Original Article

Prevalence and genetic characterization of *Cryptosporidium* in pre-weaned cattle in Urmia (Northwestern Iran)

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Abstract

Introduction: *Cryptosporidiosis* is a zoonotic disease causing digestive problems in pre-weaned calves. Considering the zoonosis of the parasite and its importance in veterinary medicine, we evaluated the prevalence and genotyping of *Cryptosporidium* spp. in diarrheic pre-weaned calves in the northwest of Iran.

Methodology: A total of 100 stool samples of the infant calves with diarrhea were collected from industrial and conventional livestock farms in Urmia City. All the samples were tested with acid-fast staining, ELISA, and PCR. Positive samples of the PCR method were sequenced to determine the *Cryptosporidium* species. The obtained results were compared for the mentioned methods based on statistical factors, sensitivity, specificity, positive and negative predictive values, as well as duration of the experiment and the costs of testing.

Results: The results of this study showed that the prevalence of *Cryptosporidium* spp. in diarrheic infant calves in Urmia city was 5%, and *C. parvum* species of *Cryptosporidium* was detected in all the sequenced samples. According to the findings of the current study, the most appropriate method for the detection of the parasite is the ELISA that has a higher sensitivity and predictive value than acid-fast staining method and should be used in veterinary laboratories.

Conclusions: In the current investigation, *C. parvum* was identified as the only infectious agent in the region and could be the main cause of human infection. More studies are needed to find the source of infection for establishing the control measures.

Key words: *Cryptosporidium*; prevalence; genetic characterization; sensitivity; specificity.

Introduction

Cryptosporidium is a zoonotic protozoan parasite infecting the gastrointestinal tract of a wide range of vertebrates, including humans, livestock, wild animals, and birds [1]. The presence of *Cryptosporidium*-infected animals, especially domestic ones, poses as a serious risk factor to human health. The infection of this parasite is mainly transmitted from animals to humans by resistant oocytes.

Currently, *Cryptosporidium* infection in cattle have been associated with four main species, i.e. *C. parvum*, *C. bovis, C. andersoni*, and *C. ryanae*; however, species such as *C. suis*, *C. hominis*, *C. serpentis*, *C. xiaoi*, *C. ubiquitum*, *C. meleagridis*, *C. muris*, and *C. felis* have been identified. *C. andersoni* gives rise to mucosal destruction of the abomasums in cattle, but *C. parvum*, *C. bovis*, and *C. ryanae* often cause atrophy of villus, shortening of microvillus, and destruction of the intestine, which leads to diarrhea [2-4]. *C. parvum* generally infects human and cattle, whereas *C. andersoni* and *C. bovis* have infrequently been detected in humans [5,6]. Hence, infected cattle are regarded potentially significant reservoirs of *Cryptosporidium* for human infections. *C. parvum* is chiefly detected in pre-weaned [7], while *C. ryanae* and *C. bovis* are found in post-weaned [8]. Meanwhile, *C. andersoni* have been reported to primarily infect adult calves [9]. Other species such as *C. felis* [10], *C. scrofarum* [11], *C. suis*-like genotype [12], *C. suis* [12], *C. hominis* [12,13], *C. ubiquitum* [14], and *C. meleagridis* [15] have also been isolated from cattle; therefore, pre-weaned cattle are the most leading factor of infection for humans. Contamination with this protozoan in calves is highly important in terms of economic damages; in Iran, calf casualties are virtually 16%, of which 75% is related to diarrhea [16,17].

Diverse approaches have already been suggested for the detection of *Cryptosporidium*. The majority of the approaches encompass direct examination of stained fecal smears under microscope [18]. The modified acid-fast staining is broadly applied for clinical diagnosis owing to its simplicity and cost

effectiveness. However, it displays relatively low sensitivity with feces [19,20]. Although several immunological techniques using antibodies or antigens have hitherto been developed, all of them are more expensive than conventional staining methods. Nonetheless, their sensitivity and specificity appear to be the same [21]. PCR-based methods are additional valuable techniques for detecting *Cryptosporidium* spp. in clinical samples. The PCR technique has high sensitivity and specificity and can detect organisms such as *Cryptosporidium*, just with a single cell. Nucleotide amplification methods that target genes encoding the oocyst wall protein, the small-subunit of rRNA, β-tubulin, TRAP-C1, TRAP-C2, ITS1, polythreonine repeat, unknown DNA sequences, mRNA of heat shock proteins, and dihydrofolate reductase are successful for detecting and differentiating *Cryptosporidium* parasites [22]. Partial sequencing of *Cryptosporidium* small subunit (SSU) rRNA gene has routinely been applied (in 86% of publications) to genotype *Cryptosporidium* in the environment (i.e. water and invertebrate sources), wild and domestic animals, and human samples. Multiple copies of the SSU rRNA gene throughout the genome render this marker significantly more sensitive than single-copy genes. PCR amplification and sequence analyses of the amplicon allow genetic characterization and differentiation between *Cryptosporidium* spp. that assists in finding the possible and actual risk sources to human or animal health. The prevalence of cryptosporidiosis has been reported to be high among renal transplant recipients in Urmia (11.5%) [23], suggesting that the area is highly contaminated with the oocysts of the parasite. The present study aimed to investigate the rate of infection with *Cryptosporidium* spp. in pre-weaned calves in Urmia city, West Azerbaijan province, Iran. Additinally, the diagnostic methods viz the acid-fast staining, molecular method, and ELISA were compared in terms of statistical factors, duration of the laboratory experiment, and the cost-effectiveness of testing, to determine the superior method for the detection of *Cryptosporidium* in the infected calves.

