in the United States (Santin et al., 2004; Xiao et al., 2007; Santin et al., 2008), Portugal (Alves et al., 2003), Canada (Trotz-Williams et al., 2006), Spain (Quilez et al., 2008), Argentina (Tomazic et al., 2013), Brazil (Do Couto et al., 2014), Japan (Wu et al., 2003; Aita et al., 2015), Poland (Kaupke and Rzeżutka, 2015) and Colombia (Avendaño et al., 2018), Slovenia (Soba and Logar, 2008). However, other studies in Egypt (Amer et al., 2010, 2013; Naguib et al., 2018), Turkey (Taylan-Ozkan et al., 2016) and Italy (Díaz et al., 2018) have reported the presence of other *Cryptosporidium* spp. (*C. bovis*, *C. ryanae* and *C. andersoni*) in pre-weaned calves with predominance being *C. parvum*. Conversely, *C. bovis* is the most prevalent species in pre-weaned dairy calves in China (Cai et al., 2017) and Western Australia and New South Wales (Ng et al., 2011).

C. parvum has been also reported as a component of the flora

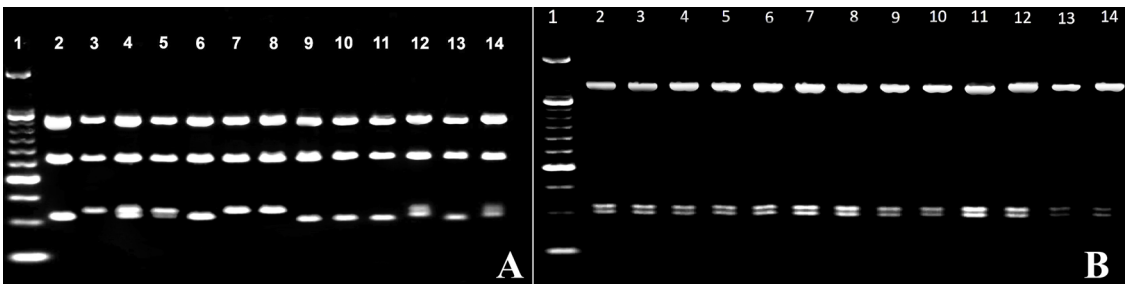

Fig. 1. PCR-RFLP genotyping of *Cryptosporidium* single oocysts isolated from calves' faecal samples after digestion with *Ssp*I and *Vsp*I restriction enzymes. **A**: *Ssp*I restriction patterns; lane 1: DNA ladder (50bp), lanes 2, 6, 9, 10, 11, 13: *C. parvum* A gene (108, 254 and 449bp), lanes 3, 7, 8: *C. parvum* B gene (119, 254 and 449bp). Lanes 4, 5, 12: *C. parvum* A and B genes, lane 14: *C. parvum* positive control. **B**: *Vsp*I restriction patterns; lane1: DNA ladder (50bp), lanes 2–6, 11, 12, 13: *C. parvum* A gene (102,104 and 628bp), lanes 7, 8, 9, 10: *C. parvum* B gene (102, 104 and 625bp) and lane 14: *C. parvum* positive control.

Table 2
Nucleotide and amino acid substitution in the non-repeat regions of *gp*60 gene sequences of single *C. parvum* oocysts subtype IIaA15G2R1 isolated from dairy calves in this study compared with calf and human IIaA15G2R1 subtype in the GenBank. F (phenylalanine), L (leucine), G (Glycine), E (Glutamic acid), V (Valine), R (Arginine), S (Serine), D (Aspartic acid), N (Asparagine).

C. parvum subtype	Codon position						
	12	66	302	465	604-606	625	667
IIaA15G2R1 (LE1: MK099816)	TTC (F)	GGC (G)	GAA (E)	GTT (V)	AGA (R)	AGT (S)	GAT (D)
IIaA15G2R1a (CTD32: MK099847)	TTG (L)	GGC (G)	GAA (E)	GTT (V)	AGG (R)	AGT (S)	GAT (D)
IIaA15G2R1b (CTD33: MK099848)	TTC (F)	GGC (G)	GAA (E)	GTT (V)	GGA (G)	GGT (G)	GAT (D)
IIaA15G2R1c (CTD35: MK099850)	TTC (F)	GGC (G)	GAA (E)	GTT (V)	AGA (R)	AGT (S)	AAT (N)
IIaA15G2R1d (S38: MK099853)	TTC (F)	GGG (G)	GAA (E)	GTC (V)	AGA (R)	AGT (S)	GAT (D)
IIaA15G2R1e (S39: MK099854)	TTC (F)	GGC (G)	GTA (V)	GTT (V)	AGA (R)	AGT (S)	GAT (D)
IIaA15G2R1(calf Iowa II: XM627480)	TTC (F)	GGC (G)	GAA (E)	GTT (V)	AGA (R)	AGT (S)	GAT (D)
IIaA15G2R1(calf: DQ630518)	TTC (F)	GGC (G)	GAA (E)	GTT (V)	AGA (R)	AGT (S)	GAT (D)
IIaA15G2R1(calf: AF164495)	TTC (F)	GGC (G)	GAA (E)	GTT (V)	AGA (R)	AGT (S)	GAT (D)
IIaA15G2R1(human: AY738190)	TTC (F)	GGC (G)	GAA (E)	GTT (V)	AGA (R)	AGT (S)	GAT (D)

