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**Original Article**

# Prevalence and Molecular Identification of *Cryptosporidium* Spp. in Pre-Weaned Dairy Calves in Mashhad Area, Khorasan Razavi Province, Iran

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***Abstract***

***Background:*** *Cryptosporidium parvum* is a zoonotic pathogen transmissible from a variety of animals to humans and is a considerable public health concern. Dairy cattle have been identified in numerous reports as a major source of environmental contamination with this pathogen. The aim of study was to detect and isolate the *Cryptosporidium* spp. from fecal samples of naturally infected pre-wean calves in the Mashhad area

***Methods:*** Overall, 300 fecal specimens from 1 to 30 days pre-weaned calves were collected from 10 farms in the Mashhad area the capital center of the Khorasan Razavi Province, Iran and microscopically examined for *Cryptosporidium* spp. All infected samples were also analyzed using nested –PCR. A polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of the small-subunit (SSU) rRNA gene was also used to detect and identify *Cryptosporidium* spp. in PCR- positive samples.

***Results:*** Eighty five (28.3%) of the specimens were positive for *Cryptosporidium* spp. The prevalence of *Cryptosporidium* spp. in 8-14 days old and diarrheic calves were significantly higher than other groups. Restriction digestion of the PCR products by *SspI*, *VspI* restriction enzymes and sequence analysis revealed the presence of *C. parvum* bovine genotype in all isolates.

***Conclusions:*** Our results suggest that pre-weaned calves are likely to be an important reservoir of zoonotic *C. parvum*.

## Introduction

*Cryptosporidium* spp. is an important intestinal protozoa in man and animals (1). Cryptosporidiosis can cause diarrheal illness in humans, which may be severe in immunocompromised individuals. Transmission usually occurs through the direct fecal-oral route or through oocysts contaminated water or food. However, the potential for zoonotic transmission of *Cryptosporidium* spp., particularly with respect to cattle and other livestock, has generated considerable interest in recent years (2-4).

Some livestock, such as cattle, sheep, and goats, harbor *Cryptosporidium* species that are infectious for humans. The use of molecular diagnostic techniques in the characterization of *Cryptosporidium* spp. has led to increased recognition of the diversity of parasites infecting humans and animals and role of animals in the transmission of human cryptosporidiosis (5).

Studies of cryptosporidiosis in dairy cattle have identified several *Cryptosporidium* species in cattle (6). Previous studies demonstrated that cattle could be infected with 10 different *Cryptosporidium* species or genotypes at least; nevertheless, *C. parvum*, *C. andersoni*, *C. bovis*, and *C. ryanae* were commonly detected in cattle worldwide. All of them were found in the different age groups, especially *C. andersoni* (7, 8); Although dairy cattle have been considered to be a major host for *C. parvum*, only pre-weaned calves are frequently infected with this species (6), while *C. bovis* and *C. ryanae* are considered predominantly infectious to post-weaned calves (9, 10).

Economic losses due to cryptosporidiosis of neonatal calves depend on the *Cryptosporidium* species/genotypes causing the infection. While *C. parvum* infection is caused diarrhea, growth retardation, dehydration and losses to the dairy industry in terms of increased labour and veterinary costs associated with calf morbidity and, occasionally, mortality (11), calves are infected with other *Cryptosporidium* species or genotypes exhibit no overt clinical signs (8, 12). Although previous studies demonstrated that some animals were important zoonotic reservoirs of *Cryptosporidium* in humans (13), and cattle could be infected with 6 out of 15 human pathogenic *Cryptosporidium* species (14), only *C. parvum* is important for the public health in relation to cattle management.

At least 11 different *C. parvum* families (IIa-IIl) with many subtypes have been described on the basis of sequence analysis of a 60-kDa glycoprotein (GP60) from humans and other mammals including cattle (15). The objective of this study was to detect and isolate the *Cryptosporidium* spp. from fecal samples of naturally infected pre-wean calves in the Mashhad area, the capital of the Khorasan Razavi Province, Iran and to determine the genetic characterization of these isolates.