Methodology
Sample collection
For this study, 100 stool samples were collected from diarrheic pre-weaned calves in 10 conventional and industrial livestock farms from October 2017 to March 2018. The samples in a plastic container were transferred to the Parasitology Laboratory at Urmia Medical School on the same day of collection.

Laboratory evaluation
Due to the impossibility of performing the methods simultaneously, each diarrheal stool sample was divided into three parts: one part for the diagnosis of *Cryptosporidium* oocytes by the acid-fast staining method of Ziehl-Neelsen, one part for PCR method (kept in 70% alcohol test tube), and one part for the ELISA method (kept in saline at -20ºC).

Ziehl-Neelsen staining method
The sedimentation method of formalin-ethyl acetate was first carried out for all the samples. To perform the method, 7 mL of formalin was poured into each test tube, 2-3 ml of the diarrheal stool was removed by Pasteur's pipette and added to the tubes, and three milliliters of ethyl acetate was added. The tubes were then shaken vigorously to mix formalin, stool, and ethyl acetate. After five minutes of centrifugation of the mixture at 450 ×g, four layers were formed. The layer from top to bottom contained ethyl acetate, fat and stool wastes, formalin, and the sediments containing oocytes. The first three layers were discarded, and a smear was prepared on a glass slide and dried at room temperature. The air-dried, methanol-fixed slides were then stained by Ziehl-Neelsen method. Briefly, the slides were stained with carbol fuchsin for 15 minutes, decolorized with 50% alcohol for 3 - 5 seconds, and finally washed with water. The samples were decolorized with 1% sulfuric acid until the disappearance of red color and then rinsed with water. After the slides were dried, the samples were stained with methylene blue for one minute and then washed with water. The slides were evaluated under a microscope with the magnification of 1,000, and *Cryptosporidium* oocytes were identified (4 - 6 microns, red cells with black granules in the blue background). The duration of acid-fast staining method to detect *Cryptosporidium* was 30 minutes [24].

PCR method
The DNA was extracted using a commercial kit (Yekta Tajhiz Azema, Iran) according to the manufacture's instruction. Forward and reverse primers designed for the multiplication of *SSU rRNA* gene of *Cryptosporidium* spp. were as follows: Forward primer: 5'GACATATCATTCAAGTTTCTGACC3' and Reverse primer: 5'CTGAAGGAGTAAGGAACAACC3'. To perform the experiment, 1 μL of each forward and reverse primer, 3 μL of extracted DNA, 12.5 μL of Master Mix, and 7.5 μL of sterile distilled water were added to the micro-tubes, and the experiment was performed with a thermocycler device with the following program: 2 min

incubation at 94°C to denature double-stranded DNA, 35 cycles of 60 s at 58°C (annealing step), 120 s at 68°C (extension step), and 45 s at 94°C (denaturing step). PCR was finally accomplished with the additional extension step for 7 minutes at 68°C.

The PCR product was loaded on 1% agarose gel and electrophoresed using 1% solution of TBE for 20 minutes. Finally, digital images were taken using a gel imaging system. *Cryptosporidium* spp. bands with the length of 830 bp were identified. Positive PCR products were sequenced, and the sequences were visualized using the Chromas software version 2.6 and compared to those registered in the GenBank using Basic Local Alignment Search Tool (BLAST) software. Duration of DNA extraction of *Cryptosporidium* and PCR method for the detection of this parasite was eight hours.

