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Short Communication

Molecular and Parasitological Study of *Cryptosporidium* Isolates From Cattle in Ilam, West of Iran

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Abstract

Background: Cryptosporidiosis is one of the most important parasitic infections in human and animals. This study was designed for survey on the prevalence of *Cryptosporidium* infection in farms of Ilam, west of Iran, using parasitology method and genotyping by Nested PCR-RFLP.

Methods: Fecal samples of 217 cattle were collected fresh and directly from the rectum of cattle. All of the samples were examined by microscopic observation after staining with modified Ziehl-Neelsen (MZN). Genomic DNA extracted by using EURx DNA kit. A Nested PCR-RFLP protocol amplifying 825 bp fragment of 18s rRNA gene conducted to differentiate species and genotyping of the isolates using *Ssp*I and *Vsp*I as restriction enzymes.

Results: The prevalence of *Cryptosporidium* infection in cattle using both methods is 3.68%. Most of the positive cattle were calves under six months. Species diagnosis carried out by digesting the secondary PCR product with *Ssp*I that *C. parvum* generated 3 visible bands of 448, 247 and 106 bp and digested by *Vsp*I restriction enzyme generated 2 visible bands of 628 and 104bp. In this investigation all of the positive samples were *Cryptosporidium parvum.*

Conclusion: *C. parvum* (bovine genotype) detected in all positive cattle samples in Ilam, west of Iran. The results of the present study can help for public health care systems to prevention and management of cryptosporidiosis in cattle and the assessment of cattle cryptosporidiosis as a reservoir for the human infection.

Introduction

Cryptosporidium is a coccidian parasite of humans, domestic animals, and wide range of other vertebrates (1, 2). This parasite cause disorders of the digestive, respiratory systems and mainly diarrhea, which is particularly serious in neonatal and immunosuppressed individuals among its mammalian hosts and lead to poor health of infected animals and significant economic losses (2-5). However, the infection is self-limiting in healthy individuals (4). Water and food sources are important for transmission of this parasite (6). In addition, the disease can also be transmitted by person-to-person, aerosols and infected animals (3, 6). *C. parvum* and *C. hominis* are the main species causing cryptosporidiosis (7-9).

Cattle are one of the major sources of *C. parvum*, which causes neonatal diarrhea in calves (10, 11) and for zoonotic contamination including waterborne outbreaks (12). According to the previous investigations, bovine cryptosporidiosis is widespread and the range of the prevalence had been reported in different studies 14 to 80 percent (13). Although in different countries, several studies have been conducted on prevalence of *Cryptosporidium* species (14-16), however little is known on the incidence of the parasite in Iran. Some information has been published about bovine and human cryptosporidiosis in Iran and epidemiological studies have not yet been conducted on a wide scale and in large number of hosts (17).

The diagnosis of this disease is generally based on the observation of oocysts in stool samples under light microscope, but this method have less sensitivity for epidemiological studies and not appropriate to determine the species of parasite. Recently, suitable methods such as molecular techniques are used for these purposes (18, 19). Polymerase chain reaction (PCR) and restriction fragment length polymorphism technique (RFLP) of molecular methods have been successfully used for isolation and genotyping of *Cryptosporidium* in different samples (20).

As regards the health and economic importance of cryptosporidiosis, this study was designed for survey on the prevalence of *Cryptosporidium* infection in farms of Ilam, west of Iran, using parasitology method and species identification (genotyping) of oocysts based on nested PCR of the 18S rRNA gene, combined with restriction fragment length polymorphism (RFLP) analysis.

Materials and Methods

Sample collecting and processing

Overall, 217 cattle fecal samples were collected fresh and directly from the rectum of cattle during May to August 2013. The age of the cattle was categorized into <6 months (78 samples), 6-12 months (78 samples) and 12-24 months (61 samples). Samples mixed with 2.5% potassium dichromate (8) and passed through 4 layer gases. All fecal samples were examined microscopically for the presence of *Cryptosporidium* oocysts after concentration by formalin–ether technique and staining with modified Ziehl-Neelsen (MZN). Finally, positive samples in microscopic observation and 25 negative samples selected for molecular study.

DNA extraction

DNA purification kit (EURx, Poland) was used for genomic DNA extraction from the *Cryptosporidium* oocysts according to manufacturer instruction.