## Materials and Methods

### *Study population*

The study was performed in the northeast region of Iran, in Mashhad area, the capital city of Khorasan Razavi Province. The city is located at 36.20° latitude and 59.35° east longitude, in the valley of the Kashaf River near Turkmenistan, between the two mountain ranges of Binalood and Hezar-Masjed. The city benefits from the proximity of the mountains and having very cold winters. Mashhad has more than 500 dairy farms with an estimated 70,000 cows of mostly Holstein/-Friesian breed.

### *Sample collection and coprodiagnosis*

In the present study, 10 industrial dairy herds were selected. The cow breed in all these farms was Holstein/Friesian, and in all farms, calves were separated from their dams after they received colostrums and were housed in individual pens until weaning at 3 months of age. Approximately 10 g of fecal

specimens were collected from the rectum of 300 randomly selected 1–30 days pre-wean calves with or without diarrhea in 10 farms. All the stool samples were previously tested for the presence of oocysts of *Cryptosporidium* spp. using the Ziehl –Neelsen staining technique (24, 25 ,36). The samples positive in the coprodiagnostic test were stored at 4 ˚C in 2.5% potassium dichromate (16).

### Oocysts purification

The positive samples for *C. parvum*-like oocysts stored in 2.5% potassium dichromate were submitted again to the flotation–concentration method using disposable material and the floated material used in the diagnosis was washed from the slide and the glass coverslip using 1 ml of TE (Tris–EDTA pH 8.0), in a disposable Petri dish, placed in a 1.5 ml microtube, and later submitted to DNA extraction (16).

### DNA extraction from oocysts

The purified oocysts were cleared by centrifugation at 12,000 × g for 5 min, and then ressuspended in 200 ml TE–SDS (Tris–HCl 10 mM, EDTA 1mM, SDS 1%). After that, the oocysts suspension was submitted to seven freeze and thaw cycles in liquid nitrogen and dry bath at 65˚ C, respectively (17). DNA was extracted using a DNA isolation kit (MBST, Iran) based on a selective binding of nucleic acids to a silica-based membrane, according to the manufacturer's instructions.

### Nested-PCR

Nested polymerase chain reaction (PCR) was used to amplify a fragment (826– 864 bp) of the SSU rRNA (18S) gene using two sets of oligonucleotide primers as described previously(18,19). PCR products were visualized on 2% agarose gel after ethidium bromide staining.

### RFLP using SspI and VspI restriction enzymes

For detection up to species level of *Cryptosporidium*, RFLP analysis using restriction enzymes *SspI* and *VspI* (Fermentas Life Sciences, Lithuania, Vilnius) of nested-PCR products were performed (18). Products were analyzed by gel electrophoresis in 0.8% agarose containing ethidium bromide (0.5 µg/ml) (17). The positive *Cryptosporidium* bovine species yields three distinct bands at 449 bp, 254 and 108 bp after the digestion of 2nd PCR products with *SspI*, also the common genotype yields two distinct bands at 104 bp and 628 bp after the digestion of 2nd PCR products with *VspI* (18).

### Sequencing

All amplicons of Nested-PCR products that have different RFLP patterns were sequenced, and SSU rRNA gene sequence was compared with sequences of *Cryptosporidium spp*. and the obtained sequences were confirmed for their uniqueness by performing BLAST with the NCBI nucleotide database (http://www.ncbi.nih.gov).

### Statistical analysis

The prevalence of Cryptosporidium infection in Pre-weaned dairy calves was compared based on the different age groups, sex and diarrheic or none- diarrheic groups. The Chi-square test was used to analyze the data and differences were considered significant when $P < 0.05$. Statistical analysis was performed using SPSS software (Ver. 20).

