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Detection of *Cyclospora, Microsporidia and Cryptosporidium* by direct microscopy and PCR in stools specimens in Northwest, Iran

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Abstract

This study was designed to detect the presence of *Cyclospora, Microsporidia and Cryptosporidium* by several different methods. A total of 1825 fecal samples submitted to the Central Parasitology Laboratory of Tabriz were assessed by conventional methods of light microscopy, formalin-ether concentration technique, staining technique and PCR method. The frequency of *Cryptosporidium* was 0. 0%, 15%, 3% and 2% and *Microsporidia was* 0. 0%, 3%, 1% and 0% by methods of microscopy, formalin-ether concentration, staining and PCR, respectively. *Cyclospora* was not found in all the stool samples examined. The frequencies of *these three protozoa in* our study were less than our expectations. Lack of infection with *Cyclospora* can be good news for local health care providers. More investigations especially in at risk populations is needed for health policy making.

Keywords: *Cryptosporidium; Cyclospora; Microsporidia,* stool samples, Iran

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Introduction

Many protozoa can be spread by food or water, but this study concentrates largely on *Cryptosporidium*, *Cyclospora* and *Microsporidia*, on their possibility to cause water and food borne diseases. One of the important causes of youth diarrhea which is usually disregarded is *Cryptosporidium*. It exists as two morphologic stages: trophozoite (an actively reproducing form) and oocyst that is a resting phase and is responsible for their spread and ecological survival (Carpenter et al., 1999). In both developing and developed societies, cryptosporidiosis is a public health problem. It uses the majority of its impact on children's health and malnutrition in developing and waterborne outbreaks in developed countries (Xiao et al., 2004).

Microsporidia are often found as opportunistic pathogens in people who have any kind of immunodeficiency and often cause chronic diarrhea, weakening diseases rather than fatal infections but is responsible for 30 to 50% of all cases of diarrhea in people with AIDS (Kotler and Orenstein, 1998). They share a common basis with fungi (Keeling, 2003), and diagnosis of microsporidiosis can be managed by finding of spores in patient material by microscopy or by PCR (Peek, 2005). No treatment is yet accessible, and protective procedures (including the use of physical or chemical methods) should be performed to demolish or inactivate infective spores existent in the

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environment (Sparfel et al., 1997; Dowd et al., 1998). *Cyclospora* also causes a self-limiting diarrhea and humans are the only hosts that *C. cayentanensis* uses and like *Cryptosporidium and Microsporidia,* more severe forms of the disease can occur in patients with immunodeficiency such as those with AIDS (Türker et al., 2004). In the developed countries, cyclosporiasis is regarded a rising disease of public health and has been considered a cause of traveler's diarrhea (Ho et al., 2002). Using up of undercooked or raw foods and the fast transport of fresh fruits exposing more customers to parasite (Orlandi et al., 2002). Infection is spread through the oral-fecal way and the oocysts that are passed are not, yet, immediately infectious so this differentiates *Cyclospora* from *Cryptosporidium,* since *Cryptosporidium* oocysts are instantly infectious upon release from the host (Mansfield and Gajadhar, 2004).

Our study was carried out to survey of frequency of *Cryptosporidium, Cyclospora and Microsporidia with* conventional laboratory methods in an endemic area, Tabriz, Eastern Azerbaijan, Iran, during 2009 to 2010.

Materials and Methods

From 2009 to 2010, 1825 human fecal samples submitted to the Central Parasitology Laboratory of Tabriz were assessed by conventional methods (light microscopy), formalin-ether concentration technique, staining technique and PCR method. First the direct microscopy examination and then without delay, the formalin-ether concentration technique was managed. For simple microscopy, briefly one drop of Lugol's Iodine was put on the microscopic slide and then a small portion of fresh stool (stored at the refrigerator temperature) was added and observed at 40X.

About 10 ml of formalin (10%) was added to approximately one gram of stool and then mixed well. Fecal suspension was strained through a funnel/filter device into a 15 ml conical centrifuge tube and about 7 ml of stool mixture was filtered. Then 3 ml of ether was added and centrifuged at 2000xg for two minutes. After centrifugation 4 layers resulted (Ether, fecal debris and fat, discolored formalin and fecal sediment). Debris was loosened by ringing with an applicator stick and the top three layers were decanted and iodine stained mounts were prepared.

