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 Zeihl-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 andersoni* 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 andersoni* 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. andersoni* 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 Zeihl-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 ACCTGTTCCCACTCAATG for primary PCR and GTAGATAATGGAAGAGATTGTG 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 *RsaI* 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 *RsaI* enzyme showed that 21 of 26 isolates (80.8 %) were *C. parvum* and 5 (19.2 %) isolates were *C. andersoni* (figure 2).

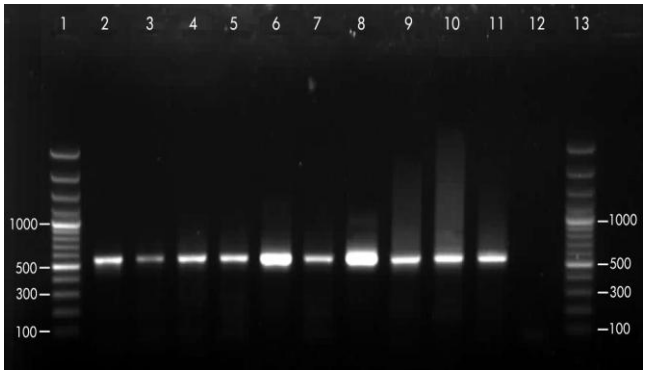

Figure 1. Molecular diagnosis of *Cryptosporidium* spp. by a nested PCR based on COWP gene. (Lanes 1, 13) 100 bp DNA marker, (lanes 2-11) PCR product , (lane 12) negative control

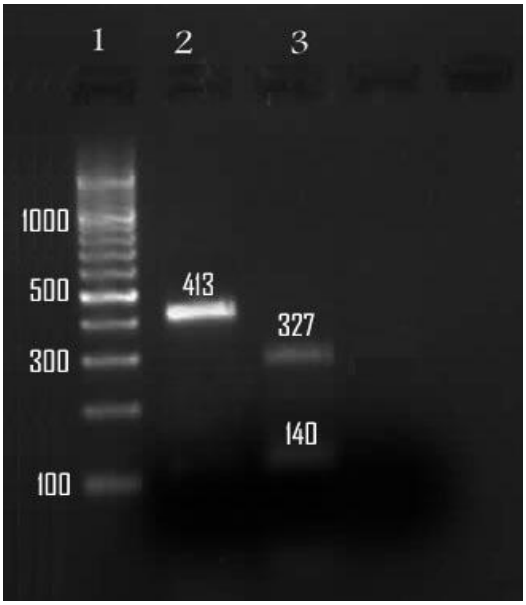

Figure 2. Gel electrophoresis of *Cryptosporidium* species with COWP gene based on PCR-RFLP technique by digestion of the secondary PCR products in cattle isolates with *Rsa*I enzyme
Lane 1: 100 bp molecular Marker; lane 2: *C. parvum*; Lane 3: *C .andersoni*

The RFLP patterns of the 553 bp *COWP* secondary PCR products digested with *RsaI* 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. bovis* 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.

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.

ACKNOWLEDGEMENTS

This study has been financially supported by Department of Parasitology, Shahid Beheshti University of Medical Sciences, Tehran, Iran and Gastroenterology and Liver Diseases Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. This paper resulted from part of the PhD research of Akbar Keshavarz.

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