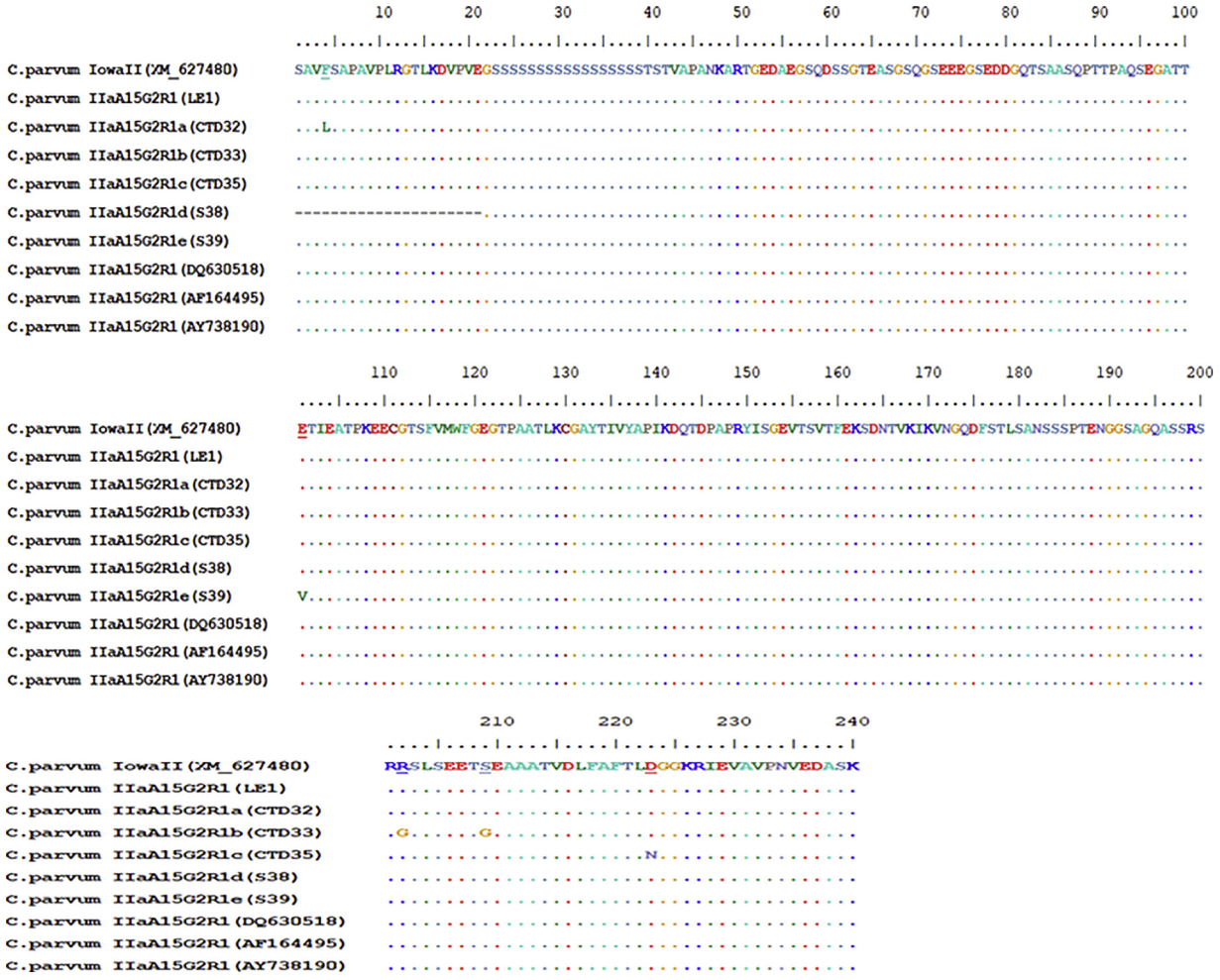

Fig. 2. The deduced amino acid sequences of *gp*60 gene of *C. parvum* subtype IIaA15G2R1 isolated from calves in this study in comparison with reference *gp*60 gene sequences of *C. parvum* subtype IIaA15G2R1 of bovine and human origin retrieved from the GenBank. Dots indicate amino acid positions that are identical to the corresponding *gp*60 gene sequence. Phenylalanine 4, glutamic acid 101, arginine 202, serine 209 and aspartic acid 223 in which mutations occur are indicated by the solid bars.

associated with neonatal calf diarrhea (Quilez et al., 2008; Ichikawa-Seki et al., 2015; Meganck et al., 2015). This substantiated the findings of this investigation; where all studied calves had watery stool and were infected with *C. parvum*.

