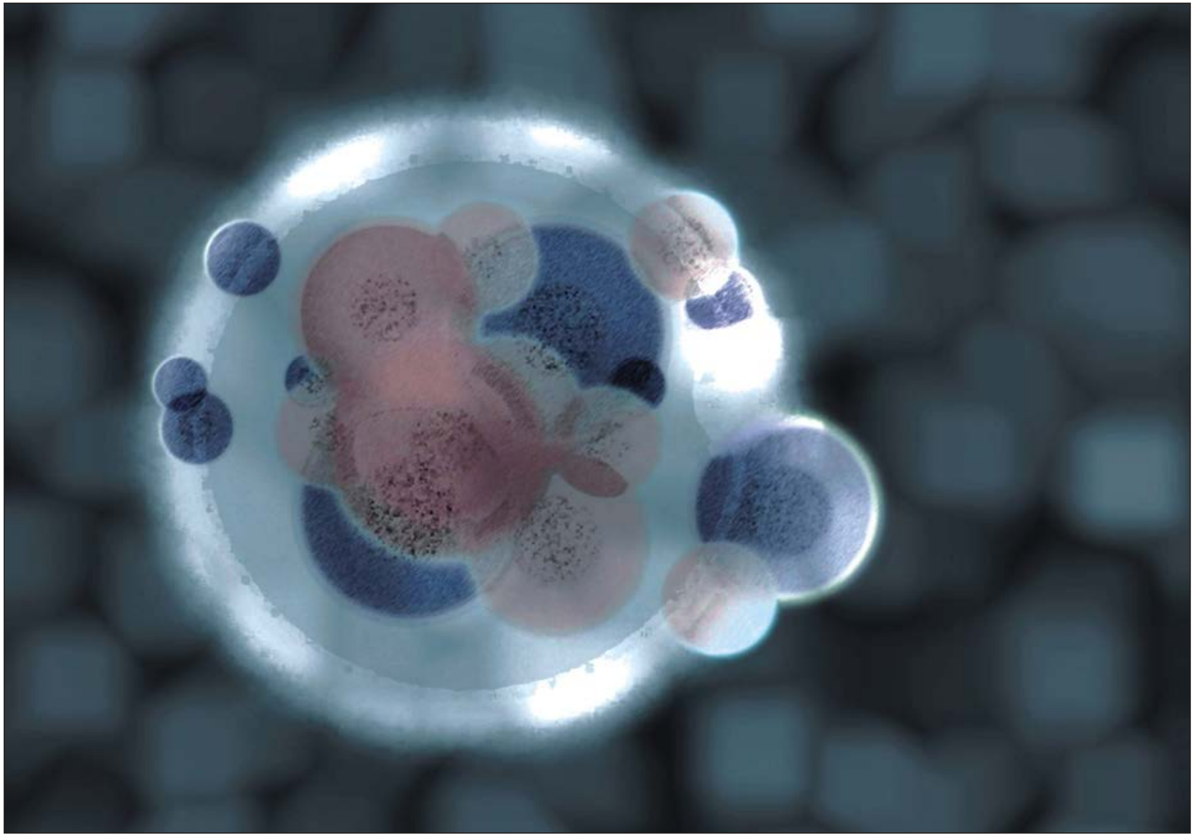

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Molecular Characterization of *Cryptosporidium* Isolates from Cattle in a Slaughterhouse in Tabriz, Northwestern Iran

[1]Esmaeel Fallah, [1]Behroz Mahdavi Poor, [1]Rasol Jamali,
[1]Karim Hatam Nahavandi and [2]Mohammad Asgharzadeh
[1]Department of Parasitology, School of Medicine,
[2]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Islamic Republic of Iran

