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Short Communication

Cryptosporidium Infection in Patients with Gastroenteritis in Sari, Iran

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Abstract

Background: Cryptosporidiosis is a common coccidian parasite infection in patients with diarrhea that has worldwide distribution especially in developed countries. Therefore, the aim of this study was to determine the occurrence of *Cryptosporidium* infection in patients with gastroenteritis admitted to hospitals of Mazandaran University of Medical Sciences by parasitological and molecular methods in Sari, Iran.

***Methods*:** Stool samples were collected from 348 patients with gastroenteritis admitted to the hospitals of Medical University in the Sari and Ghaemshahr cities in Mazandaran Province, Northern Iran in 2010-2011. Oocysts of *Cryptosporidium* identified using Formalin-Ether concentration method and stained by Aacid-fast staining (AFS) and Auramine phenol fluorescence (APF). Genomic DAN extracted from microscopically positive samples and nested PCR -RFLP by using SSU rRNA that identifies of the species of cryptosporidium.

***Results*:** In 348 patients with gastroenteritis, the most clinical symptoms were diarrhea, nausea, vomiting, dehydration, fever and weight loss. 2.3% (8 cases) of diarrheal samples tested by both microscopy and molecular methods were positive for the presence of cryptosporidium. Nested PCR products yielded unique bands of 846 bp, correspond to cryptosporidium. Species diagnosis carried out by digesting the secondary PCR product with *Sph*I restriction enzyme, which noted 3 clearly bands of 449, 254, and 108 bp correspond to *Cryptosporidium* spp.

***Conclusion*:** The results of present study on *Cryptosporidium* spp. in this area can make a background data for control programs and further molecular analyses. Thus, further work needs to determine the origin of *Cryptosporidium* species in this area.

Introduction

In recent decades, opportunistic protozoa of the genus *Cyclospora*, *cryptosporidium* and *Toxoplasma*, and enteric pathogen protozoa such as *Giardia intestinalis* and *Entamoeba histolytica*, create the most concerns throughout the world as food-borne diseases and development of diseases in humans and animals (1, 2).Protozoan parasites of the genus *Cryptosporidium* are small coccidia that can infect gastrointestinal and respiratory epithelial cells of vertebrates (3).This protozoan is an intracellular extracytoplasmic monoxenic parasite and is considered as one of the most important pathogenic water and food transmitted zoonotic parasite (2,4,5,6). *Cryptosporidium*, from the late twentieth century, has been known worldwide as an important causative agent of endemic and epidemic diarrhea and affects mostly children (generally under 6 years old) and immunocompromised patients in developed countries (7-10). *Cryptosporidium parvum* and *C. hominis* are the most common species of the parasite that can infect human and transmit through fecal - oral or contaminated drinking water routs (9, 10).

Moreover, in last decade, *Cryptosporidium* has been introduced as a new pathogenic agent in people with normal immune systems in addition to who are immunocompromised; this case illustrates the importance of further extensive studies on any aspects of the parasite and the disease in different regions especially in patients with gastroenteritis, children and people with immune deficiency (11). Prevalence of cryptosporidiosis in patients with diarrhea is more prevalent than the other people and *C. parvum* is known as the third or fourth cause of long-term diarrhea in children that is associated with weight loss (12, 14). In Iran, the prevalence of the parasite in several studies in recent years in patients with gastroenteritis, AIDS and students was from 0.1% to 7.7% (16, 17, 26). The reported prevalence of the parasite in patients with gastroenteritis in Mazandaran was 0.1 to 4.1% (8, 12). However, in the years 2007-2008 Nahravanian et al. could not find the parasites in stool samples of patients with gastroenteritis in Mazandaran Province (12).

Current common methods for detection of *Cryptosporidium* are including concentration techniques and modified acid-fast staining (3). Recently, the use of molecular biology techniques to determine the species and genotypes of *Cryptosporidium* and as an alternative to conventional methods for detection of the parasite in clinical specimens have been increased (5, 17, 18). The SSU rRNA gene has a high sensitivity for the detection of *Cryptosporidium* and today this gene has been widely used (19, 20). So far, few studies have been conducted on the prevalence of *Cryptosporidium* in the Mazandaran Province.

Considering to the increasing importance of intestinal protozoa in recent years, detection of them by scientific parasitological methods in-patient with diarrhea, children, and immunosuppressed patient is a priority. Therefore, the aim of this study was to determine the occurrence of *Cryptosporidium* infection in patients with gastroenteritis admitted to hospitals of Mazandaran University of Medical Sciences by using parasitological and molecular methods (PCR-RFLP) in Sari, Iran.