Several epidemiological studies have assessed the importance of dairy calves as a reservoir for *C. parvum* and their role in the zoonotic transmission of cryptosporidiosis using molecular subtyping tools. The *gp*60 is the most polymorphic gene marker used for subtyping *C. parvum* because of its sequence heterogeneity. It has a tandem trinucleotide repeat that codes for amino acid serine at the 5´ end of the gene (TCA, TCG or TCT) which categorizes *C. parvum* into different subtype families with several subtypes within each subtype family (Sulaiman et al., 2005; Xiao, 2010).

In this study, all sequenced *C. parvum* individual oocysts were the same subtype IIaA15G2R1. This subtype is zoonotic and was the only subtype previously identified in dairy calves in Maryland, USA (Santin

et al., 2008). However, other studies in the United States (Xiao et al., 2007), Canada (Trotz-Williams et al., 2006), Italy (Díaz et al., 2018), Slovenia (Soba and Logar, 2008), Portugal (Alves et al., 2003) and Japan (Wu et al., 2003; Aita et al., 2015) reported the presence of other *C. parvum* subtypes in pre-weaned calves with subtype IIaA15G2R1 being the most prevalent. Consequently, human cryptosporidiosis has been primarily related to the zoonotic *C. parvum* subtype IIaA15G2R1. The contribution of calves in the zoonotic transmission of *C. parvum* subtype IIaA15G2R1 has been documented in the United States (Peng et al., 2003; Feltus et al., 2006), Japan (Wu et al., 2003), Portugal (Alves et al., 2003), Slovenia (Soba and Logar, 2008) and Tunisia (Rahmouni et al., 2014**)** by subtyping *C. parvum gp*60 from calves and humans. All these studies found the zoonotic subtype IIaA15G2R1 in both calves and humans in the studied area.

The observed mutation in the *gp*60 sequences of *C. parvum* subtype IIaA15G2R1 in the current study suggested that this locus is under selective pressure (Feng et al., 2013). Synonymous and non-synonymous mutations in the non-repeat region of the *gp*60 sequences of *C. parvum* belonging to the same subtype has been reported in former studies (Sulaiman et al., 2005; Amer et al., 2010). There was 99.7–100% identity between *C. parvum* subtype IIaA15G2R1 *gp*60 gene sequences in this study and IIaA15G2R1 subtype previously identified among pre-weaned calves in the United States (GenBank accession Nos.: DQ630518, AF164489 and AF164495), Colombia (GenBank accession No. MF142042), Egypt (GenBank accession No. AB514090), Australia (GenBank accession No. MG516788), Canada (GenBank accession No. DQ192503), Poland (GenBank accession No. KP997149), Portugal (GenBank accession No. AY166804), Japan (GenBank accession No. AY167589) and the reference *C. parvum* subtype IIaA15G2R1 Iowa II calf isolate (GenBank accession No. XM627480). Interestingly, our *gp*60 gene sequences shared 99.7–100% identity with subtype IIaA15G2R1 previously identified in humans in United States (GenBank accession Nos.: AF164496, DQ640630 and JX575582), United Kingdom (GenBank accession No. HQ005737), Japan (GenBank accession No. AY167592), Slovenia (GenBank accession No. AM988865), Spain (GenBank accession No. KY499051), Lebanon (GenBank accession No. KM215754), Kuwait (GenBank accession No. AY738190) and Mexico (GenBank accession No. KY990917).

5. Conclusions

This study highlighted that the *SSU* rRNA based nested PCR and PCR-RFLP in combination with *gp*60 sequencing are specific tools for determining the true distribution of species and subtypes of individual *Cryptosporidium* oocysts in the sample. Thereby, these tools are helpful in epidemiological investigations for elucidating the frequency, dynamic and sources of *Cryptosporidium* infection, as well as transmission patterns and zoonotic potential in endemic and non-endemic regions. This study confirmed that pre-weaned calves are an important potential sources and reservoirs for zoonotic *C. parvum,* especially the zoonotic subtype IIaA15G2R1. The similarity between *C. parvum* subtype IIaA15G2R1 identified in calves in this study and subtype IIaA15G2R1 previously identified in humans in United States and other different geographic regions confirms the role of calves in the zoonotic transmission of cryptosporidiosis. Future studies with individual oocysts at other genetic loci may provide more information about the genetic structure of *Cryptosporidium* through the examination of individual sporozoites recovered from oocysts and through the careful examination of the oocysts shed by animals and people to understand the potential of genetic recombination in areas where zoonotic transmission occurs.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments

We would like to thank the veterinarians in the Ambulatory and Production Medicine, College of Veterinary Medicine, Cornell University for their assistance in collecting faecal samples.

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