Abstract: The aim of the present study was to determine *Cryptosporidium* species and genotypes in cattle by molecular methods. Fecal samples were collected from 104 adult cattle in a slaughterhouse in Tabriz, Northwestern Iran during the period from June to July 2007. Initial identification of cryptosporidiosis was carried out by formalin-ether concentration and Kinyoun acid fast staining method. Genomic DNA was extracted from microscopically positive samples and nested PCR was performed to amplify the partial small-subunit rRNA gene of *Cryptosporidium* that were subsequently digested by restriction enzymes to determine the *Cryptosporidium* species and genotypes present. In this study *Cryptosporidium* parasites were found in 10.5% (11 cases) adult cattle. Among 11 analyzed isolates, two different species of *Cryptosporidium* were identified; 64% (seven cases) of isolates belonged to *C. andersoni* and 36% (four cases) to the potentially zoonotic species of *C. parvum* bovine genotype. The results of present study showed that two species of *Cryptosporidium*, *C. andersoni* and *C. parvum* bovine genotype are responsible for cattle cryptosporidiosis in this region and the existence of *C. parvum* bovine genotype suggest that there is a potential risk of zoonotic transmission of *C. parvum* bovine genotype infection between cattle and human, likely by means of contaminated water or food, or through direct contact in the farmers and veterinary staff.

Key words: *Cryptosporidium*, cryptosporidiosis, cattle, small-subunit rRNA, nested PCR

INTRODUCTION

Cryptosporidium spp. is a common protozoan parasite that mainly infects the gastrointestinal tract of wide range of vertebrates including humans and cattle throughout the world (Dillingham *et al*., 2002; Thompson *et al*., 2005; Sunnotel *et al*., 2006). It is responsible for significant diarrhea diseases affecting mostly children (Ramirez *et al*., 2004) and immunocompromised individuals, especially in HIV positive patients (Hunter and Nichols, 2002).

Now, according to biological and genetic characterization, *Cryptosporidium* spp. has been shown to composed of 16 species (Sunnotel *et al*., 2006). Two species of *Cryptosporidium* have been reported commonly from cattle (Santin *et al*., 2004); *Cryptosporidium parvum* (bovine genotype or genotype 2) is recognized as one of the most common causing neonatal diarrhea and *Cryptosporidium andersoni* can cause moderate-to-sever impairment of weight gain, decreased feed efficiency and reduced milk production. Recently *C. bovis* (formerly named bovine B genotype) and deer-like genotype were identified in cattle (Thompson *et al*., 2005; Slapeta, 2007).

Cryptosporidium parvum (bovine genotype) represents a zoonotic risk and domestic livestock, predominantly cattle acts as a major reservoir for human infection through direct contact or contamination of drinking water (zoonotic transmission) (Tzipri and Ward, 2002; Hunter and Thompson, 2005).

Identification of *Cryptosporidium* to specie and genotype level is important to determine the possible source of infection in outbreaks and risk factors associated with the transmission (Ramirez *et al*., 2004). 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* species and genotypes in clinical and environmental samples (Xiao *et al*., 1999, 2000, 2001, 2004).

Corresponding Author: Behroz Mahdavi Poor, Department of Parasitology, Faculty of Medicine,
Tabriz University of Medical Sciences, Golgasht Ave., Tabriz, Islamic Republic of Iran
Tel: +984113364665 Fax: +984113364665

There are not enough data on the molecular identification of the species infecting cattle in Iran. In the present study, we used SSUrRNA-based nested PCR-RFLP technique to characterize cattle infective species and genotypes of *Cryptosporidium* parasites and determine the potential risk of cattle cryptosporidiosis as a zoonotic infection in Tabriz city in northwest part of Iran.

MATERIALS AND METHODS

Fecal samples: Total of 104 rectal fecal samples were collected from cattle (age > two years old) in a slaughterhouse in Tabriz, northwestern Iran during the period from June to July 2007. Specimens were concentrated by formalin-ether concentration method and stained with Kinyoun acid fast method and examined with light microscope under oil immersion lens. Eleven microscopically positive specimens containing *Cryptosporidium* oocysts were selected and diluted in 2.5% potassium dichromate solution and filtered through gauze to remove large particles and stored at 4°C for further analysis several weeks later (Meamar *et al*., 2006; Mendonca *et al*., 2007).

