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The profile of inflammatory factors in dairy calves with *Cryptosporidium* infection

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Abstract

The present study was conducted to investigate the detection and identification of *Cryptosporidium* species via molecular techniques and evaluate the serum concentrations of inflammatory factors in *Cryptosporidium* species. The fecal samples (n = 256) were collected from pre-weaned (≤ 2.00 months) calves and the positive samples were identified utilizing Ziehl-Neelsen staining. Nested species-specific multiplex PCR (nssm-PCR) and restriction fragment length polymorphism (RFLP) were used to identify the species and sub-species. The serum concentrations of IL-1β, IL-6, IL-12, TNF-α, and IFN-γ were also assessed. The results revealed that 10.54% of samples were positive. The results of Nested-PCR showed that 92.59% of the samples were positive for *C. parvum* while 7.41% were positive for *C. andersoni*. The results of RFLP confirmed 92.59% of the samples for *C. parvum*, 3.70% for *C. muris / C. andersoni*, and 3.70% for *C. muris*. The serum concentrations of IL-1β, IL-6, IL-12, TNF-α, and IFN-γ were significantly higher in the infected calves compared to those in healthy calves. However, the serum concentration of IFN-γ was significantly higher in the calves infected with *C. parvum* while the serum concentrations of TNF-α and IL-6 were significantly higher in those infected with *C. andersoni.* In conclusion, *C. parvum* was prevalent in the region and the calves demonstrated inflammatory responses to *Cryptosporidium* species.

Introduction

Cryptosporidium genus belongs to opportunistic protozoans of Apicomplexa phylum and causes infection in gastrointestinal and respiratory systems of certain mammalians.[1] Cryptosporidiosis is commonly transmitted by the consumption of contaminated feed sources.[2] Species of *Cryptosporidium parvum, C. bovis, C. andersoni,* and *C. ryanae* have been detected in bovine cryptosporidiosis.[3] The *C. parvum* mainly causes infection, diarrhea, progressive dehydration, weight loss, delayed growth, and occasional death in pre-weaned calves.[4] Innate and adaptive immunities have important roles in protecting body against cryptosporidiosis.[5] Cytokines are the main compounds of the immune system, which play an important role in signal transduction between cells and regulate the immune responses.[6] Interleukin 1β (IL-1β), interleukin 6 (IL-6), tissue necrosis factor-α (TNF-α), and interferon gamma (IFN-γ) are involved in the inflammatory responses.[7] Interleukin-12 (IL-12) promotes antiparasitic, antimicrobial, and antitumor activity of the macrophages and natural killer cells.[8,9]

Different methods have been employed for detection of *Cryptosporidium.* Microscopic method is the most common method for the identification of *Cryptosporidium* using the stool samples.[10] Herein, modified Ziehl-Neelsen (ZN) staining technique was used for the detection of intestinal cryptosporidiosis. Molecular techniques have further sensitivity compared to microscopy methods. Molecular methods are used for genotyping and subtyping of *Cryptosporidium*.[11] The amplification of small-subunit ribosomal RNA (SSU rRNA) with Nested-PCR and restriction fragment length polymorphism (RFLP) are sensitive molecular techniques for the detection of *Cryptosporidium* DNA in the host's fecal and environmental samples and are utilized in *Cryptosporidium* genotyping.[12] Quantitative PCR (qPCR) is a technique for the detection and quantification of *Cryptosporidium* in both fecal and environmental samples, specifically for human pathogenic species *C. parvum* and *C. hominis*.[13]

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Knowing Information about the inflammatory responses in the infections with *Cryptosporidium* species may help to control and prepare agents for the treatment of cryptosporidiosis. Thus, the present study was conducted to identify *Cryptosporidium* species with molecular tools in infected calves and investigate their relations with inflammatory factors.

Materials and Methods

Ethical standard. All the procedures were approved by Ethical Committee of Science and Research Branch, Islamic Azad University (Tehran-Iran) and with Ethical number (IAUSR, 1398, 407).

Sampling and identification of *Cryptosporidium* oocytes. The fecal samples (n = 256) were collected from rectum of pre-weaned (≤ 2 months) calves from all over Semnan province (Iran) from April to September 2019. Table 1 represents the studied farms. The samples were stored in sterile plastic containers at 4.00 ˚C until microscopic examination and genomic DNA extraction.

Ziehl-Neelsen staining. Hot and cold procedures were used for ZN staining of the fecal smears as reported by Rekha *et al.*[14]

DNA extraction. To extract oocyte DNA, QIAGEN QIAamp 180 - 220 DNA stool mini kit (QIAGEN, Düsseldorf, Germany) was employed.