Polymerase Chain Reaction (PCR)

To amplify a fragment of 18S rRNA gene, Nested-PCR was performed using outer primers: (forward: 5'-AACCTGGTTGATCCTGCCAGTAGTC-3' reverse: 5'-TGATCCTTCTGCAGGTTCACCTACG-3') for 1350 bp am-

plicon and inner primers: (forward: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' reverse: 5'- AAGGAGTAAGGAACAACCTCC-A-3') for a 825 bp PCR product (5) in a total volume of 25 µl contained 1 µl DNA template, 2.5 mM PCR buffer, 0.2 mM of each dNTP, 2.5 mM Mgcl2, 10 pmol of each primer and 2.5 U of Taq DNA polymerase under the following conditions: 94˚C for 5 min as initial denaturation, followed by 35 cycles of 94˚C for 30 s, 68˚C for 90 s and 72˚C for 90 s with a final extension of 10 min at 72˚C. Amplicons were visualized by electrophoresis in 1.5% agarose gel and stained in ethidium bromide solution for 10 min and photographed under UV light.

Restriction Fragment Length Polymorphism (RFLP)

The restriction fragment analysis for species- and strain-specific diagnosis of *Cryptosporidium* parasites was performed by digestion of the secondary PCR product with *Ssp*I (Fermentas) for species diagnosis and *Vsp*I (Fermentas) for genotyping of *C. parvum,* under condition recommended by the supplier. The digested products were fractionated on a 2% agarose gel electrophoresis and visualized by ethidium bromide staining and photographed using UV transilluminator. The species were characterized according to Xiao et al. (21).

Results

The prevalence of *Cryptosporidium* infection in cattle using both methods was 3.68% (8 out of 217). There was no significant difference between the rate of the infection in calves less than 6 months and those between 6-12 months of age ($P>0.05$) but in adults was significantly different from those less than 6 months of age ($P<0.05$). After genomic DNA extraction, nested-PCR was conducted for amplification of 1350 bp and 825 bp fragments in the first and second PCR reaction respectively (Fig. 1). Most of the positive cattle were calves under six months. Species diagnosis was carried out by digesting the secondary PCR product with *Ssp*I (Fig. 2).

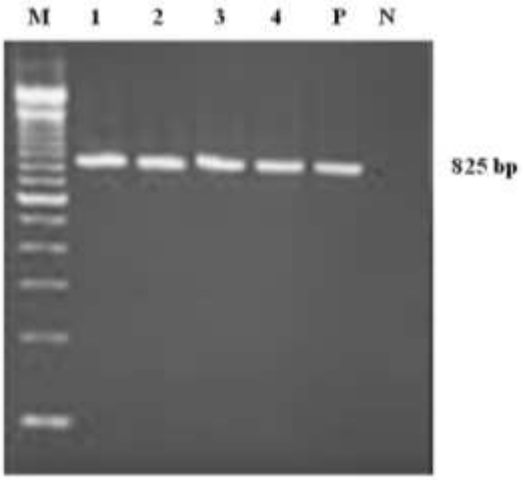

Fig.1: Agarose gel electrophoresis of 18S rRNA secondary PCR products of *Cryptosporidium* isolates. Lane N: negative control (without template DNA), Lane P: positive control (*C. parvum)*, Lanes 1-4: samples from cattle, M: 100bp DNA size marker

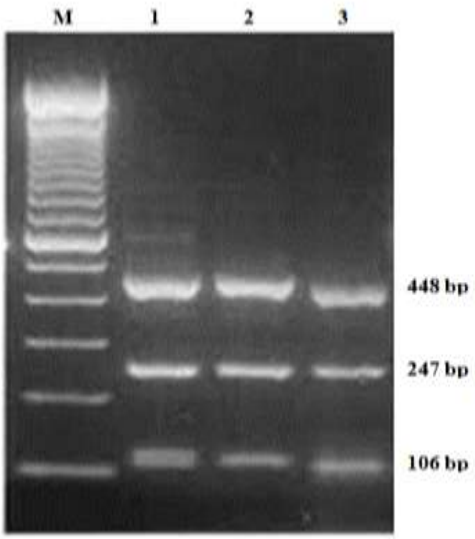

Fig. 2: Profile of the RFLP analysis for differentiation of *Cryptosporidium* species in calve fecal samples based on 18s rRNA using *Ssp*I restriction enzyme that recognized *C. parvum* in all of the positive samples. Lanes 1-3: digested secondary PCR products of the samples, M: 100bp DNA size marker

C. parvum generated 3 visible bands of 448, 247 and 106 bp. Secondary PCR product was digested by *Vsp*I restriction enzyme (Fig. 3). *C. parvum* generated 2 visible bands of 628 and 104bp. All of the positive samples were *C. parvum*.