Staining

The Trichrom staining technique for *Microsporidia* and the Cold Kinyoun Acid Fast for both *Cyclospora* and *Cryptosporidium* was applied to fecal smears of sediments, so after being mounted, slides were examined by microscope with a 100X oil immersion objective and evaluated on the basis of physical appearance of protozoa (Gharagozlou, 1997; Read et al., 2004; Carmen et al., 2008).

DNA extraction

About 300 µl stool was diluted in 150 µl buffer TE 10:1 (Tris, 10 mM, pH=8; EDTA, 1 mM, pH=8, SDS 10% 10.60 µl) and 8 µl Proteinase K (20 mg/ml) added and after vortexing, incubated at 60°C overnight. Followed by 100 µl NaCl (5M) and 80 ml CTAB/NaCl (Cetyl trim ethyl ammonium bromide) added and incubated at 65°C for 10 minutes. After that 700 Ml Chloroform/isoamylalcohol 24:1 attached and after vortexing for 20 seconds, centrifuged at 11000 x g for 8 minutes. The upper aqueous layer was separated to another sterile tube, and about 420 µl of ispropanol was appended and after mixing, put in refrigerator -20°C for 30 minutes and centrifuged at 12000 x g for 5 minutes. At this stage, upper aqueous layer was discarded and 1 ml of 70% cold ethanol was added and following mixing centrifuged at 12000 x g for 5 minutes. Again upper layer was rejected and the micro tube was put upside down on a small tissue in room temperature for 15 minutes and after drying, sediment was diluted in 40 µl of deionized sterile water (Monis, 1996; McIntyre et al., 2000; Read et al., 2004).

The Polymerase Chain Reaction (PCR)

Cyclospora

Two primers (F1E: 5`- TACCCAATGAAAACAG TTT-3` and RB2: 5`-CAGGAGAAGCCAAGGTAGG-3`) were used to detect *Cyclospora* in stool specimens and amplification was performed to amplify fragments of the *18s* rRNA gene of *Cyclospora.* Samples were subjected to a thermal cycler consisted of 15 minutes at 95°C followed by a denaturation step at 94°C for 30 seconds, an annealing step at 53°C for 30 seconds, a primary extension at 72°C for 90 seconds and a final extension at 72°C for 10 minutes and maintained at 4°C. Products were analyzed by electrophoresis on 1% agarose gel, stained with ethidium bromide (0.5µg/ml) and visualized under a UV transilluminator (Carmen, 2008).

Cryptosporidium

Two set of primers were used to amplify *Cryptosporidium.* Amplification was performed in 2 phases via outer primers (Forward: 5´-TTCTAGAGCTAATACATGCG-3´ and Reverse: 5´-CCCATTTCCTTCGAAACAGGA-3´) designed to amplify fragments of the *SSUrRNA* gene of *Cryptosporidium* of 1325 base pairs (bp) in the primery PCR and inner primers (Forward: 5´-GGAAGGGTTGTATTTATTAGATAAAG-3´and Reverse: 5´-AAGGAGTAAGGAACAACCTCCA-3´) designed to amplify fragments of the *SSUrRNA* gene of *Cryptosporidium* of 826-864 bp in the secondary PCR. Specimens were rendered to a thermal cycler included of 4 minutes at 94°C followed by a denaturation stage at 94°C for 45 seconds, an annealing process at 52°C

for 1 minutes, a primary extension at 72°C for 45 seconds and an ending extension at 72°C for 7 minutes and result maintained at 4°C for primary PCR and 4 minutes at 94°C followed by a denaturation step at 94°C for 45 seconds, an annealing step at 51°C for 1 minutes, a primary extension at 72°C for 45 seconds and a final extension at 72°C for 7 minutes and product maintained at 4°C for the secondary PCR. Similar to *Cyclospora* result were studied by electrophoresis on a 1% agarose gel, stained with ethidium bromide (0.5µg/ml) and visualized under a UV transilluminator (McLauchlin, 1999).

Microspora

For these organisms two series of primers (the forward primer V1: 5'-CACCAGGTTGATTCTGCCT GAC-3' and the reverse one PMP2: 5'-CCTCTCCGG AACCAAACCCTG-3') were used to detect *Microspora* in stool samples and amplification was managed to amplify fragments of SSU-rDNA (the small-subunit ribosomal DNA) gene of *Microspora* specimens were put to a thermal cycler and the PCR conditions was 10 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C; a final phase of extension at 72°C for 10 minutes and product was maintained at 4°C and were analyzed by electrophoresis on a 1% agarose gel, stained with ethidium bromide (0.5µg/ml) and envisaged under a UV transilluminator (Sing et al., 2001; Samie et al., 2007; N´estor et al., 2005).