Material and Methods

This study was a cluster randomized descriptive - cross-sectional study. Stool sampling was conducted for 348 patients with gastroenteritis admitted to the hospitals of Mazandaran University of Medical Sciences (in the cities of Sari and Ghaemshahr) in Mazandaran Province in 2010-2011.The samples were stored in fixative (10% formalin) and transported to Parasitological Research Laboratory, and assessed for the presence of oocyst of the parasite using concentration method (Forma-

lin- Ether) and stained by acid-fast staining (AFS) and auramine phenol fluorescence (APF) for detection of *Cryptosporidium* oocysts. By light microscopy, oocysts of *Cryptosporidium* are seen as round pink - red objects on a pale green background by the method of Kinyoun acid fast staining (8,10,12,16,19).

DNA extraction

All 348 samples were repeatedly (five times) 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 possible PCR inhibitors. DNA extracted from purified oocysts by 5 cycles of freezing liquid nitrogen for, followed by thawing at 98 °C for 1 min to disrupt the oocyst wall (21, 30). DNA extracted by DNA mimi Kit (Bioneer, Daejeon, Korea) according to the manufactures protocol.

PCR and Restriction Fragment Length Polymorphism (RFLP)

The method of nested PCR and Restriction Fragment Length Polymorphism (RFLP) by using a small-subunit rRNA gene (15, 23, 24) was used for identification of the species of *Cryptosporidium* spp. In initial PCR a product of 1325 bp of the gene was amplified using a set of primers including forward: 5´-TTCTAGAGCTAATACATGCG-3´ and reverse: 5´-CCCATTTCCTTCGAAACAGGA-3´. The thermal cycling condition in primary PCR was including an initial hot start at 94 °C for 4 min following 35 cycles each consisting of 45 sec at 94 °C, 1 min at 52 °C and 45 sec at 72 °C were and a final extension step at 72 °C for 7 min.

The primers used the nested PCR were forward: 5´- GGAAGGGT TGTATTTA TTAGA TAA AG-3´and reverse: 5´-AGGAGTAAGGAACAACCTCCA-3´. This primer set amplifies a range of 826-864 bp fragments depending on species of the parasite (16, 28, 30). The cycling condition for the nested PCR was identical to the primary PCR with exception of the annealing temperature that was 55 °C. The PCR products visualized with electrophoresis on 1.5% agarose gels stained with Ethidium bromide by UV transillumination (15, 30). Based on PCR product, to differentiate of *Cryptosporidium parvum* from the other species, restriction enzyme of *SspI* (Fermentase, Lithuania) was used for restriction digestion for differentiation of *Cryptosporidium* spp. under condition recommended previously. Visualization of the digested products was performed through 2% agarose gel electrophoresis and Ethidium bromide (0.5 µg mL^{-1}) staining using UV transillumination (15, 16, 23, 25).

Results

In 348 patients with gastroenteritis, distribution of the patient in age groups was 36.8% more than 40 years old, 32.7% were 21-40 years old and 32.8 were in range of 10-20 years old. 53.2% of cases were male. Totally, 2.3% (8 cases) of diarrheal samples were positive for the presence of C*ryptosporidium* by both microscopy and molecular methods. In the 348 patients with gastroenteritis (with diarrhea), the most clinical symptoms were nausea (19.8%), vomiting (14.1%), dehydration (19%), fever (19%) and weight loss (14.1%).

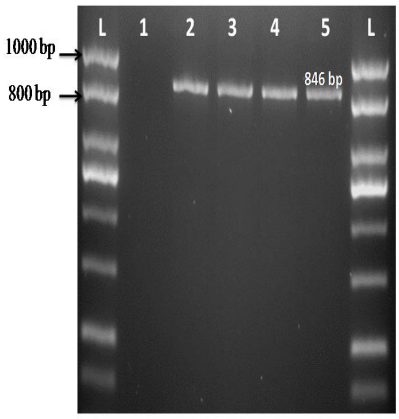

Fig.1: Nested PCR amplified SSU rRNA fragments of *Cryptosporidium*, Line 1: Negative control, line 2-5: Positive samples , DNA bands with size 845 bp. L: DNA ladder (1000 bp)

The region SSU rRNA based nested PCR-RFLP were used to characterize and corroborate of *Cryptosporidium* parasite. The stool samples of 348 patients with gastroenteritis were examined by light microscopy and nested PCR-RFLP methods. Nested PCR products yielded unique bands of 846 bp, correspond to *Cryptosporidium* (Fig. 1).Species diagnosis carried out by digesting the secondary PCR product with SspI restriction enzyme. We observed three clearly; bands of 449, 254, and 108 bp correspond to *Cryptosporidium* spp. (C. *parvum or C.hominis* genotypes) (Fig. 2).