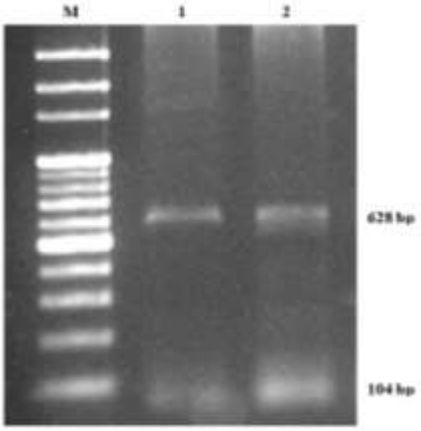

Fig. 3: Profile of the RFLP analysis for differentiation of *Cryptosporidium* genotypes in calve fecal samples based on 18s rRNA using *Vsp*I restriction enzyme that recognized *C. parvum* bovine genotype in all of the positive samples. Lanes 1, 2: digested secondary PCR products of the samples, M: 100bp DNA size marker

Discussion

The Present study is the first report on the prevalence and molecular characterization of *Cryptosporidium* in cattle in Ilam, west of Iran.

The rate of positive results obtained 3.68% that is lower than the other similar studies such as Karanis et al. (22), Radfar et al. (23) and Xiao et al. (21) with the positive rates 18.1%, 18.9% and 88.4% respectively. Koyama et al. recorded a low prevalence (1.5%) of *Cryptosporidium* infection in adult cattle in Japan (24). Gow and Waldner also reported 1.1% in beef cows and 3.1% in other cows at western Canadian cow-calf herds (25). Much of the studies indicate that bovine cryptosporidiosis has a low prevalence in adults and acts as asymptomatic carries of infection so will be a source of infection for younger animals. A number of factors, including climatic conditions, locations of sampling, volumes of samples, diversity of animals in the areas, ecosystems, season and some of technical difficulties for the recovery of *Cyptosporidium* oocysts may contribute in these variations (3). It was observed that the majority of the animals between 1-6 months of age were found to have cryptosporidiosis caused by *C. parvum*, compared to those above six months and one year of age as the similar observations were also presented by other investigators (26-29).

Not only calves, but also adult cattle were found to be excreting the oocysts. Thus, asymptomatic adult cattle can be regarded as playing the role of a reservoir for *Cryptosporidium* that might lead to the infection of human or other domestic animals. Our findings showed that there was no significant difference between the rate of the infection in calves less than 6 months and those between 6-12 months of age, while the infection rate amongst adults was significantly different from those less than 6 months of age. Digestion of secondary PCR product with *Vsp*I and *Ssp*I showed the presence of *C. parvum* bovine genotype in 8 cases (100%) in cattle isolates. The other similar studies of *Cryptosporidium* positive cattle in few countries all of isolates were belonged to *C. parvum* (30-32). But in some studies, *C. parvum* and *C. andersoni* were found in adult cattle (20, 33). Our findings indicated that *C. parvum* is responsible for cattle cryptosporidiosis in this region.

Based on the results of this study, bovine genotype of *C. parvum* is the predominant genotype in the cattle's of this region and indicates that cattle must be considered as a major risk for transmission and may be contribute as a public health concern because the bovine genotype of *C. parvum* takes in to accounts for most cases of human cryptosporidiosis in Iran (20, 31).

Conclusion

C. parvum (bovine genotype) was detected in all positive cattle samples in Ilam, west of Iran.

Domestic animals particularly cattle can play an important role in transmission of *Cryptosporidium* oocyst to human. The results can help for public health care systems to prevention and management of cryptosporidiosis in cattle and the assessment of cattle cryptosporidiosis as a reservoir for the human infection.

Acknowledgments

The authors declare that there is no conflict of interest.

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