DNA extraction: About 200 mg of fecal material that stored in 2.5% potassium dichromate were washed in a solution of phosphate buffered saline (pH = 7.2) and centrifuged at 14000 x g for 10 min in 4°C to remove potassium dichromate and other possible PCR inhibitors. This process was repeated for five times. The pellets were subjected to ten freeze-thaw cycles (three min in liquid nitrogen followed by three min at 65°C) to disrupt the oocyst wall (Kostrzynska *et al*., 1999). DNA was extracted by using the modified proteinase K, SDS and CTAB method (Van Soolingen *et al*., 1994).

The extracted DNA pellet was resuspended in 20 μL of TE buffer and stored at -20°C before its application in PCR.

PCR and Restriction Fragment Length Polymorphism (RFLP): The species of *Cryptosporidium* oocysts in fecal samples were identified by using a small-subunit rRNA-based nested PCR and Restriction Fragment Length Polymorphism (RFLP) analysis described previously (Xiao *et al*., 1999, 2000, 2001, 2002).

An initial PCR product of 1325 bp was amplified using outer primers:

Forward: 5′-TTCTAGAGCTAATACATGCG-3′
Reverse: 5′-CCCATTTCCTTCGAAACAGGA-3′

Each PCR mixture (40 μL) contained 100 μM of each dATP, dTTP, dGTP, dCTP, 0.5 μM of each forward and reverse primer, 3 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, 20 mM Tris-Cl, 50 mM KCl, 0.4 μg bovine serum albumin and 50-200 ng of DNA template.

For primary PCR a total of 35 cycles each consisting of 45 sec at 94°C, 1 min at 52°C and 45 sec at 72°C were performed with initial hot start at 94°C for 4 min and a final extension step at 72°C for 7 min.

For the second round of amplification, the inner primers used were:

Forward: 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′
Reverse: 5′-AAGGAGTAAGGAACAACCTCCA-3′

Depending on species and genotype this primer set amplifies a range of 826-864 bp fragments (Xiao *et al*., 2001, 2004).

Each PCR mixture (40 μL) contained 100 μM of each dATP, dTTP, dGTP, dCTP, 0.5 μM of each forward and reverse primer, 2 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, 20 mM Tris-Cl, 50 mM KCl and 50-200 ng DNA template (primary PCR product).

For the secondary PCR, cycling conditions were identical to the conditions used for primary PCR except that the annealing temperature was 55°C.

Both positive (*Cryptosporidium* DNA) and negative controls (containing all PCR reagents but no DNA template) were included in each PCR to validate results.

Secondary PCR products were visualized after electrophoresis on 1% agarose gels and stained in an ethidium bromide solution (0.5 μg mL^{-1}) and recorded by UV transillumination (Frederick *et al*., 2002).

Restriction digestion was carried out by using *SspI* and *VspI* (Fermentase, Lithuania) restriction enzymes under condition recommended by the supplier, to differentiated *Cryptosporidium* sp. and *C. parvum* genotypes.

Because *C. andersoni* and *C. muris* had identical *SspI* and *VspI* restriction pattern, *DdeI* (Biolabs, New England) digestion was performed to differentiate *C. andersoni* from *C. muris* under manufacture recommendation (Xiao *et al*., 2001, 2004).

Digestion products were visualized under UV light after 2% agarose gel electrophoresis and ethidium bromide (0.5 μg mL^{-1}) staining and gels were recorded by using UV transillumination (Frederick *et al*., 2002).

The species and genotypes were characterized according to restriction patterns that previously described (Xiao *et al*., 2004).

RESULTS AND DISCUSSION

In this study, *Cryptosporidium* oocysts were detected in 11 adult cattle (10.5%). DNA amplification of *Cryptosporidium* positive samples yielded products of the expected size about 830 bp in the nested PCR analysis of the SSUrRNA gene.

