Original Article

Genetic Characterization of *Cryptosporidium* spp. among Children with Diarrhea in Tehran and Qazvin Provinces, Iran

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Abstract

**Background:** *Cryptosporidium* is an intracellular apicomplexan parasite that infects a wide range of vertebrates including humans. Cryptosporidiosis is a major cause of diarrhea in children with and without human immunodeficiency virus (HIV) infection in developing countries. More recently, the molecular methods for identification of morphologically indistinguishable species have been developed. The aim of this study was to determine the characterization of various species of this coccidian among children with diarrhea by using molecular methods.

**Methods:** Fecal samples were collected from 1263 children with diarrhea who referred to Pediatrics Medical Centers in Qazvin and Tehran, two central provinces of Iran. Initial identification of *Cryptosporidium* was carried out by Ziehl-Neelsen acid-fast staining method of stool samples. DNA was extracted from positive microscopically samples and were subjected to a two step nested PCR-RFLP based on SSU-rRNA gene.

**Results:** Out of 1263 collected samples, 31 (2.5%) were found to be contained *Cryptosporidium* oocysts. RFLP analysis showed that 80.6% of the positive isolates were *C. parvum*, 16.1% *C. hominis* and 3.2% had mix infection pattern of both *C. parvum* and *C. hominis*.

**Conclusion:** Our results showed that the zoonotic pattern of transmission is predominant and has considerable significance in epidemiology of cryptosporidiosis in the study areas.

**Keywords:** Cryptosporidium, Genotyping, SSU-rRNA, Children, Diarrhea

Introduction

*Cryptosporidium* is a protozoan parasite that infects the gastrointestinal tract of a wide range of vertebrates including humans, domestic and wild animals and also birds (1). Cryptosporidiosis is a major cause of diarrhea in children with and without human immunodeficiency virus (HIV) infection in developing countries (2). Diarrhea is usually self-limiting in healthy host. However, it can be life-threatening in children and immunocompromised individuals (3). Transmission is through the faecal-oral route, following direct or indirect contact with *Cryptosporidium* oocysts via person to person, zoonotic, waterborne, food borne or airborne contact (4).

More recently, genetic characterization of *Cryptosporidium* spp. at polymorphic loci has facilitated the development of molecular approach to study of the epidemiology of cryptosporidiosis (5). Currently 18 *Cryptosporidium* species are recognized as valid: *Cryptosporidium parvum* and *C. hominis* are the species predominantly found in human but other spe-

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cies including *C. meleagridis, C. felis, C. canis, C. suis, C. muris, C. andersoni, C. baileyi* and two cervine and skunk genotypes of *Cryptosporidium* have been frequently reported in humans (6).

In different parts of Iran, there are several studies which have documented the prevalence of *Cryptosporidium* spp. from 1 to 7% in human, mainly based on microscopically examination (7-10). However, in one study the genotypes of *Cryptosporidium* species were identified in HIV positive patients (11). In the other study 867 faecal samples were examined by modified Ziehl–Neelsen technique and the prevalence of *Cryptosporidium* spp. was 2.7%. RFLP analysis showed that 70.8% of all cases were *C. parvum* and 29.2% were *C. hominis* (12).

The aim of this study was to identify the species of *Cryptosporidium* in children with diarrhoea by using PCR- RFLP method based on SSU-rRNA gene to better understanding of the epidemiology and transmission of infection in the study regions.

**Materials and Methods**

**Sample preparation:**
Fecal samples were collected from 1263 children with diarrhea whom referred to Pediatrics Medical Centers in Qazvin and Tehran provinces in Iran from December 2005 to September 2007. *Cryptosporidium* oocysts were seen in samples concentrated by formalin–ethyl–acetate sedimentation and stained with a modified Ziehl–Neelsen technique (1). The positive *Cryptosporidium* spp. isolates were preserved in 2.5% potassium dichromate and kept at 4°C until used for DNA extraction.

**DNA extraction:**
About 300 µl of fecal suspension was washed with distilled water three times to remove trace of dichromate and genomic DNA extracted by 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. The oocysts were frozen in liquid nitrogen and their thawing was carried out at 90°C in water bath.

**PCR-RFLP analysis**
A two–step nested PCR protocol was used to amplify an 826-864 bp fragment of the SSU-rRNA gene using two sets of oligonucleotide primers: 5’-TTC TAG AGC TAA TAC ATG CG-3’ and 5’ -CCC ATT TCC TTC GAA ACA GGA -3’ for primary PCR and 5’-GGA AGG GTT GTA TTT ATT AGA TAA AG-3’ and 5’-CTC ATA AGG TGC TGA AGG AGT A-3’ for secondary PCR as described previously (13). For the restriction fragment length polymorphism (RFLP) analysis, seven µl of the secondary PCR product were digested in a total volume of 20 µl sample at 37°C overnight by using 2U of *Ssp*I restriction enzyme (Fermentase, life science, Cat No ER0771) for species diagnosis and 2U of *Vsp*I enzyme (Fermentase, life sciences, Cat NoER0911) for genotype identification of *C. parvum*. The digested products were fractionated on the 2% gel by ethidium bromide staining.

**Sequencing:**
Nested-PCR products containing SSU-rRNA gene of two isolates were directly sequenced with appropriate primers in both directions. The PCR samples that were found to contain single bands on the agarose gels were treated with a Pre-Sequencing kit (USB Corporation, Cleveland, Ohio) before sequencing. The PCR products were then sequenced using an Applied Biosystems (ABI) Terminator Cycle Sequencing Ready Reaction kit (BigDye® Terminator V3.1 Cycle Sequencing Kit) on an ABI 3130xl Genetic Analyzer. The sequences obtained were manually edited and aligned using gene-ruler software (version 3.05).