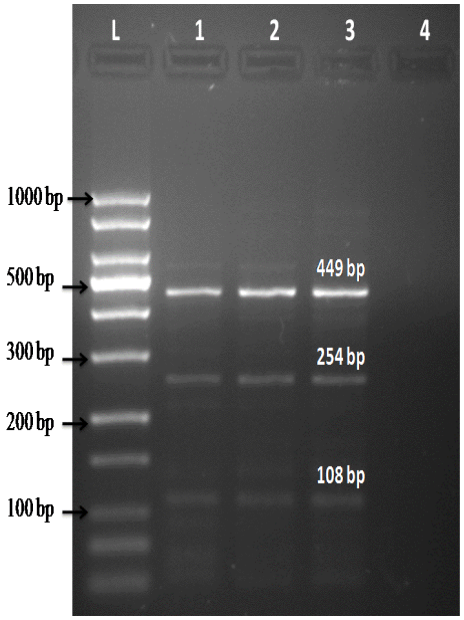

Fig.2: Digestion of secondary PCR product with *SspI* restriction enzyme. Line 1-3: Digestions product of known *Cryptosporidium* Spp (449, 254 and 108 bp). Line 4: Negative control L: DNA ladder (1000 bp)

Discussion

The purpose of the current study was to determine and confirm the occurrence of *Cryptosporidium* infection in patients with gastroenteritis by using molecular methods. The first major finding was that 2.3% (8 cases) of diarrheal samples tested by both microscopy and molecular methods were positive for the presence of *Cryptosporidium*. Today, enteric opportunistic coccidian, including *Cryptosporidium*, are considered as important infective factors in HIV positive patients, and more recently, in transplant recipients treated with corticosteroids and even in healthy people with normal immune system (26).There are different reports on prevalence of *Cryptosporidium* species among people in the world. Predominate species of the parasite in Kenya, Thailand, Peru, South India, Malawi and South Africa in children or HIV positive adults was *C. hominis* (27). This finding is compatible with the previous parasitological and molecular studies in Iran and UK, Kuwait, Portugal, France, North America, and the Netherland (28-30).

In Iran, there are many evidences showing *Cryptosporidium* in human (31-33). Most of these reports are concentrated on children with diarrhea or mental retardants and people with immune suppression or HIV +. Prevalence of this parasite in various parts of Iran based on microscopic examination of fecal samples was 4.1% in west, 7% in southeastern, 2.2% in south, and 7.7% in north-west (21). In a study conducted in Mazandaran Province in Iran on 142 samples of feces (64 HIV + and 78 HIV-) by methods of concentration (using formalin - ether) and modified Kinyoun acid fast staining, in 4.9% of HIV+ samples and 5.2% of HIV- samples *Cryptosporidium* was detected. (34). In another study on 362 stool samples of children with mental disabilities no cases of *Cryptosporidium* were observed (34). Other study on 614 stool samples of children under 3 years old with or without diarrhea in rural and urban area showed *Cryptosporidium* in 10.4% of the cases (36). The prevalence of the infection in rural children are about twice as urban children, and in children with diarrhea is about three times as in children without diarrhea (36). None of the healthy children HIV- that was investigated in two separate studies in Dakar and Jakarta had been infected with *Cryptosporidium* (37). Only 1.5% of stool samples of 206 patients with HIV + assessed by Kinyoun acid fast staining was positive for *Cryptosporidi-*

um in Iran (26). In Riyadh, Saudi Arabia, stool samples of 136 patients with suppressed immune system were assessed with modified trichrome and Ziehl – Neelsen, *Cryptosporidium* was detected in more than 8.1% of cases (38).

In this study, similar results were obtained with both the microscopic and molecular methods. All 8 positive cases diagnosed by microscopic methods also were detected by PCR technique. This similarity indicates that if the microscopic method, including staining with Ziehl – Neelsen, be done correctly and accurately can be considered as a valuable diagnostic method for detection of intestinal coccidian. Additionally, molecular characterization of *Cryptosporidium* in fecal samples would be beneficial in providing better insight on the possible transmission dynamics of parasite. Due to the wide range of traditional animal husbandry, special climate, and high humidity, Mazandaran Province is at high risk for zoonotic diseases including cryptosporidiosis.

Conclusion

Detection of *Cryptosporidium* Spp. in this study can reveal the importance of transmitting the infection to human (children, immunocompromised patients, farmers, and veterinarians) with the parasite through direct contact and contamination of food and water with oocysts of the parasite in north of Iran. According to the life cycle of the parasite isolated in present study, direct or indirect contact with human and animals could be the main modes of transmission of the parasite to human. Present results can help public health care system for preventing and managing of cryptosporidiosis in human. In this study, we confronted with limit of the lack of epidemiological data for analyzing various determinant of distribution of the disease in provincial level. Further work needs to be done to establish molecular, parasitological, and epidemiological investigations on the origin of *Cryptosporidium* Spp. in this area.

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