Restriction analysis of the secondary PCR products with the restriction enzymes *SspI* (Fig. 1) and *VspI* (Fig. 2) showed the presence of *C. parvum* bovine genotype in four cases (36%) and *C. muris/C. andersoni* in seven cases (64%) in cattle isolates.

For differentiation of *C. andersoni* from *C. muris*, *DdeI* digestion of the secondary PCR products were done. Digestion of the PCR products of *C. andersoni* yields four bands at 20, 156, 186 and 470 bp that three bands were visible on an agarose gel (Xiao *et al*., 2001). The results showed all strains belong to *C. andersoni* (Fig. 3). Mixed infections were not detected.

The present study represents the first report on the molecular characterization of *Cryptosporidium* species in cattle in Tabriz, Iran. The actual number of infected cattle is probably further underestimated because only one fecal sample was collected per animal. If that sample was identified as negative during a period when the cattle were experiencing intermittent oocyst excretion, the cattle would be considered negative (Fayer *et al*., 2007).

This study finding confirms that *C. andersoni* and *C. parvum* (bovine genotype) are responsible for cattle cryptosporidiosis in this region and *C. andersoni* is more prevalent than *C. parvum* (bovine genotype) in adult cattle in this area. This result expected because *C. parvum* primarily infects the intestine of young calves (predominantly calves less than 2 months) whereas *C. andersoni* infects the abomasums of juvenile and adult cattle (Santin *et al*., 2004; Thompson *et al*., 2005).

Few repots on cattle cryptosporidiosis are available in Iran (Meamar *et al*., 2007; Nouri and Toroghi, 1991). In

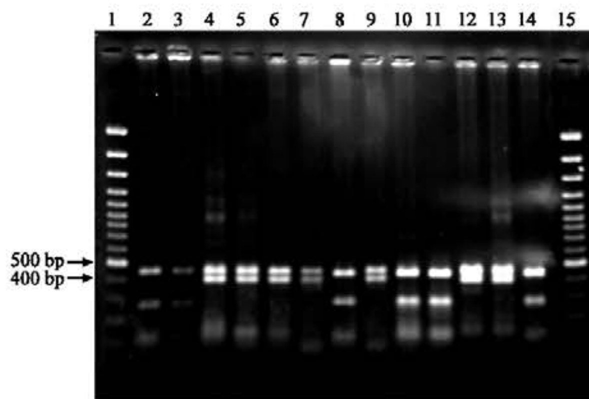

Fig. 1: Digestion of secondary PCR product with SspI. Lane 1 and 15: 100 bp DNA ladder; Lane 2 and 3 digestions product of known *C. parvum* specie; Lane 4 through 7, 9, 12 and 13: *C. andersoni/muris* (385 and 448 bp) and Lane 8, 10, 11 and 14: *C. parvum* (449, 254 and 108 bp)

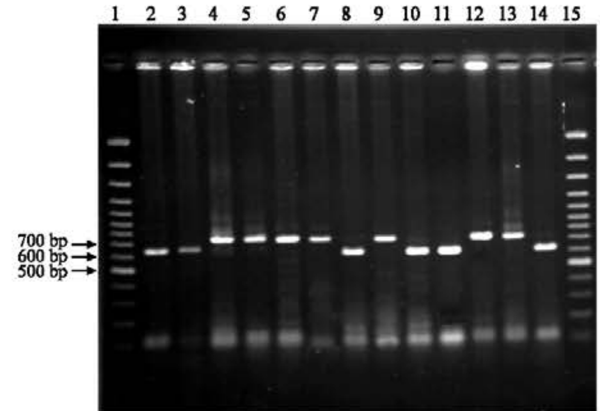

Fig. 2: Digestion of secondary PCR product with VspI. Lane 1 and 15: 100 bp DNA ladder; Lane 2 and 3 digestions product of known *C. parvum* bovine genotype; Lane 4 through 7, 9, 12 and 13: *C. andersoni/muris* (731 and 102 bp) and Lane 8, 10, 11 and 14: *C. parvum* bovine genotype (628 and 104 bp)

