

Genotyping of *Cryptosporidium* spp. in clinical samples: PCR-RFLP analysis of the *TRAP-C2* gene

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ABSTRACT

Aim: The aim of the present study was to determine the species and genotypes of *Cryptosporidium* spp. among children with diarrhea by PCR- RFLP using the *TRAP-C2* gene.

Background: *Cryptosporidium* is a globally distributed protozoan parasite and one of the most common causes of infection and diarrhea in humans.

Patients and methods: Four hundred and sixty nine stool samples were collected from children less than 12 years with diarrhea who had been referred to Pediatrics Medical Centers in Gazvin provinces. The presence of *Cryptosporidium* oocysts was determined by Ziehl-Neelsen acid fast staining, then, genomic DNA was extracted from positive samples and nested PCR-RFLP was performed to amplify the *TRAP-C2* gene.

Results: The overall prevalence of *Cryptosporidium* infection in children was 2.5 %. Results of nested PCR amplification showed that of 12 positive children samples, 10 (83.3%) were belonged to *C. parvum*, followed by *C. hominis* in 1 (8.3%) and mixed infection in 1 isolate (8.3%).

Conclusion: This study showed that *Cryptosporidium parvum* (the zoonotic genotypes) is more prevalent than other *Cryptosporidium* species in children from this area. This suggests that zoonotic transmission is the main mode of transmission of *Cryptosporidium* infection in Iran.

Keywords: *Cryptosporidium*, Genotypes, *TRAP-C2* gene.

(Gastroenterology and Hepatology From Bed to Bench 2011; 4(1): 29-33).

Introduction

Cryptosporidium is a protozoa infection which is a cause of diarrhea in children and immunocompromised patients throughout the world. Currently 18 *Cryptosporidium* species have been recognized. *Cryptosporidium parvum* and *C. hominis* are the species predominantly found in humans. Other species found in a variety of hosts includes *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris*, *C. andersoni*, *C. baileyi* (1).

In recent years, researchers have developed PCR-based techniques for detection and identification of *Cryptosporidium* spp. These techniques are based on the polymorphic nature of the *Cryptosporidium* oocyst wall protein (*COWP*), the dihydrofolate reductase (*DHFR*), thrombospondin related adhesive protein 1 (*TRAP-C1*), thrombospondin-related adhesive protein 2 (*TRAP-C2*), internally transcribed spacer 1 (*ITS1*), polythreonine repeat (*Poly-T*), small-subunit (*SSU*) rRNA genes and undefined genomic sequences among strains that infect humans and most animals at (2-6). Previous studies of cryptosporidiosis in Iran have been performed using microscopy (7-12) and

Received: 12 July 2010 Accepted: 4 October 2010

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recently studies of *Cryptosporidium* molecular characterization have been conducted (13-14).

In this study we identified the genotypes of the *Cryptosporidium* isolates from clinical samples using polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) analyses of the *TRAP-C2* gene (Thrombospondin-Related Adhesive Protein of *Cryptosporidium*-2).

Materials and Methods

Sampling

A total of 469 fecal samples were collected from children with diarrhea who had been referred to Pediatric Medical Centers in Ghazvin, Iran. *Cryptosporidium* oocysts were identified in samples after concentration by formalin-ethyl-acetate sedimentation and staining with a modified Zeihl-Neelsen technique. The positive *Cryptosporidium* spp. isolates were preserved in 2.5% potassium dichromate and kept at 4°C until used for DNA extraction.

DNA extraction

Approximately 300 µl of fecal suspension was washed three times with distilled water to remove trace of dichromate and then genomic DNA was extracted using the DNAzol kit according to the manufacturer's instructions (Invitrogen, life technologies, Cat. No 10503-027, USA) with the addition of three freeze-thaw cycles (10 minutes) after resuspending the DNA samples in lysis (to rupture the *Cryptosporidium* oocysts). The oocysts were frozen in liquid nitrogen. Thawing was carried out at 90° C in water bath.

TRAP-C2 gene amplification

Nested PCR was used to amplify a fragment (266-366 bp) of the *TRAP-C2* gene using two sets of oligonucleotide primers: CATATTCCTGTCCCTTGAGTTGT and TGGACAACCCAAA TGCAGAC for primary PCR and GGTAATTGGTCACGA and

CCAAGTTCAGGCTTA for secondary PCR, as described previously (2, 5). PCR products were visualized on 1% agarose gel after ethidium bromide staining.

RFLP analysis

For restriction fragment length polymorphism (RFLP) analysis of secondary PCR products, we used restriction enzymes *BstEII*, *HaeIII* for *TRAP-C2* gene. The restriction digestion products were visualized by electrophoresis on 2% agarose gel after ethidium bromide staining.

Results

In this study *Cryptosporidium* isolated from children with diarrhea in Gazvin province of Iran were characterized by PCR-RFLP. From all isolates thought to possibly contain *Cryptosporidium*, oocysts were detected and then stained by modified Ziehl-Neelsen method.