Results

A total of 1825 stool specimen were collected and on the basis of microscopic examination, 0.00% were positive for *Cryptosporidium*, *Cyclospora and Microsporidia* in all of the stool samples (Table 1). The Concentration technique and staining detected 15 (0.8%) and 3 (0.2%) positive for *Cryptosporidium*, 3

Table 1: Comparison of the frequency of *Cyclospora cayetanensis, Microsporidia and Cryptosporidium* by direct microscopy and molecular methods among 1825 specimens submitted to Tabriz Central Lab, Eastern Azerbaijan, Iran (2009-2010)

Method percentage	Organism	Frequency	
Direct microscopy	*Cryptosporidium*	0	0.0%
	Cyclospora	0	0.0%
	Microsporidia	0	0.0%
Concentration technique	*Cryptosporidium*	15	0.8%
	Cyclospora	0	0.0%
	Microsporidia	3	0.2%
Staining	*Cryptosporidium*	3	0.2%
	Cyclospora	0	0.0 %
	Microsporidia	1	0.05%
	Cryptosporidium	2	0.1%
Molecular methods	*Cyclospora*	0	0 .0 %
	Microsporidia	0	0.0%

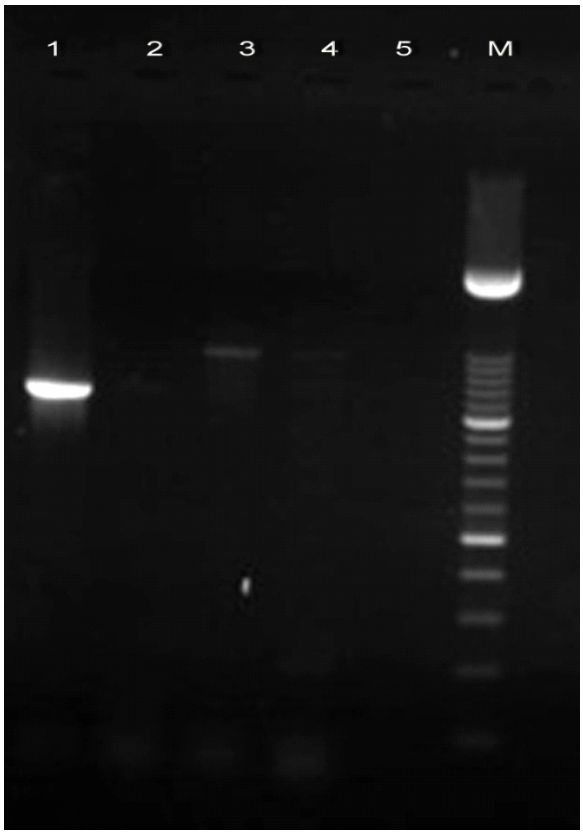

Fig. 1: Ethidium bromide-stained agarose gel of PCR products (1325 bp) of *Cryptosporidium* in specimens submitted to Tabriz Central Lab, Northweststst, Iran (2009-2010). Lane M, 1000 bp DNA ladder; Lane 1 *Cryptosporidium* and lane 2 Negative control.

(0.2%) and 1 (0.05%) positive for *Microsporidia* while *Cyclospora* was not found in any of the samples (Table 1). PCR assay was used for accurate detection of *Cryptosporidium*, *Cyclospora* and *Microsporidia* genus in the samples and as the table 1 shows 2(0.1%) were positive for *Cryptosporidium* and *Cyclospora* and *Microsporidia* were not discovered in any of the specimens even in the molecular methods. When DNA was extracted from whole feces, the 18 rRNA (of *Cyclospora*), SSUrRNA gene of *Cryptosporidium* and SSU-rDNA gene (of Microspora) was amplified by the specific primers. After agarose gel electrophoresis, on the basis of their patterns, bands with expected size of *Cryptosporidium* was observed but neither *Cyclospora* nor *Microsporidia* was seen by molecular methods. (Fig. 1).

