Molecular Identification of Cryptosporidium sp. in the Cattle Stool Samples in Ardabil City, Northwestern Iran

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Abstract: Cryptosporidium parvum is a protozoan parasite that is now recognized as one of the leading causes of diarrhea in cattle. Surface waters contaminated with human and animal feces serve as main source for epidemic spread of Cryptosporidium parasites. In this study, we used a small-subunit rRNA-based Pcr-restriction Fragment Length Polymorphism (RFLP) technique to determine the prevalence and to characterize species of Cryptosporidium parasites in cattle’s in Ardabil city in Iran. Among 107 samples examined, 19 samples showed positive results. Restriction pattern analysis showed C. andersoni as the most common species with 14 cases; followed by C. parvum, bovine genotype, with 5 cases. Our results confirm that zoonotic transmission can be occur in the study region.

Key words: Cryptosporidium sp., PCR-RFLP, cattle, Ardabil, sample, Iran

INTRODUCTION

Cryptosporidium parasites are prevalent causes of long-lasting and life-threatening diarrheal diseases among immunocompromised patients (Leoni et al., 2006; Meamar et al., 2006; Gatei et al., 2003; Cama et al., 2006). Cryptosporidium sp. colonize human and animals (Hunter and Thompson, 2005; Thompson et al., 2005) and cause mild diarrhea in healthy people especially in children (Hamedi et al., 2005; Nath et al., 1999). Five species of Cryptosporidium parasites including: C. parvum human genotype (previously known as C. hominis), C. parvum bovine genotype, C. parvum dog genotype, C. meleagrisidis, C. felis and C. suis (pig genotype) have been found in human so far (Sunnotel et al., 2006; Xiao et al., 2002a). Molecular typing tools have indicated that 2 human and bovine genotypes of C. parvum are responsible for the most outbreaks (Caccio, 2005; Sulaiman et al., 1998). The transmission is directly fecal-oral or by water and food contaminated with Cryptosporidium oocysts, with as 10 viable oocysts needed for infection (Olkhuysen et al., 1999). Outbreaks of cryptosporidiosis occur as consequences of human and animal feces contaminated water consumption (Brandonisio, 2006; Fayer, 2004; Rush et al., 1990; Thompson et al., 2005). Resistance to chlorine disinfectants (Korich et al., 1990; King and Monis, 2006) and small infectious dose of Cryptosporidium parasites (Chappell et al., 2006) has made them a potential hazard to water supplies, such that Cryptosporidium sp. can been important public health concern.

Recent molecular characterization of Cryptosporidium from wildlife indicated that most animals are infected with host adopted species or genotypes (Xiao et al., 2002b). Thus, identification of human-infective parasites is the mainstay in epidemiologic studies of Cryptosporidium.

Immunofluorescence assay was extensively used for the identification of Cryptosporidium in environmental and clinical samples (Lechevallier et al., 1995; Stibbs and Ongerth, 1986). This method works based on detection of genus specific antigens on the surface of organism and only provides detection on genus level (Yu et al., 2002). PCR technique together with other genetic tools, such as Restriction Fragment Length Polymorphism technique (RFLP) has successfully been used in detection and differentiation of Cryptosporidium genotypes in fecal samples (Patricia Neira-Otero et al., 2005; Jae-Hwan Park et al., 2006).

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MATERIALS AND METHODS

In the present study, we used SSUrRNA-based nested PCR-RFLP technique to characterize and determine distribution of Cryptosporidium sp. and genotypes in cattle stool samples collected from animal husbandries in Ardabil city in Northwest part of Iran.

Cattle stool samples and samples processing: From March 2005 to August 2005, 107 diarrheal stool samples were collected from cattle's. Stool Samples mixed with 2.5% potassium dichromate (Coupe et al., 2005) and passed trough 4 layer gasses. All of the samples were stained with modified Ziel-Neelsen and the positive samples (19 from 107) for Cryptosporidium were stored at 4°C for DNA extraction and further study.

DNA extraction: Samples centrifuged at 12000 × g for 4 min to collect oocysts. The pellets then were subjected to 8 freeze-thaw cycles and DNA extraction carried out by using CTAB method (Frederick et al., 1999). The extracted DNA was dissolved in 100 µL of TBE buffer.

