Molecular characterization of bovine Cryptosporidium using Cryptosporidium oocyst wall protein (COWP) gene

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ABSTRACT

Cryptosporidium is a globally distributed protozoan parasite and one of the most common causes of infection and diarrhea in humans and cattle. The aim of the present study was to determine the species of Cryptosporidium among cattle with diarrhea by a nested PCR-RFLP technique at Cryptosporidium oocyst wall protein (COWP). Fecal samples from 158 calves aged 1-20 weeks were collected from 10 dairy farms in Qazvin province, Iran. Initial identification of Cryptosporidium was carried out by Ziehl-Neelsen acid-fast staining method of stool samples. DNA was extracted from 26 (16.45 %) positive microscopically samples and Cryptosporidium genotypes were determined. Cryptosporidium parvum were identified in 80.8% of the positive samples and, Cryptosporidium andersonii in 19.2%. In conclusion the use of COWP primers could be sensitive enough to conduct a routine detection study. The nested PCR method using the COWP gene sequence can be an alternative diagnostic method to identify infected with Cryptosporidium and its genetic diversity.

Keywords: Genotypes; bovine Cryptosporidium; COWP gene; cattle; Iran.

INTRODUCTION

Cryptosporidium is responsible for significant diarrheal diseases in both developing and developed countries [1]. Recent studies suggest that at least four Cryptosporidium species or genotypes including Cryptosporidium parvum, Cryptosporidium bovis, Cryptosporidium andersonii and Cryptosporidium ryanae infect cattle. Cryptosporidium ryanae identified as a Cryptosporidium deer-like genotype. The occurrence of these Cryptosporidium spp. in cattle is age-related [2-6], and at least two species of Cryptosporidium are commonly detected: C. parvum, dominant in pre-weaned calves (<2 months), and C. andersonii infecting older calves and adult cattle[2, 7]. C. bovis and C. ryanae are considered to be predominant in post-weaned calves (2–11 months)[2,3].

In the recent years, researchers have developed PCR-based techniques for detection and identification of Cryptosporidium spp. One genetic locus which allows differentiation between Cryptosporidium isolates is the COWP gene, and genotyping methods based on PCR/restriction fragment length polymorphism (RFLP) analysis of a fragment of this gene have been described previously [8-10]. There are several molecular studies which have documented the prevalence of Cryptosporidium in human and cattle base on 18s rRNA, 18s SSU rRNA, TRAP-C2 and GP60 genes [1, 9, 11-16]. In the present study, we identified the genotypes of the Cryptosporidium isolates from Iran, using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analyses of the COWP gene locus.

MATERIALS AND METHODS

Samples collection

Total of 158 stool specimen from calves with mean age±SD 12.3±4.22 (months) were collected from 2005 to 2007 in Iran and Cryptosporidium oocysts were recorded positive by modified Ziehl-Neelsen staining. The positive Cryptosporidium spp. isolates were preserved in 2.5% potassium dichromate and kept at 4°C until used for DNA extraction. Total DNA was extracted from all positive specimens using a DNAzol kit according to the manufacturer’s instructions (Invitrogen, life technologies, Cat. No
10503-027, USA) with addition of three times 10 minutes freeze-thaw cycles after resuspending in lysis buffer in order to rupture the Cryptosporidium oocysts.

**Nested PCR**

Nested PCR was used to amplify a fragment of the COWP gene using two sets of oligonucleotide primers: TGTCCTCCAGGTACTACA and ACCTGTTCCTCACTCAATG for primary PCR and GTAGATAATGAGAGATTGTG and GGACTGAAATACAGGCATTATCTTG for secondary PCR, as described previously [8]. Primary amplification was carried out in 32 cycles of 94°C for 45 s, 40°C for 45 s, and 72°C for 1 min, with an initial denaturation at 94°C for 3 min and a final extension at 72°C for 7 min. For secondary amplification, 32 cycles of 94°C for 35 s, 40°C for 35 s, and 72°C for 1 min were used, with identical initial denaturation and final extension conditions. Then PCR products were visualized on 1% agarose gel after ethidium bromide staining. For RFLP analysis of secondary PCR products, we used restriction enzyme Rsal restriction enzyme (Fermentas life sciences) to digest 1 mg of the secondary PCR products of the SSU rRNA gene. The restriction digestion products were visualized by electrophoresis on 2% agarose gel after ethidium bromide staining.

