

## Original Article

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

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(Received 1 May 2008; Accepted 11 Sep 2008)

## 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 Zeihl-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 *Cryptosporidium 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-

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 diarrhea 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 re-suspending 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 *SspI* restriction enzyme (Fermentase, life science, Cat No ER0771) for species diagnosis and 2U of *VspI* 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 generunner 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).

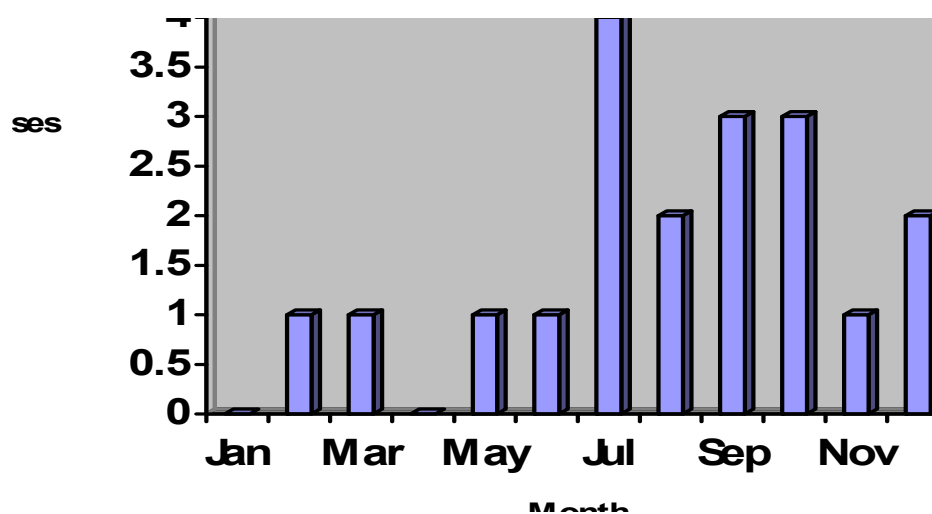
The SSU-rRNA gene fragment was successfully amplified from all positive samples. RFLP analysis of the nested PCR products by using two enzymes showed that 25 out of 31 isolates (80.6%) were *C. parvum*, 5 (16.1%) *C. hominis* and one isolate (3.2%) showed mix infection pattern of *C. parvum* and *C.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

**Table 1:** Prevalence of *Cryptosporidium* spp. in two provinces

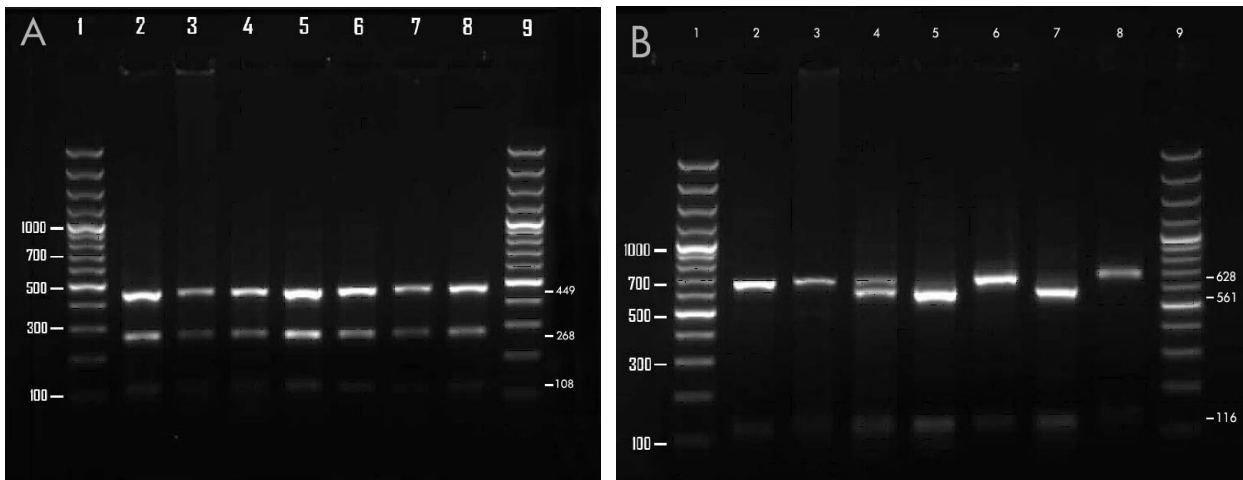
Province	Examined No.	Positive No.	%
Qazvin	469	12	2.55
Tehran	794	19	2.40
Total	1263	31	2.45

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

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



**Fig 1:** The seasonal distribution of cryptosporidiosis cases among the study population(n=19)



**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 epide-

miology 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

We thank the staff of Pediatric Medical Centers of Qazvin and Tehran for their cooperation.

This project was supported financially by Research Division of Shahid Beheshti University (M.C) through Cellular and Molecular Biology Research Center. The authors declare that they have no Conflict of Interests.

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