The nucleotide sequence data of *C. parvum* and *C. hominis* have been submitted to the Genebank/EMBL/DDBJ database under accession numbers AB434889 and AB434890.
Results

Over a period of 24 months from December 2005 to September 2007 a total of 1263 fecal samples were collected from children less than 12 years old with diarrhea. Among the 1263 patients (46.2% male and 53.7% female) included in this study, microscopic examinations of the specimens revealed the presence of Cryptosporidium spp. in 31 (2.45%) of the samples (Table 1). Genotyping of these isolates in children showed that the frequency of C. parvum, C. hominis and mixed infection with both of them were 25, 5 and 1%, respectively.

There was no significant difference between infection rate and gender. The highest seasonal prevalence was seen during the July to October (Fig. 1).

The SSU-rRNA gene fragment was successfully amplified from all positive samples. RFLP analysis of the nested PCR products by using two enzymes showed that 25 out of 31 isolates (80.6%) were C. parvum, 5 (16.1%) C. hominis and one isolate (3.2%) showed mix infection pattern of C. parvum and C. hominis (Table 2). The bonds of each species after using SspI and VspI enzymes are shown in Fig 2.

<table>
<thead>
<tr>
<th>Province</th>
<th>Examined No.</th>
<th>Positive No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qazvin</td>
<td>469</td>
<td>12</td>
<td>2.55</td>
</tr>
<tr>
<td>Tehran</td>
<td>794</td>
<td>19</td>
<td>2.40</td>
</tr>
<tr>
<td>Total</td>
<td>1263</td>
<td>31</td>
<td>2.45</td>
</tr>
</tbody>
</table>

Table 1: Prevalence of Cryptosporidium spp. in two provinces

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR size(bp)</th>
<th>SspI digestion</th>
<th>VspI digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parvum</td>
<td>848</td>
<td>449, 268, 108, 12, 11</td>
<td>628, 116, 104</td>
</tr>
<tr>
<td>C. hominis</td>
<td>851</td>
<td>449, 268, 111, 12, 11</td>
<td>561, 116, 104, 70</td>
</tr>
</tbody>
</table>

Table 2: RFLP analysis of the SSU-rRNA gene of various Cryptosporidium spp.

Fig 1: The seasonal distribution of cryptosporidiosis cases among the study population (n=19)
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Fig. 2: PCR-RFLP of Cryptosporidium based on SSU-rRNA gene
lane 1 and 9, 100 bp marker; lane 2, 3, 6 and 8, C. parvum; lane 4: mix infection with both genotype. Lane 5 and 7, C. hominis (SspI panel A, VspI panel B).

Discussion

In the developing countries, the association of Cryptosporidium with acute and persistent diarrhea in children is well recognized. In Iran most of the earlier studies of Cryptosporidium were based on microscopic examination of fecal samples. According to the previous studies, the prevalence of parasite in various parts of Iran was 4.1% in west, 7% in southeastern, 2.2% in south, and 7.7% in northwest (7-10).

In the present study the prevalence of Cryptosporidium in children was 2.5% out of 1263 diarrheic stool samples. Higher rates of infection were reported in Egypt (17%), Uganda (5.9%), Kenya (25%), Turkey (3.5%), Pakistan (10.3%) and 8.2% in Indonesia (14-19).

One of our important findings was the seasonal occurrence of Cryptosporidium infection with a peak transmission during July to October (Fig. 1). This peak was coincide with the hot and dry season and also was similar to that observed elsewhere including; Peru, Guatemala, United States and Gaza (16).

Developing of molecular techniques for identifying morphologically indistinguishable species has a key role for understanding of the epidemiology of this protozoan parasite. Even though the prevalence of cryptosporidiosis in tropical countries is high, limited studies have been conducted to characterize Cryptosporidium species from human at molecular level. Molecular methods have shown that most cases of human cryptosporidiosis are mainly caused by C. parvum and C. hominis (previously known as the human genotype or genotype H). This species is found almost exclusively in human, whereas C. parvum (genotype bovine or genotype C) is usually found in livestock animals as well as humans (20).

A recent genotype analysis of 17 cases of Cryptosporidium among HIV positive and negative patients in Iran showed that 76% of isolates had C. parvum and 24% of the isolates belonged to C. hominis (11).

In our study the RFLP analysis indicated that C. parvum with a rate of 80.6% is the predominant species in children. It had a close agreement with the previous study in HIV samples in Iran (11), as well as other countries such as UK, Kuwait, France, Switzerland and the Netherland (21-25). In the other hand, C. hominis is predominant in other nations such as Bra-
zil, Kenya, Malawi, United State, Thailand, Japan and South Africa (26-29).

The prevalence of these species varies in different region of the world, geographic variation occurs also within a country (30). Leon analyzed 2414 samples in the United Kingdom and detected 41.7% of them as C. hominis, 56.1% as C. parvum and 0.9% of all cases as mixed infection (31). Wielinga also analyzed 91 positive sample in the Netherlands and found that the prevalence of C. hominis, C. parvum and mixed infection with both species were 70%, 19% and 10%, respectively (25). Although some authors have found another species such as C. felis, C. canis and C. meleagridis in human but we did not detect them (16).

In conclusion our results 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.

Acknowledgment

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