Journal of Animal and Veterinary Advances 7 (3): 246-250, 2008
ISSN: 1680-5593
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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 immunicompromised 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. meleagriadis*, *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 (Okhuysen *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 bean 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 gases. 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* oocyts in stool samples were identified by using a small-subunit rRNA-based nested PCR described previously (Xiao *et al*., 2001; 2000). For primary PCR, oligonucleotid primers: 5'- TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA-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 $MgCl_2$, 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'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-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 $MgCl_2$, 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 *Ssp*I and *Vsp*I (Fermentase, Lithuania) restriction enzymes under condition recommended by the supplier. *C. andersoni* and *C. muris* sp. were further differentiated by digestion with *Dde*I (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 etidium bromide staining and recorded by UV transillumination (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: SUrRNA-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 *Vsp*I and *Ssp*I 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

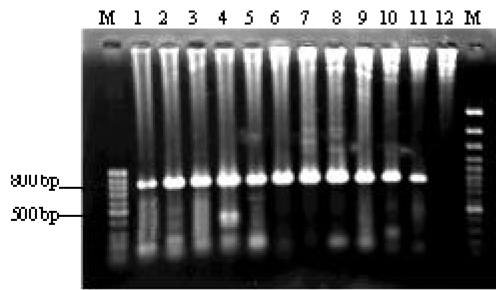

Fig. 1: Ethidium bromide-stained 1% agarose gel with the small-subunit rRNA-based secondary PCR products of cattle stool samples. Lanes 1 through 10, DNA from *Cryptosporidium* oocysts in samples 826-864 bp, lane 11 positive control, lane 12 negative control and lanes M L and M R 50 and 100 bp DNA ladders

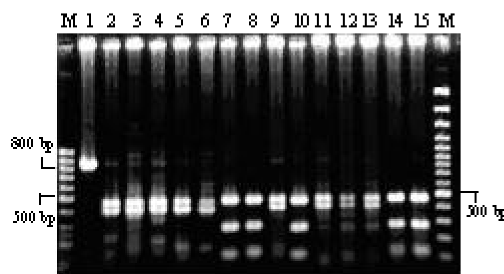

Fig. 2: Differentiation of *Cryptosporidium* sp. and genotypes in cattle stool samples with the small-subunit rRNA-based PCR-restriction fragment polymorphism technique. Secondary PCR product was digested by *Ssp*I restriction enzyme. Lane 1 undigested secondary PCR products, Lanes 2 through 6, 9, 11 and 12, *C. andersoni/muris* 385 and 448 bp: Lanes 7, 8, 10 and 15, *C. parvum* 449, 254 and 110 bp: lanes 13 and 14 digestion products of known *C. andersoni/muris* and *C. parvum* sp., respectively and lanes $M_{(L)}$ and $M_{(R)}$ 50 and 100 bp DNA ladders, respectively

digesting secondary PCR products with *Dde*I (Xiao *et al*., 2001, 2000). Digestion of *C. andersoni* yields 4 bands at 20, 156, 186 and 470 bp and *C. muris* yields 5 bands at 20, 156, 186, 224 and 247 bp. Digestion patterns of *C. andersoni* and *C. muris* give 3 and 4 visible bands on agarose gel, respectively (Xiao *et al*., 2004). The results showed all strains belong to *C. andersoni* no *C. muris* sp. was detected. *C. andersoni* was prominent genotype with 14 (73.68 %) cases followed by *C. parvum* bovine genotype with 5 (26.3 %) cases.

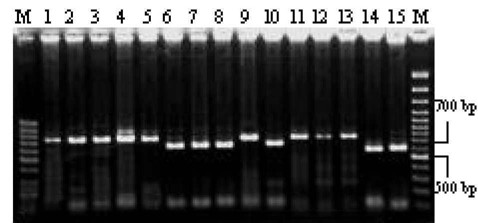

Fig. 3: Differentiation of *Cryptosporidium* sp. and genotypes in cattle stool samples with the small-subunit rRNA-based PCR-restriction fragment polymorphism technique. Secondary PCR product was digested by *Vsp*I restriction enzyme. Lanes 1 through 5, 9,11 and 12, *C. andersony/muris* 731 and 102 bp; lanes 6 through 8,10 and 15 *C. parvum* bovine genotype 628 bp, 104 bp, lanes 13 and 14, digestion products of known C. *andersoni/muris* and *C. parvum* bovine genotype, respectively and lanes $M_{(L)}$ and $M_{(R)}$ 50 and 100 bp DNA ladders

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* oocysts in stool samples from children with diarrhea (Mohammadi *et al*., 2007), Theses findings further emphasis on the importance of zoonotic cycle in cryptosporidiosis.

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