PCR-restriction fragment length polymorphisms: The species of Cryptosporidium oocysts in stool samples were identified by using a small-subunit rRNA-based nested PCR described previously (Xiao et al., 2001; 2000). For primary PCR, oligonucleotide primers: 5'-TTCTAGAGCTAATACATGC-3' and 5'-CCCATTTCTTCGAAACAGGA-3' with expected amplicon size of 1325 bp was used. The PCR amplification reaction mixtures contained 100 µM of each deoxynucleoside triphosphate, 0.5 µM of each forward and reverse primer, 3 mM MgCl₂, 5 U of Taq DNA polymerase, 10 µL PCR reaction buffer (Fermentase, Lithuania), 0.4 µg bovine serum albumin (Sigma, USA) and 5 µL DNA template in total volume of 50 µL reaction mixtures. Cycling parameters were 4 min at 94°C hot start, (initial heat activation step), followed by 35 cycles of 45 sec at 94°C, 1 min at 52°C and 45 sec at 72°C, with a final extension of 7 min at 72°C. In addition positive control (Cryptosporidium DNA, a gift from Dr. Meamar, Tehran University of Medical Sciences) and negative controls (No template DNA) were included in each PCR to validate results. The false negative PCR results were ruled out by adding the Cryptosporidium DNA in samples produced negative results.

The secondary PCR was performed using oligonucleotid primers: 5'-GGAAGGGTTGTATATATGGATATAAG-3' and 5'-AAGGAGTAAAGGAACCACTCCA-3' according to previous works (Xiao et al., 2001; 2000). Depending on the species and genotypes this primer set amplifies a range of 826-864-bp amplicon size (Xiao et al., 2000, 2004). The reaction mixture contained 100 µM of each deoxynucleoside triphosphate, 0.5 µM of each forward and reverse primer, 2 mM MgCl₂, 2.5 U of Taq polymerase, 2.5 × PCR reaction buffer and 2 µL DNA template (primary PCR product). The amplification condition was identical to the primary PCR except that the annealing temperature was 55°C.

To differentiate Cryptosporidium sp. and C. parvum genotypes, the RFLP analysis were performed by digesting secondary PCR product with SspI and VspI (Fermentase, Lithuania) restriction enzymes under condition recommended by the supplier. C. andersoni and C. muris sp. were further differentiated by digestion with DdeI (Biolabs, New England) restriction enzyme under manufacture recommendation (Xiao et al., 2000, 2001). Digested products were separated on a 2% agarose gel and visualized by ethidium bromide staining and recorded by UV transilluminator (Frederick et al., 1999). The species were characterized according to previously published restriction patterns (Xiao et al., 2004) and for confirmation of the species, the restriction pattern of secondary PCR product compared with patterns produced by digestion of known Cryptosporidium species DNA (a gift from Dr. Meamar, Tehran University of Medical Sciences).

RESULTS AND DISCUSSION

PCR amplification: SSUrRNA-based nested PCR method has been found to be more sensitive and specific in detection of Cryptosporidium in water (Xiao et al., 2000; Jiang et al., 2005; Quintero-Betancourt et al., 2003) and human or animal fecal samples (Meamar et al., 2006; Guyot et al., 2001; Sulaiman et al., 2005). In our study, all of 19 samples produced positive PCR amplification by nested PCR (Fig. 1). As shown by the results, oocyst of both C. andersoni and C. parvum genotypes were identified in cattle's by PCR-RFLP analyses.

In the waterborne outbreaks in England, human cryptosporidiosis was found to be primarily caused by drinking water contamination and C. parvum was identified as the causative parasite species (McLauchlin et al., 2000).

Restriction pattern analysis: Digestion of secondary PCR products with VspI and SspI showed the presence of C. andersoni/muris and C. parvum bovine genotype (Fig. 2 and 3). Digestions with the mentioned enzymes produce the identical patterns for the C. andersoni and C. muris sp. (Fig. 2 and 3). They were differentiated by
CONCLUSION

In conclusion from public health importance view, among the identified Cryptosporidium parasites, C. parvum bovine genotype is potentially human-infective and may contribute as a public health concern (Chappell et al., 2006; Sterling, 2000; Xiao et al., 2002). As our previous studies showed the presence of the C. parvum and C. andersoni in environmental water resources (Mohammadi et al., 2007) and high prevalence of human-infective C. parvum oocyst in stool samples from children with diarrhea (Mohammadi et al., 2007), these findings further emphasize on the importance of zoonotic cycle in cryptosporidiosis.

REFERENCES