From the 469 samples, a total of 12 *Cryptosporidium* isolates were characterized. The *TRAP-C2* gene fragment of all isolates was successfully amplified from 12 positive samples. RFLP analysis of the nested PCR products by using two enzymes (*BstEII*, *HaeIII*) showed that 10 out of 12 isolates (83.3%) were *C. parvum*, 1 (8.3%) *C. hominis* and one isolate (8.3%) showed mix infection pattern of *C. parvum* and *C. hominis* (Fig1 and Fig 2).

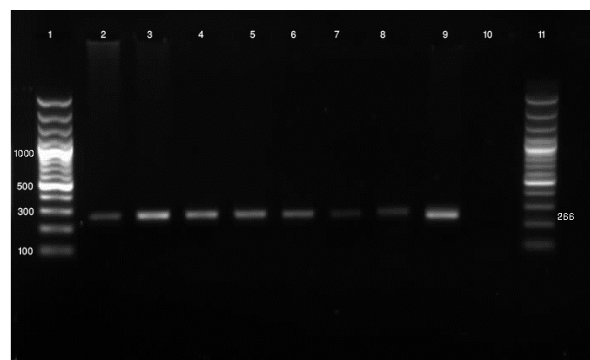


Figure 1. Molecular diagnosis of *Cryptosporidium* spp. by a nested PCR based on *TRAP-C2* gene. Lane 1: 100 bp DNA marker, Lane 2-9: PCR product, Lane10: negative control

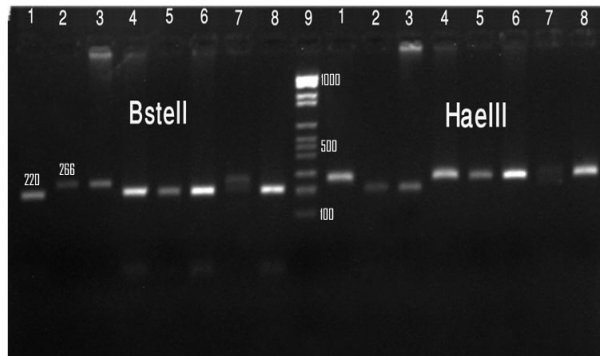


Figure 2. Gel electrophoresis of *Cryptosporidium* species with *TRAP-C2* gene based on PCR-RFLP technique by digestion of the secondary PCR products in clinical isolates with *HaeIII*, *BstEII* enzyme. Lane 1, 4, 5, 6, 8: *C. parvum*, Lane 2, 3: *C. hominis*, Lane 7: mixed infection both genotype

Discussion

In developing countries, the association of *Cryptosporidium* with acute and persistent diarrhea in children is well recognized. To date, a variety of genotypic markers are available to differentiate some species and subspecies within the genus *Cryptosporidium*. Nested-PCR/RFLP for *TRAP-C2* gene has been reported as a recognized technique for the differentiation of strains of *Cryptosporidium*. The ability to genotype each of the 12 *Cryptosporidium* isolates in this study reflects the specificity and sensitivity of the PCR-RFLP method, as described previously (2, 4-5).

High rates of infection have been reported in Egypt (17%), Uganda (5.9%), Kenya (25%), Turkey (3.5%), Pakistan (10.3%) and 8.2% in Indonesia (14-19). Previous studies performed in children within Iran have reported a prevalence of 2.4 % in Tehran, 11.6% in Kermanshah province and 2 % in Shahrekord province (1, 8-9).

A recent genotype analysis of *Cryptosporidium* among HIV positive and negative patients in Iran using 18s rRNA gene showed that 76% of isolates was *C. parvum* and 24% of the isolates was belonged to *C. hominis* (12).

In the present study the prevalence of *Cryptosporidium* in children was 2.25 %. In our study the RFLP analysis indicated that *C. parvum*,

with a rate of 83.4 %, is the predominant species in children. Our study is therefore in agreement with a previous similar study performed in Iran (1); and the results are comparable to other studies performed in countries such as the United Kingdom, Kuwait, France, Switzerland and the Netherlands (20-24).

The predominance of *C. parvum* in humans may be due to high prevalence of bovine cryptosporidiosis in this area. By comparison *C. hominis* is the predominant species found in similar studies in other nations such as Brazil, Kenya, Malawi, the United States, Thailand, Japan and South Africa (25-28). We can conclude that the *C. parvum* is the predominant cause of *cryptosporidium* infection in Iran.

Furthermore the use of *TRAP-C2* primers could be an alternative diagnostic method to identify human infection with *Cryptosporidium*. Information generated from this diagnostic approach would be useful, not only in diagnosis and identification of the sources of contamination, but also in controlling the disease.

Further molecular characterization on human and animals is needed, to increase our knowledge about *Cryptosporidium* and its transmission.

Acknowledgements

We acknowledge the very helpful assistance of Prof. Lihua Xiao from Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Services, U.S.

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