## Results

*Cryptosporidium* spp. oocysts were microscopically in 28.3% (85/300) of fecal samples. Fecal samples were classified according to the consistency as diarrheic (45/300) and non-diarrheic (255/300). Diarrhea was recorded in 100% (45) of the positive samples. The prevalence of *Cryptosporidium* spp. infection in diarrheic calves was significantly higher than non-diarrheic calves ($P<0.05$) (Table.1), Also the highest frequency of infection was seen in 8-14 days old group ($P<0.05$).

**Table.1:** Prevalence of *C. parvum* infections by different risk factors in the pre-weaned calves

| Risk factors | Total | Infection rate | | *P* value |
|---|---|---|---|---|
| **Gender** | | No. | **(%)** | |
| Male | 145 | 46 | 31.72 | *P*>0.05 |
| Female | 155 | 39 | 25.16 | *P*>0.05 |
| Age | | | | |
| 1-7 days | 45 | 17 | 37.77 | |
| 8-14 days | 48 | 28 | 58.33 | *P*<0.05 |
| 15-21 days | 55 | 17 | 30.90 | |
| 22-30 days | 152 | 23 | 15.13 | |
| Stool Consistency | | | | |
| diarrheic | 45 | 45 | 100 | *P*<0.05 |
| Non- diarrheic | 255 | 40 | 15.68 | |

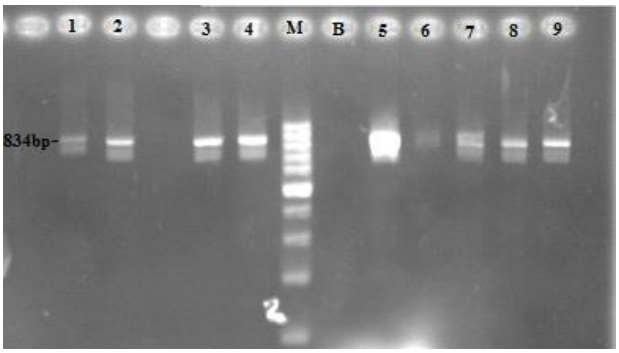


**Fig.1:** Ethidium bromide-staining 1% agarose gel with small-subunit rRNA – based secondary PCR products of calve stool samples. Line B, negative control. Lane M, 100bp 100 bp molecular marker, Line 5, positive control and other lines, DNA from *Cryptosporidium* oocyst in samples 826-834bp

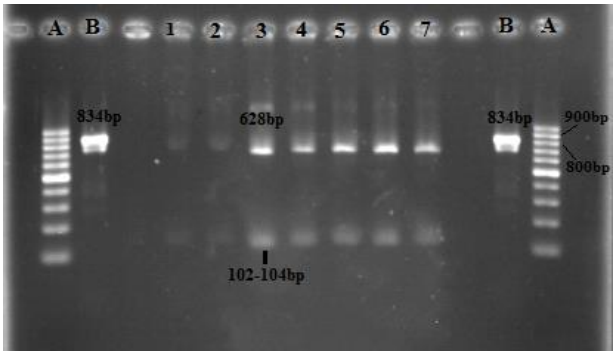


**Fig. 2:** Gel electrophoresis of *Cryptosporidium* SSU rRNA. PCR–RFLP products resulting from digestion of the nested-PCR products (secondary PCR product). with *VspI* restriction enzyme. Line A, 100 bp molecular marker. Line B, nested-PCR product. Line 1through 7, *C. parvum*