Discussion

Throughout the world, intestinal parasite infections are observed, with high prevalence rates in many areas and the prevention and control of them are now

possible than ever before. From parasite infections agents, *Cryptosporidium*, *Cyclospora* and *Microsporidia* are important intestinal parasites worldwide. In the lifecycle of these parasites, carrier patients act as a source of infection by continuously excreting the oocyst (and or spore) stage in their stools. Several species identified in the *Cryptosporidium* genus can parasitize many animals and are the cause of zoonotic infections affecting humans including *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. cuniculus* and *C. meleagridis* meanwhile human cryptosporidiosis is caused by *C. parvum* and *C. hominis* and a number of molecular assays have been developed for their specific detection in stool and environmental samples (Xiao et al., 2001; Chalmers et al., 2009; Kvac et al., 2009). In clinical laboratories, Acid Fast staining methods as a current diagnostic test for detecting of *Cryptosporidium* are most frequently used meanwhile immunofluorescence microscopy, for most sensitivity and specificity is the method of choice (Kaplan et al, 2002). Regarding microsporidiosis, five genera (*Encephalitozoon* spp., *Enterocytozoon* spp., *Pleistophora* sp., *Septata* spp., and *Nosema* spp.) and unspecified *Microsporidia* (referred to by the common word Microsporidium) have been related with human infection, which comes into view to reveal mainly in immunocompromised persons. They are intracellular spore-forming protozoal microorganisms owned by the phylum *Microspora* with a rich verity of hosts, including mainly invertebrates and all classes of vertebrates (Weber et al., 1994).

Routine detection of *Microsporidia* is microscopic examination of specimens such as stool, urine, duodenal aspirates, sputum, nasal discharge, bronchoalveolar lavage fluid, and conjunctival smears. Final identification is made by using the electron microscopy or immunofluorescence technique (Weber et al, 1994). With relation to *Cyclospora cayetanensis*, it is a cyst-forming protozoan that causes a self-limiting diarrhea and the only host *C. cayentanensis* uses are humans. Infection is transmitted through the oral-fecal route and due to its small size and intracellular habitat, detection of *C. cayetanensis* can be very hard. Microscopic detection in stool samples of oocysts and demonstration of oocyst sporulation are two usual methods for diagnosis and since finding out of these three microorganisms is so difficult, there is an apparent need to carry out amplification by polymerase chain reaction (Türker et al., 2004; Mansfield., 2004). In the present study, that specimens were from people referred to Central Parasitology Laboratory of Tabriz, the occurrence of *Cryptosporidium* were 0.0%, 15%, 3.0% and *Microspora* were 0.0%, 3.0%, 1.0% by methods of microscopy, formalin-ether concentration and staining, correspondingly. Subsequently, PCR test was performed well with human fecal specimens and in terms of *Cryptosporidium* detected (less infection than concentration and staining methods, Table 1) and *Cyclospora* and *Microspora* were not found in all the stool samples examined. An investigate from the north Santiago (Chile) in 1995, showed a prevalence of 0.4% for *Cryptosporidium* and most infections were detected in rainy and mildly cold period. In this report with analysis of 4892 stool samples, it is concluded that *Cryptosporidium* prevalence increased in hospitalized children and HIV infected adults meanwhile infections are uncommon in healthy outpatients (Mercado, 1995). In another study from Karachi (Pakistan), stool specimen from children with extended diarrhea were examined by wet mounts beside with modified acid fast staining and examination revealed that Out of 300 samples, five (1.7%) were diagnosed as positive for *Cryptosporidium*. Also it was noted that modified acid fast staining of fecal samples is a main, non-invasive and practical diagnostic method in identification of *Cryptosporidium* (Shoaib et al., 2003).

In the other study in Peshawar (Pakistan), stool samples from young children (not over 5 years of age) presented with diarrhea were gathered and wet mount preparation and modified Ziehl-Neelsen staining were used for detection of oocysts in fecal specimens. *Cryptosporidium* oocysts were found in 18 (9.0%) out of 200 children suffering from diarrhea (Shahina et al.., 2010). In a controlled study, in Jakarta (Indonesia), *Cryptosporidium* was identified in 15% samples, who were HIV infected with CD4 (Idris et al., 2010).

Some attempts that have been performed prior to our study in the other parts of our country, to determine the frequency of the parasites, showed wide-ranging results. A sample of 412 cattle from Kerman province (South of Iran), showed a cryptosporidiosis prevalence of 18.9% and in this study 4 isolates of *C. andersoni* and 8 isolates of *C. parvum* were found for the first time in Iran by using molecular techniques and by this report, *Cryptosporidium* infection was common in cattle of Kerman (Fasihi, 2006). A cross-sectional study conducted by Hamedi indicated that in children referred to Bandar Abbas Pediatric Hospital in southeastern Iran, the prevalence of *Cryptosporidium* infection was 7.0% and there were also a significant association between *Cryptosporidium* infection and underweight children (Hamedi et al., 2005). In Mazandaran province (north of Iran) 142 stool samples (64 HIV+/AIDS patients and 78 non-HIV infected individuals) collected and screened for intestinal parasites and results showed a frequency of 2.5% for *Cryptosporidium* spp (Daryani et al., 2009). In Esfahan (Center of Iran) the prevalence rate of *Cryptosporidium* infection in hemodialysis patients and 2 control groups were assessed and according to this report, twelve (11.5%) dialysis patients were infected with *Cryptosporidium* and this was significantly higher than 4 (4.4%), and 5 (3.6%)

