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SSU- rRNA Gene Analysis of *Cryptosporidium* spp. in HIV Positive and Negative Patients

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Abstract

Cryptosporidium is an apicomplexan parasite of humans and a wild range of domestic as well as wild animals. An 833-bp fragment of the 18S-rRNA gene was used to identify *Cryptosporidium* spp. recovered from children and adult patients, in human immunodeficiency virus (HIV) positive and negative patients in Iran. Initial identification of cryptosporidiosis was carried out by Ziehl-Neelsen acid-fast staining method of stool samples. The samples, then, were identified specifically by nested PCR, targeting the most polymorphic region of the 18S-rRNA gene. The genotype encountered was detected by restriction endonuclease digestion of the nested-PCR product. Among 17 analyzed isolates, two different genotypes of *Cryptosporidium* were identified; 24% of the isolates belonged to *C. parvum* human genotype, and 76% to the potentially zoonotic species of *C. parvum* bovine genotype. The results of the present study showed that in contrast to HIV negative individuals, HIV positive individuals were more likely to be infected with zoonotic genotypes of the parasite; it was also confirmed the fact that zoonotic transmission of the parasite in Iran was as frequent as the transmission of anthroponotic origin. These outcomes are helpful for researchers to establish the corresponding prevention and treatment measures.

Keywords: Cryptosporidium*, SSU-rRNA gene, HIV, Iran*

Introduction

Cryptosporidium is an emerging human and animal enteropathogen. Nowadays, cryptosporidiosis has been reported from over 40 countries, in both immunocompetent and immunocompromised patients. The most severe clinical infections are observed in individuals with acquired immune deficiency syndrome (AIDS) in whom cryptosporidiosis induces watery diarrhea, leading to electrolyte imbalance and cachexia (1). However, it is now recognized that the infection is common in children aged 1-5 yr, causing long-term diarrheal illness associated with shortfalls in linear growth and weight gain (2, 3). Cryptosporidiosis can appear as a zoonosis or as an anthroponosis disease. The transmission is directly fecal-oral or by water and food contaminated with *Cryptosporidium* oocysts, with as few as ten viable oocysts needed for infection (4). Two distinct *C. parvum* genotypes, referred to as the human (genotype 1 or genotype H) and cattle (genotype 2 or genotype C) genotypes, have been recognized to be responsible for human cryptosporidiosis for a while ago (5). The various biological and genetic differences led to propose a new species, *C. hominis*, to denote the human genotype (6). Recent studies suggest that *C. felis* and *C. meleagridis* may also cause diarrhea in humans (7, 8). *Cryptosporidium* spp. isolated from different regions of the world has different

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antigens, virulence, infectivity, and sensitivity to drugs and disinfectants (1, 9). Therefore, to design control programs, it is necessary to know the species and genotypes of *Cryptosporidium* occurring in each region. To date, there is no data available on the molecular epidemiology of *Cryptosporidium* in Iran, and the main species and genotypes involved in the infection, in humans or animals remain unknown. However, studies on human cryptosporidiosis have shown prevalence of 3-7% in Iran (10, 11).

Therefore, the current study was undertaken to detect the genotypes of parasites from the fecal specimens of sporadic human cryptosporidiosis cases in Iran.

Materials and Methods

Parasite Isolates Fecal specimens were collected from children aged 1-12 yr and adult patients, in HIV positive and HIV negative patients, diagnosed according to clinical symptoms consistent with cryptosporidiosis. From each specimen an aliquot was processed using the standard formalin-ethyl acetate concentration procedure and examined microscopically for the presence of C*ryptosporidium* oocysts on modified acid-fast Ziehl-Neelsen stained slides. Then, the positive fecal samples for *Cryptosporidium* were diluted in 2.5% dichoromate solution and kept at 4 ºC until subsequent molecular procedures. The intensity of infection was quantified by the number of oocysts counted in a 20 µl volume of concentrated stool specimen: 1+ (1-50 oocysts), 2+ (51-150 oocysts), and 3+ (>150 oocysts). Firstly, clinical and epidemiological information were obtained from the original forms filled for every individual and further information was added after interview with the patients and completing the standard questionnaires.

DNA extraction Stool specimens in potassium dichromate were washed five times in cold distilled water to remove traces of the preservative, prior to DNA extraction. All *Cryptosporidium* DNA used in this study were extracted by the QIAamp DNA Stool Mini kit (QIAgen, Hilden, Germany) according to manufacturer's instructions. DNA samples were, then, stored at -20 °C until later analysis.