ELISA method

The experiment was performed using a commercial ELISA kit (*Cryptosporidium* 2nd Generation [Fecal], USA). Six samples were randomly removed due to the limitation of ELISA plate wells. To conduct the experiment, positive and negative controls were poured into wells 1 and 2, respectively, and buffer solution was added to all the wells. Afterward, samples were added to each well individually, except for negative and positive control wells, and incubated at room temperature for one hour. The plates were rinsed with washing solution, and then the conjugated enzyme was added to all the wells. The plates were eventually incubated at room temperature for 30 minutes. The wells were washed with washing solution, and 100 μL of chromogenic solution was added to each well, and the plate was incubated at room temperature for 10 minutes. In the end, 100 μL of stop solution was added to each well, and the optical densities were read by an ELISA reader device. The optical density above 0.08 was considered as positive for *Cryptosporidium*

antigens. The duration of the ELISA method took 2.5 hours to detect *Cryptosporidium* antigens.

Statistical analysis

In the current study, acid-fast staining, PCR, and ELISA methods were compared in terms of sensitivity and specificity, as well as negative and positive predictive values [25]. PCR method was considered as the gold standard.

$$Specificity = \frac{FalseNegative}{FalsePositive + TrueNegative} \times 100$$

$$Sensitivity = \frac{Truenegative}{FalseNegative + TruePositive} \times 100$$

$$Negativepredictivevalue = \frac{TrueNegative}{FalseNegative + TrueNegative} \times 100$$

$$Positivepredictivevalue = \frac{TruePositive}{FalsePositive + TruePositive} \times 100$$

Results

Stool samples under study were collected from 10 (four industrial and six conventional) livestock farms, where the health status of animals varied from inappropriate to good. Five samples were positive for *Cryptosporidium*: two from Band, one from each of Karim Abad, Rahim Abad, and Shorkandy farms. Based on the data from Table 1, the percentage of *Cryptosporidium* infection prevalence in pre-weaned calves suffering from diarrhea in Urmia city was 5%.

Among 100 stool samples, Ziehl-Neelsen staining method detected five positive cases, while three cases were confirmed by PCR; two cases were false-positive

Table 1. The location of sampling from diarrheic calves, the type and health status of the livestock farms, and *Cryptosporidium*-positive cases using different methods.

Location of the livestock farm	Type of the livestock farm	Health status	PCR method	Staining method	ELISA method
Khangah Sorkh	conventional	Good	-	-	-
Karim Abad	conventional	Inappropriate	+	+	+
Rahim Abad	conventional	Proper	+	+	-
Saatluo	conventional	Proper	-	+	-
Shorkandy	industrial	Inappropriate	+	-	+
Arnesa	industrial	Good	-	-	-
Dastgerd	industrial	Good	-	-	-
Band	conventional	Inappropriate	+ +	+ -	+ +
Gara-aghaj	conventional	Inappropriate	-	+	-
Par	industrial	Good	-	-	-

and two cases were false-negative. ELISA method identified four positive cases, which all were affirmed by PCR. Therefore, one case was false-negative, but there was no false-positive cases. The results are summarized in Table 1.

In this work, Ziehl-Neelsen staining and ELISA were compared with PCR method regarding the statistical parameters. Sensitivity, specificity, positive predictive value, and negative predictive value for the staining method and ELISA were 60%, 97.89%, 60%, and 97.89%, as well as 80%, 100%, 100%, and 98.8%, respectively.

Five *Cryptosporidium*-positive isolates in PCR method were sequenced by Kawsar Biotech Company (Iran) based on the Sanger method. Sequence results showed that all the five isolates were *C. parvum* and registered in the GenBank under the accession numbers MK426792, MK426793, MK426794, MK426795, and MK426796.

Discussion

Cryptosporidium infection has been reported by numerous countries so that the prevalence rates of the infection in the cattle in India, Japan, France, China, Argentina, and Turkey were 26.15%, 33%, 39%, 2.55%, 8%, and 7.5%, respectively [26-31]. In the present study, the prevalence rate of the infection in pre-weaned calves suffering from diarrhea in Urmia city was 5%. The results of the present study in terms of *Cryptosporidium* infection prevalence was relatively close to those obtained from Isfahan and Ilam cities, which were 6.25% and 3.68%, respectively [32,33]. Previous studies, however, showed a higher prevalence of *Cryptosporidium* infection in Urmia (22.3%), as well as Hamadan (12.8%), and East Azerbaijan provinces (41.1%) [17,34,35]. In another study by Heidarnegadi *et al.* [36] in Khuzestan province in different seasons of the year, from August 2009 to April 2011, the infection was detected in 14.5% of human samples and 51.9% in pets [36]. Mirzai *et al.* [35] revealed that the prevalence of *Cryptosporidium* infection in Urmia city was higher in winter than in the warm season. However, in Nourmohammadzadeh *et al.*'s [17] and Heidarnegadi *et al.*'s [36] studies, the percentage of contamination in spring and summer was higher than the other seasons. In the current study, the percentage of *Cryptosporidium* infection among diarrheic calves in Urmia reduced due to various reasons, including sampling season, increasing the awareness of livestock keepers, and monitoring the health of livestock farms, especially industrial ones. Moreover, the rate of *Cryptosporidium*

infection in industrial livestock farms was less than the conventional ones.