cases in the 2 control groups respectively (Seyrafian et al., 2006). Our results show that in compare with the other regions of Iran, the prevalence of *Cryptosporidium* in Tabriz (with an average of 3.6% by various methods) is not as much of we predicted. About the other microorganism, *Microsporidia* spp, that has appeared in recent years as an opportunistic infectious agent with an ever-present distribution, the clinical infection differ broadly, from an asymptomatic to weight loss, watery diarrhea, abdominal cramps, mainly in immunosuppressed patients (Didier, 2005). In Brazil, stool samples and cultures were collected from ninety-eight patients (with rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis) and healthy control patients and positive tests for *Microsporidia* were significantly higher in all types of rheumatic disease patients (Aikawa., 2011).

During a case control study that was conducted in 103 outpatients in Venesuela, using unconcentrated formalin-fixed stools examined by Weber's chromotrope-based staining method, *Microsporidia* were detected in 14 (13.6%) of 103 patients (Bonilla et al., 2006). In a similar study, in Italy occurrence of Microsporidial infections was 1.8% and it seems many factors such as socio-economic conditions may influence parasitosis prevalence (Marangi et al., 1995). Microsporidiosis is common in children under 5 years of age, particularly those who live in developing countries (Tumwine et al., 2005) or who are HIV positive (Leelayoova et al., 2001; Wanachiwanawin et al., 2002). In a report from Uganda (an African country) from 2002 through 2003, a total of 243 children under 60 months of age with persistent diarrhea were assessed and results showed an association between reduced weight gain and microsporidiosis (Siobhan et al., 2009). In our country in Khoramabad (west of Iran), a study conducted by (Fallahi et al., 2007) indicated that 276 fecal samples of HIV+/AIDS patients, submitted to medicine center were assessed by microscopy and Formalin-ether concentration techniques and prevalence of *Microsporidia* spp was 5.5%, whereas, in contrasts with our study where infection rate of *Microsporidia spp* is too low and is 0.01%, however, our samples were from non-HIV infected individuals. In terms of *Cyclospora*, which have been clearly recognized as important causes of diarrhea during childhood (Griffiths, 1998; Looney, 1998), in a study from Tanzania (Africa) by Cegielski, 59 fecal specimens were investigated and 2 cases of all samples were positive for *Cyclospora* (Cegielski et al., 1999). In another study from a pediatric hospital of Havana City (Cuba) a total of 401 stool specimen (113 pediatric patients with and 288 children without diarrhea) were collected and observed on the basis of microscopic examination and results showed a frequency of 5 (4.4 %) *C. cayetanensis* only in the group of children with diarrhea (Núñez et al., 2003). In Iran, there have been few studies directing this subject, however according to a study that was conducted to determine the prevalence of intestinal parasitic infections in renal transplant recipients, none of investigated specimens were positive to *C. cayetanensis* infection (Azami et al., 2010) and these results are similar to our findings, as this microorganism was not found in all the stool samples examined. Recently, a study conducted by Rezaeian et al. (2000) indicated an uncommon distribution of this parasite in Iran, with only two cases reported so far. Also the prevalence of Cyclosporiasis was studied in Tehran in 2002-2003 by direct smear, formalin-ether concentration and staining techniques, and according to this report, among 420 stool samples from children under 10 years of age with diarrhea, *C. cayetanensis* was not detected in any of the stool samples and similar to our work, absence of infection with *C. cayetanensis* was apparent (Nikmanesh et al., 2006). Fortunately it seems that *Cyclospora* infection is very rare and in spite of larger volume size of stool (1825 samples), we did not find the abovementioned microorganism in Tabriz. This study showed that the parasitic infection with *Cryptosporidium, Cyclospora and Microsporidia* in Tabriz city is not a major health problem, because the results of these parasites was low by microscopy and PCR methods and additional studies are needed to determine whether single or mixed infections occur in other geographical regions in Iran.

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