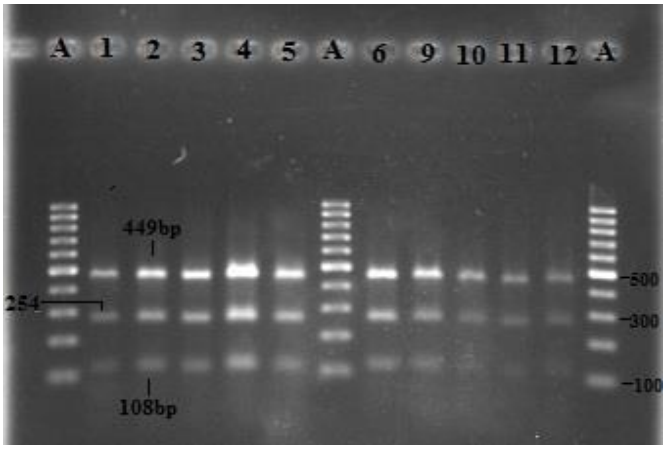


**Fig. 3:** Gel electrophoresis of *Cryptosporidium* SSU rRNA. PCR–RFLP products resulting from digestion of the nested-PCR products (secondary PCR product). with *SspI* Restriction enzyme. Line A, 100 bp molecular marker./ Line 1through 12, *C. parvum*

Statistically there was no significant correlation between infection rate and sex factor ($P>0.05$).

All of the infected samples were also positive by nested PCR (Fig.1). Forty five of nested- PCR positive were selected and identified as *C. parvum* by RLFP using *SspI* and *VspI* (Fig. 2) (Fig. 3). All of the 45 positive samples have shown a similar band on gel electrophoresis. Subsequently, 15 of sequences were obtained; 100% (15/15) were *C. parvum* bovine genotype, Sequence analysis showed 100% homology to the registered in GenBank for *C. parvum* under accession number: *JX237833.* *C. parvum* was only isolated from calves aged <30 days old, with the highest peak at 58.33% detected in 8-14 age group.

## Discussion

Although many studies dealing with cryptosporidiosis of Pre-weaned calves have been published in Iran, our study represents the second report where the data is supported by molecular techniques. In the present study, the frequency of *Cryptosporidium* spp. infection was 28.3% in the pre-weaned calves. In comparison with studies conducted in other countries, this frequency rate was lower than the infection rates (48–100%) in the pre-weaned dairy calves (9, 20), but, in agreement with results of other studies (21, 22). A few studies were done on the frequency of *Cryptosporidium* spp. infection in calves in Iran. Our frequency was lower than a similar study that was done in calves in Mashhad area, Iran (24), and higher than another study in Iran. (25). In the present study the high prevalence of infection was observed in 8-14 days age group of calves and diarrheic. The result was along with the previous study that done this area (24) and other studies that reported in other countries (6, 9, 23, 26-28). All infected samples were examined with nested-PCR and confirmed the microscopic results. In the present study, all positive-PCR samples was detected such a *C. parvum* bovine genotype (100%). In some studies, it has been reported that the zoonotic *C. parvum* is responsible for the majority of *Cryptosporidium* infections in Pre-weaned calves, and only a small percentage of *Cryptosporidium* infections in post-weaned calves and heifers (6, 9, 29-31). Results of some studies have indicated that there are not significant correlations between the calf with age, oocyst excretion and the species/ genotypes of *Cryptosporidium* spp. (26,32-34). As well as the man are predominantly infected with the *C. parvum* bovine genotype and *C. hominis* via water, food and person-to-person or animal–human contact (8, 35, 36). In the present study, we used a RFLP-PCR with *SspI* and *VspI* restriction enzymes to differentiate of *C. parvum* from *C. hominis*. Our results demonstrated that *C. parvum* is the major cause of infection in the preweaned calves in Mashhad farms. This finding was in agreement with similar studies (6, 10, 35). Besides our study indicated that the bovine genotype of *C. parvum* could be the majority of *Cryptosporidium* isolates that is very important as a primary cause of human infections (13, 14, 35).

## Conclusion

In the present study, we used a microscopy and molecular examination to detect and differentiate of *Cryptosporidium* spp. in dairy farms. Our results demonstrated that *C. parvum* is the major cause of infection in preweaned calves in Mashhad farms and it could be a primary cause of human infections.

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