**RESULTS**

Out of 158 collected samples, 26 (16.45 %) were found to be contained Cryptosporidium oocysts (figure 1). The PCR-RFLP analysis of nested PCR products of Cryptosporidium based on COWP gene fragment by using Rsal enzyme showed that 21 of 26 isolates (80.8 %) were *C. parvum* and 5 (19.2 %) isolates were *C. andersoni* (figure 2).

![Figure 1](image1.png)

**Figure 1.** Molecular diagnosis of Cryptosporidium spp. by a nested PCR based on COWP gene. (Lanes 1, 13) 100 bp DNA marker, (lanes 2-11) PCR product, (lane 12) negative control.

![Figure 2](image2.png)

**Figure 2.** Gel electrophoresis of Cryptosporidium species with COWP gene based on PCR-RFLP technique by digestion of the secondary PCR products in cattle isolates with Rsal enzyme
Lane 1: 100 bp molecular Marker; lane 2: *C. parvum*; Lane 3: *C. andersoni*

The RFLP patterns of the 553 bp COWP secondary PCR products digested with Rsal showed characteristic bands of 413, 106, 34 bp for *C. parvum* and 327, 140, 86 bp for *C. andersoni* respectively.

**DISCUSSION**

Cryptosporidium genetic polymorphism is increasingly being detected, due to the extensive use of modern molecular techniques. Previous studies of bovine cryptosporidiosis in Iran have been mostly microscopic examinations [17,18], although studies of its molecular characterization have been conducted recently [1, 6, 11-16].

In our study the prevalence of Cryptosporidium in cattle was found out microscopically and then the distributions of Cryptosporidium species/genotype were determined by PCR-RFLP analysis of the PCR products of the COWP gene from positive samples. The overall prevalence of Cryptosporidium was 16.7% (26/158), and *C. parvum* being the most prevalent species (80.8 %). This result is contrast to other RFLP analysis study on 107 faecal samples which was showed that 73.7% of all cases were *C. andersoni* and 26.3 % were *C. parvum* [13]. In study by Meamar et al, *C.
parvum identified as a sole species in cattle on the basis of 18S rRNA locus in cattle [1]. Keshavarz et al. was determine the characterization of various species of this coccidian among cattle using nested PCR-RFLP based on SSU-rRNA gene and documented the first report of C. parvum and new Subgenotype of C. parvum in Iranian cattle [6]. In Spain, 47.9% of the 844 calves less than one month of age on 22 farms were found to be infected with Cryptosporidium by microscopy [19].

In Vietnam, the prevalence of Cryptosporidium overall and C. parvum in cattle in particular was 35.7% and 33.5%, respectively [20]. A compatible prevalence (20.6%) was reported in calves in Turkey [21] using the same method, but a lower incidence rate was found in pre-weaned calves in Canada [22] and the United States [23] (40.6% and 85% respectively). Kvac et al. in the Czech Republic examined a total of 7,021 fecal samples by light microscopy. Cryptosporidium oocysts were found in 1,814 (25.8%) samples, in which 561 (8%) samples were positive for C. parvum and 1,253 (17.8%) for C. andersoni [24]. The higher infection rate of C. parvum, i.e., 80.8%, as compared to that of C. andersoni (3.3%) appears to reflect the dominance of C. parvum in Iran. Due to its zoonotic nature, more attention should be paid to this parasite for the control of bovine and human cryptosporidiosis.

REFERENCES

In conclusion the use of COWP primers could be sensitive enough to conduct a routine detection study. The nested PCR method using the COWP gene sequence can be an alternative diagnostic method to identify infected with Cryptosporidium and its genetic diversity [9]. Comparison of studies in human and animal infection indicated that the zoonotic pattern is the main mode of cryptosporidiosis transmission in Iran and it is indicating that direct or indirect contact with animals is the main route of spread of infection. Further molecular characterization on human and animals is needed to be done to increase our knowledge about Cryptosporidium transmission and its epidemiology [25]. To overcome some potential limitations of this study such as lack of data on clinical findings and small number of samples, more studies should be conducted in diverse geographic area.

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