Nested species-specific multiplex PCR (nssm-PCR). Nested-PCR technique for identification of *Cryptosporidium* species was conducted with specific primers (Table 2) with a product size of 1370 bp as reported by Thomson *et al.*[15] In the first step, the reagents were 2.50 µL DNA template, 0.20 µmol of primer, 200 µmol of dNTP mix, 1.50 µmol of $MgCl_2$, 2.50 µL of PCR buffer, and 2.50 µL of Taq DNA polymerase. Thermal program was performed under 95.00 ˚C for 5 min and 30 thermal cycles, under 94.00 ˚C for 60 sec, under 72.00 ˚C for 60 sec, and the final step was carried out under 72.00 ˚C for 5 min. In the second step, all the conditions were similar to those in the first one and 1.00 µL of the first PCR product was used as a pattern for the second step following dilution in a ratio of 100 to 1. In this step, the second primers were used and a piece with a length of 241 - 840 base primer were replicated and included *C. andersoni*, C*. ryanae, C. parvum,* and *C. bovis*. Standard species were employed as the positive control while the negative control did not have any pattern DNA.

Restriction fragment length polymorphism (RFLP). To identify sub-species, RFLP technique was performed on 18S rRNA.[16] In the next step, genomic DNA was extracted and 18S rRNA was replicated with Nested-PCR. To replicate 845 nucleotides fragment, 18S rRNA was used as reported by Xiao *et al.*[16] Table 2 depicts the internal and external primer sequences.

Inflammatory responses. The blood samples (5.00 mL) were collected from the calves on the same day as the fecal sampling was carried out. They were investigated for the serum concentrations of IL-1β (detection ratio of 6.25 - 4,000 pg mL^{-1}), IL-6 (detection ratio of 2.74 - 2,000 pg mL^{-1}), IL-12 (detection ratio of 6.25 - 400 pg mL^{-1}), TNF-α (detection ratio of 0.10 - 30 ng mL^{-1}), and IFN-γ (detection ratio of 0.11 - 30.00 ng mL^{-1}) with bovine specific ELISA kits produced by Ray Biotech Co. (Norcross, USA).

Statistical analysis. The data regarding molecular and microscopy parts were analyzed with SPSS Software (version 23.0; IBM Corp., Armonk, USA) for the mean and frequencies. The calves were divided into three groups, namely healthy, infected with *C. parvum*, and infected with *C. andersoni* groups. The data were compared via Kruskal-Wallis procedure and the agreement between the methods was investigated with Kappa coefficient.

Results

Microscopic findings. Microscopy results showed that 10.54% of the samples were positive in ZN staining (Table 1).

The results of Nested-PCR. The results of Nested-PCR confirmed microscopy results (Kappa coefficient = 1.00, p = 0.000) and also showed that 92.59% of samples and 7.41% of samples were positive for *C. parvum* (303 bp) and *C. andersoni,* respectively (625 bp; Fig. 1A). The findings for each region are shown in Table 1.

The investigation of species with PCR-RFLP. The results of RFLP confirmed previous results (Kappa coefficient = 1.00, p = 0.000) and showed that 25 samples were identified with VSP enzyme (104 and 628 bp), which implicate the presence of bovine *C. parvum* and genotype A gene sub-species (Fig. 2). Our findings also revealed that Ssp I enzyme showed bands in 385 and 448 bp regions, which implicate the presence of *C. muris*/*C. andersoni* sub-species (Fig. 2C). Figure 2D represents bands at 156, 186, and 224 bp regions confirming *C. muris* sub-species.

Serum concentrations of inflammatory factors. Figure 2 demonstrates the serum concentrations of the inflammatory cytokines. As could be seen, the serum concentrations of cytokines were significantly higher in the infected calves compared to those of non-infected ones. The results confirmed the inflammatory responses in the infected calves. However, the serum concentration IFN-γ was significantly higher in the calves infected with *C. parvum* compared to that in the calves infected with *C. andersoni* ($p < 0.05$). The serum concentrations of IL-6 and TNF-α were significantly higher in the calves infected with *C. andersoni* compared to those in the calves infected with *C. parvum* ($p < 0.05$).

Table 1. The studied regions in the current study and number of positive samples in different methods.

Regions/Farms	No. of animals	No. of infected animals	
		Microscopy	Nested-PCR
Varesh Dibaj	17	2	2
Abir Abad	15	0	0
Ebrahim Mazhari	13	2	2
Ahmad Azizi	14	1	1*
Ali Khorasani	15	1	1
Gholamhossein Jaberzadeh	16	2	2
Hossein Azizian	19	3	3
Mohammad Tehrani	12	1	1*
Verkian	17	2	2
Vamerzan	12	1	1
Gol Narges	16	2	2
Ali Darabian	17	1	1
Mohammad Aminian	13	2	2
Haji Abad Golshan	14	1	1
Sharifieh	13	2	2
Abolfazl Alinezhad	19	2	2
Mohammad Reza Ardakani	14	2	2

* Asterisk shows positive samples for *C. andersoni.*

Table 2. Primers sets for different species of *Cryptosporidium* isolated with Nssm-PCR and 18S rRNA in Nested-PCR for RFLP.