In this study, three diagnostic methods of *Cryptosporidium* were compared in stool samples by considering some factors, such as cost of the methods, time of performing the experiments, sensitivity, specificity, and positive and negative predictive values of the methods, to determine the superior method for detecting the parasite. Most comparisons of *Cryptosporidium* diagnostic methods have performed in human specimens, but we compared the diagnostic methods in calves to obtain a standard and cost-effective method. Our results showed that PCR and ELISA methods were more accurate than the microscopic method. Yilmaz *et al.* [37] made a comparison between two methods, ELISA and acid-fast staining, using the stool samples of 2,000 children in Turkey, in order to detect *Cryptosporidium* parasite. The ELISA results identified *Cryptosporidium* antigens in 97 children, while acid-fast staining method found 31 positive cases, suggesting the higher sensitivity of the ELISA compared to the staining method [37]. In a study performed in 2015, Gawad *et al.* [38] collected fecal specimens from 200 diarrhea patients in Egypt for the detection of *Cryptosporidium*. All the specimens were tested with acid-fast staining, sandwich ELISA, and PCR methods; their results showed 19 (9.5%), 25 (12%), and 42 (21%) positive cases, respectively. Comparing the staining method and ELISA with PCR technique, as the gold standard, displayed the sensitivity of 45% and specificity of 100% for staining method and 57% and 96% for ELISA, respectively [38]. Our results were in line with those reported by Yilmaz *et al.* [37] and Gawad *et al.* [38].

In this study, regarding the costs and the time spent for conducting the experiments, the acid-fast staining method required less time to perform, and it was more cost-effective than ELISA and PCR, but low sensitivity and predictive value were the downsides of the method. These disadvantages increase the false-negative cases, and, therefore, the true-positive cases may be ignored. ELISA method did not need a long time to perform and had high sensitivity, which is comparable with the molecular methods, and its cost was affordable to the breeders and livestock farmers. PCR method is a time-consuming and costly experiment and is not a cost-effective test for livestock farmers, but it is more suitable for researches. According to the results of the present study, it is recommended to apply ELISA method for the detection of *Cryptosporidium* in diarrheic calves in veterinary laboratories. Using the ELISA method, as a cost-effective and sensitive method

for detecting *Cryptosporidium* in diarrheic calves, which are the potential carriers of the parasite and a possible contaminator of the environment, could prevent the destruction of community's valuable protein sources and provide significant contribution to the livestock farmers, thereby promoting the economy of the livestock farming sectors and public health.

The current work is the first research on the prevalence and molecular characterization of *Cryptosporidium* in pre-weaned cattle in West Azerbaijan province in the northwest of Iran. In this study, all the species isolates from pre-weaned cattle were identified as a *C. parvum* using PCR. Santín and the colleagues [39] found 19.2% positive cases in cattle of all ages in Meryland (USA), but in pre-weaned calves, the prevalence was 45.8%. Besides, 97% of the samples were *C. parvum*. Similar to our finding, various investigations conducted in Iran have found *C. parvum* as the dominant species of *Cryptosporidium*. Using nested PCR-RFLP of SSU rRNA (SSU 18s rRNA) gene, Saki and Asadpouri [40] showed that all the positive *Cryptosporidium* spp. cases from cattle in the southwest of Iran were *C. parvum*. Oskouei *et al.* [33] reported that all 217 fecal samples (3.68% - 8/217) from cattle in Western Iran were *C. parvum*-positive. The same result was also obtained by Asadpour *et al.* [41] in the northeast of Iran. In cattle in Qazvin province, 72.6% of positive cases belonged to *C. parvum*, followed by *C. andersoni* (17.7%), *C. bovis* (7.8%), and a new subgenotype of *C. parvum* (1.9%) [42]. Mirzai *et al.* [35] have also identified *C. parvum* and *C. andersoni* in cattle in the northwest of Iran. A number of studies in Northwestern and Northeastern China, Brazil, and Sir Lanka have demonstrated that the common incidence of *C. bovis*, *C. ryanae*, and *C. andersoni* is due to the lack or low prevalence of *C. parvum* in pre-weaned cattle, which is not in agreement with our results [15,43-45].

Conclusions

In the current investigation, we utilized microscopy, immunological and molecular methods to determine the prevalence and discernment of *Cryptosporidium* spp. in pre-weaned cattle of Urmia city, in the northwest of Iran. *C. parvum* was identified as the only infectious agent in the region and could be the main cause of human infection. More studies are needed to find the source of infection for establishing the control measures.

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