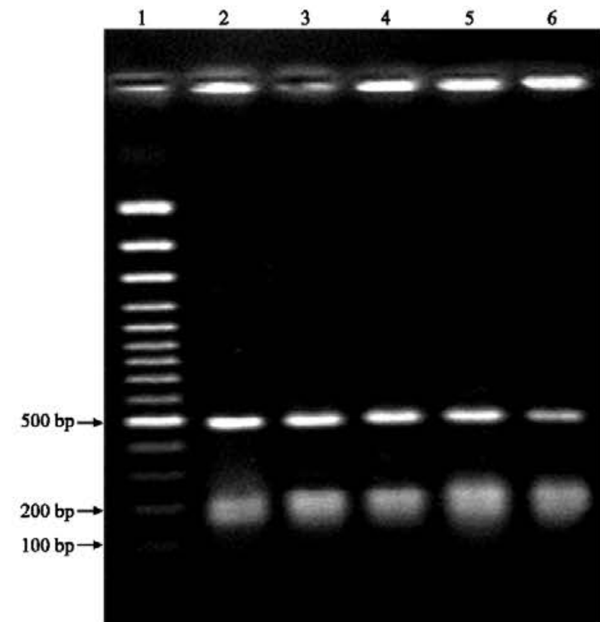

Fig. 3: Differentiation of *C. andersoni* and *C. muris* with digestion of secondary PCR product with DdeI. Lane 1: 100 bp DNA ladder; Lane 2 through 7: *C. andersoni* (470, 186 and 156 bp)

studies of *Cryptosporidium* positive cattle in this country (Meamar *et al*., 2007), India (Saha Roy *et al*., 2006) and Netherlands (Wielinga *et al*., 2007) all of isolates were belonged to *C. parvum* but in United states (Fayer *et al*., 2007) *C. parvum*, *C. andersoni* and *C. bovis* were found in adult cattle. In a similar study in Japan all of isolates from adult cattle were belonged to *C. andersoni* specie (Koyama *et al*., 2005). These findings are different from present study result. These results reflect the existence of geographic variations in the distribution of *Cryptosporidium* spp. in cattle.

In the present study existence of *C. parvum* (bovine genotype) in cattle indicate that cattle must be considered as a major risk for zoonotic transmission and may be contribute as a public health concern because the bovine genotype of *C. parvum* accounts for most cases of human cryptosporidiosis in Iran (Meamar *et al*., 2006). However our PCR-RFLP method could not differentiate sub genotypes of *C. parvum*, further molecular and biological studies are needed to distinguishing sub genotypes of *C. parvum* to improve our knowledge of parasite zoonotic transmission in this area (Meamar *et al*., 2007; Slapeta, 2007).

On the other hand, *C. andersoni* as one of the most important cause of the economic losses in dairy and beef cattle (Thompson *et al*., 2005) should be considered in this area. The only report of *C. andesoni* in humans identified three persons out of 2414 subjects with cryptosporidiosis in England between 1985 and 2000 (Leoni *et al*., 2006).

Present results might help public health care system for preventing and managing of cryptosporidiosis in cattle and the assessment of zoonotic risk of cattle cryptosporidiosis as a reservoir for the human infection.

The results of present study indicate that cattle represents a risk to farmers and veterinarians by means of direct contact and to the general human population (especially children and immunocompromised individuals) through the contamination of food and water with oocysts. Prevalence testing and molecular characterization of *Cryptosporidium* in fecal samples from veterinarians, farmers and other animal handlers would be beneficial in providing better insight on the possible transmission dynamics of parasite.

In conclusion, two species of *Cryptosporidium* were detected in cattle in Northwestern part of Iran. It is necessary that further epidemiological survey of *Cryptosporidium* species and genotypes in human and animal reservoirs and water resources be performed in this region to clarify the route of infection and providing a program for managing and tracking sources of cryptosporidiosis.

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