Primer pair	Sequence 5' - 3'	Fragment size	Species detected
AL1687 (EF) AL1691 (ER)	TTCTAGAGCTAATACATGCG CCCATTTCTTCGAAACAGGA	1,370	Genus-specific external
AL1598 (IF) AL3032 (IR)	GAAGGGTTGTATTTATTAGATAAAG AAGGAGTAAGGAACAACCTCCA	840	Genus-specific internal
CaF AL3032 (IR)	GCAAATTACCCAATCCTGAC AAGGAGTAAGGAACAACCTCCA	625	*C. andersoi*
Cr AL 3032 (IR)	TGTTAATTTTTATATCAATTCTACGG AAGGAGTAAGGAACAACCTCCA	415	*C. ryanae*
Cph F AL3032 (IR)	AGAGTGCTTAAAGCAGGCATA AAGGAGTAAGGAACAACCTCCA	305	*C.parvum*
CbF AL3032 (IR)	CTTCTTATTCCTTCTAGAATAAAAATG AAGGAGTAAGGAACAACCTCCA	241	*C.bovis*
18s rRNA internal (primer 1)	TTCTAGAGCTAATACATGCG CCCATTTCCTTCGAAACAGGA	868	-
18s rRNA external (Primer 2)	5'-GGAAGGGTTGTATTTATTAGATAAAG AAGGAGTAAGGAACAACC TCCA	864	-

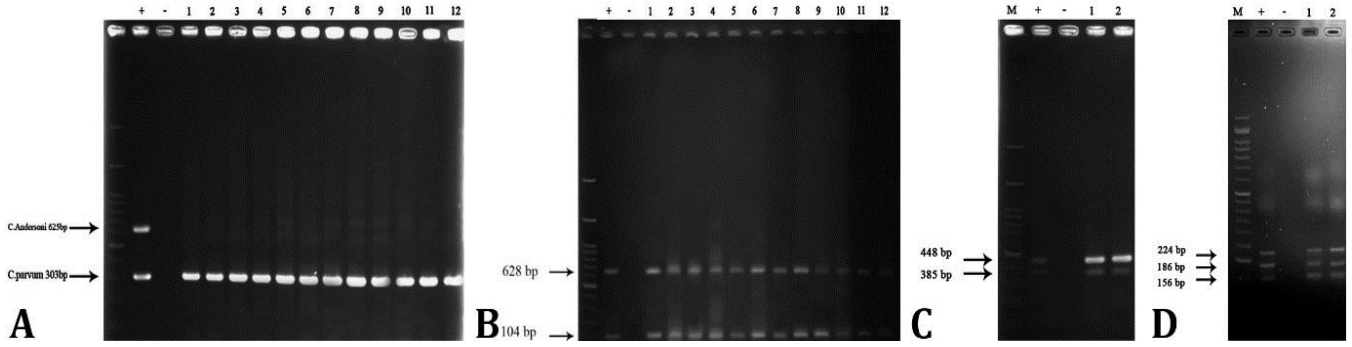

Fig. 1. A) The results of Nested-PCR in the positive samples. The first column (marker), + column (positive column), - column (negative control), columns 1 - 12 show the positive samples for *C. parvum.* **B)** The results of PCR-RFLP under VspI enzyme. The first column (marker), + column (positive column), - column (negative control), columns 1 - 12 show the positive samples for *C. parvum* bovine with genotype A gene sub-species. **C)** The results of PCR-RFLP under SspI enzyme. Columns 1 - 2 show the positive samples for *C. muris*/*C. andersoni* sub-species. **D)** The results of PCR-RFLP under Dde enzyme. Columns 1 - 2 show the positive samples for *C. muris* sub-species.

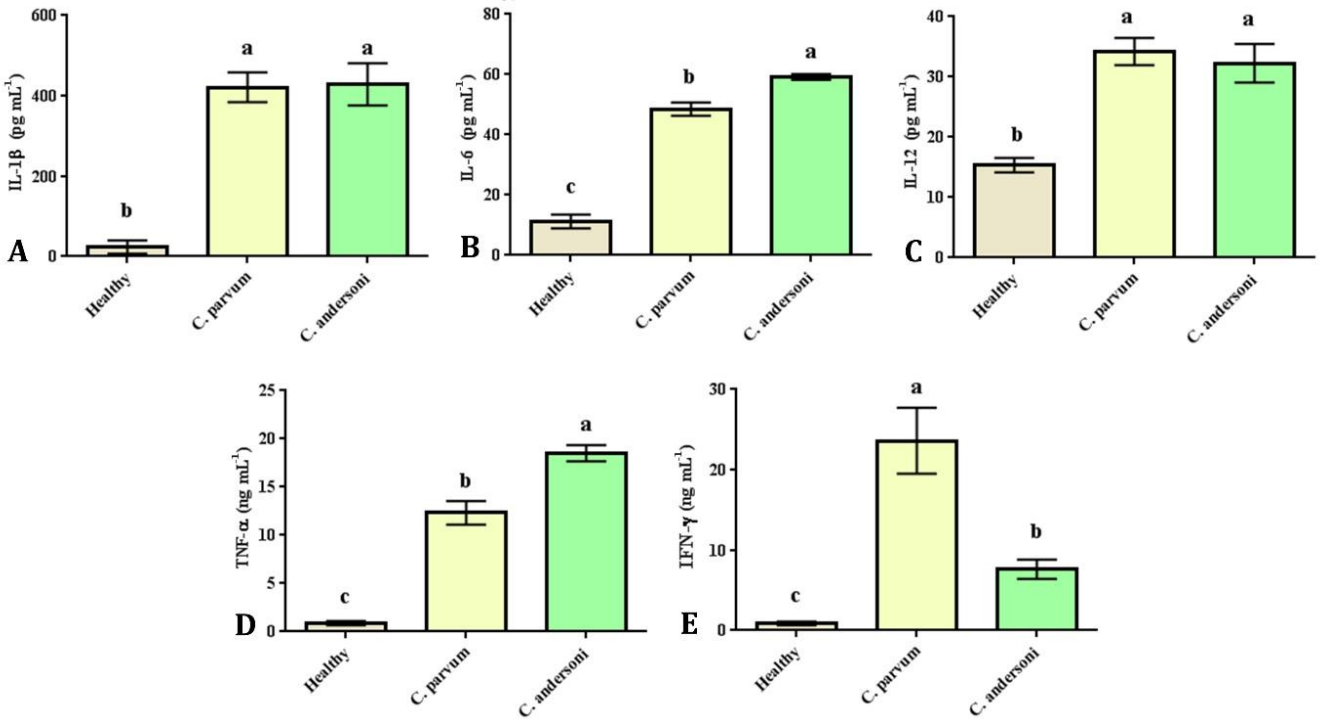

Fig. 2. The serum concentration of inflammatory cytokines.
[abc] Different letters show significant differences among the groups at $p < 0.05$.

Discussion

Microscopy results showed that some samples were positive for cryptosporidiosis. Studies have reported a prevalence rate of 3.40% to 96.60% for *C. parvum* in pre-weaned calves.[4] The disagreement between our findings and those of others could be attributed to the age of calves and applied diagnostic techniques. Fayer *et al.* reported that claves show different levels of sensitivity to cryptosporidiosis in different ages.[17] Their results indicated that Nested-PCR (Kappa coefficient = 1.00, $p = 0.000$) and RFLP techniques (Kappa coefficient = 1.00, $p = 0.000$) have the best agreement with microscopy techniques.

The results of Nested-PCR showed that most samples belonged to *C. parvum* and *C. andersoni* species. In the current study, the samples were collected from pre-weaned calves and *C. parvum* was detected as the most prevalent species. Our results showed that *C. parvum* is the most prevalent species in young calves. In addition, the RFLP results confirmed Nested-PCR technique results and separated *C. muris* from *C. andersoni*. The RFLP produces restriction patterns that are used for identification of sub-species.[18] Accordingly, different methods have confirmed the higher prevalence of infection with *C. parvum* in Iran.

The serum concentrations of IL-1β, IL-6, IL-12, TNF-α, and IFN-γ were significantly higher in the calves infected with *Cryptosporidium*. The serum concentration and expression of IL-1β are higher in the diseases and suppress appetite and increase temperature and inflammation.[19] Beheshtipour and Raeeszadeh reported an increase in the serum concentration of IL-1β in calves infected with diarrhea syndrome.[20] TNF-α and IL-1β increase the migration of leukocytes to the infection site.[8] The results here revealed that TNF-α concentration was higher in the calves infected with *C. andersoni.* The increase in IL-6 is a prognostic marker in neonatal calf diarrhea.[7] The increase in concentrations of IFN-γ and IL-12 could be attributed to the response of the marker to T lymphocytes and natural killer as an immune response. The serum concentration of IFN-γ was significantly higher in the calves infected with *C. parvum*.

Overall, microscopy results demonstrated a low rate of infection in the pre-weaned calves, which was also confirmed by molecular methods. The highest prevalence belonged to *C. parvum* followed by *C. andersoni*. The calves infected with *C. andersoni* showed higher concentrations of IL-6 and TNF-α while the serum concentration of IFN-γ was higher in the calves infected with *C. parvum*. The major limitation of the current research was the rather small size of positive samples. Nevertheless, this study opens a window for future studies.

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Conflict of interest

The authors have no conflict of interest to declare.

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