Nested-PCR amplification and RFLP analysis *Cryptosporidium* species and *C. parvum* genotype were determined by nested PCR of the SSU rRNA gene fragment and RFLP analysis as the procedure previously described by Xiao et al. (12, 13). For the primary PCR, a PCR product about 1,325 bp long was amplified by using primers 5′-TTC TAG AGC TAA TAC ATG CG-3′ and 5′-CCC ATT TCC TTC GAA ACA GGA-3′ (Alpha DNA, Quebec, Canada). Each PCR mixture (total volume, 50 µl) contained 10 µl of 10x PCR buffer, 6 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 µM, each primer at a concentration of 100 nM, 1.5 U of *Taq* polymerase (Roche, Mannheim, Germany), 5 µl of DNA template, and 0.1 µg/ µl of BSA (Promega, USA). The amplification reactions of 35 cycles were initiated by denaturation of the DNA at 94 ºC for 5 min, denaturation at 94 ºC for 45 s, annealing of the primer at 52 ºC for 45 s, and extension at 72 ºC for 1 min, with an additional 7 min extension at 72 ºC. For the secondary PCR, a PCR product 819 to 837 bp long (depending on the species) was amplified by using 1 µl of the primary PCR product and primers 5′-GGA AGG GTT GTA TTT ATT AGA TAA AG-3′ and 5′-AAG GAG TAA GGA ACA ACC TCC A-3′ (Alpha DNA, Quebec, Canada). The PCR mixture and cycling conditions were identical to primary PCR, except that 3 mM $MgCl_2$ was used in the PCR and annealing lasted 45 s at 55 ºC. Positive (DNA of *C. parvum*) and negative (water instead of DNA template) controls, were included in each amplification run. The PCR product was analyzed by electrophoresis in a 2% agarose gel (Merck, Darmstad, Germany) and visualized after ethidium bromide staining and recorded by UV illumination (Fig. 1). For the restriction-fragment analysis, 15 µl of the secondary PCR products was digested in a 20 µl

reaction mixture containing 1µl (20 U) of *Ssp*I (Promega, USA) (for species identification) or 1µl of *Vsp*I (Promega, USA) (for genotyping of *C. parvum*), 0.5 µl of bovine serum albumin and 3 µl of the appropriate 10X restriction buffer at 37 °C for 2 h, under conditions recommended by the supplier. The digest products were fractioned by 2% agarose gel containing 0.2 µg/ml ethidium bromide and visualized under UV illumination.

Results

In this study C*ryptosporidium* isolated from HIV positive patients and HIV negative patients in Iran were characterized by PCR-RFLP. In general, 17 C*ryptosporidium* isolates were characterized. Initially, for all isolates C*ryptosporidium* oocysts were detected from diarrheic stool specimens and then stained by modified Ziehl-Neelsen method. RFLP analysis of the nested-PCR products showed that 13 (76%) isolates were *C. parvum* bovine genotype and 4 (24%) that of *C. hominis* (Table 1).

Species diagnosis was performed by digesting the secondary PCR product with *Ssp*I, and differentiation of *C. parvum* genotypes by digestion with *Vsp*I (Fig. 2 and 3). Digestion of the secondary PCR products from the group *C. parvum* parasites with *Ssp*I, showed an identical restriction pattern. *C. parvum* infecting humans and bovine showed an identical restriction patterns, with three visible fragments of 108 to 111, 254, and 449 bp in size (Table 2). To differentiate human and bovine genotypes of *C. parvum* genotypes, the secondary PCR product was digested with *Vsp*I. *C. parvum* bovine genotype produced two visible bands of 628 and 104 bp, whereas the human genotype produced three visible bands of 561- 104, and 70bp (Table 2).

Table 1: Results of PCR-RFLP analysis of 17 positive stool samples used in this study

No. of isolate	Age group*	Sex	HIV status	Intensity of infection **	PCR-RFLP analysis ***
1	Adult	M	+	+ 3	*A*
2	Adult	M	+	+ 3	*B*
3	Adult	F	+	+ 2	*A*
4	Adult	M	+	+ 2	*A*
5	Adult	F	+	+ 2	*A*
6	Adult	M	+	+ 2	*A*
7	Adult	M	+	+ 2	*A*
8	Adult	M	+	+ 3	*A*
9	Child	M	-	+ 1	*A*
10	Child	F	-	+ 3	*B*
11	Child	F	-	+ 2	*B*
12	Child	M	-	+ 1	*A*
13	Child	M	-	+ 3	*B*
14	Child	M	-	+ 1	*A*
15	Child	M	-	+ 1	*A*
16	Child	M	+	+ 1	*A*
17	Adult	M	-	+ 1	*A*

* Adults: > 12 yr; Children: 1-12 yr

** +3 (severe infection), +2 (moderate infection), +1 (mild infection)

*** A: *C. parvum* bovine genotype; B: *C. hominis*

Table 2: RFLP in the SSU-rRNA gene of various *Cryptosporidium spp.* (11)

Species	PCR fragment no.	*Ssp*I digestion*	*Vsp*I digestion*
C. hominis	837	11, 12,**111, 254, 449**	**70**, 102, **104, 561**
C. parvum	834	11, 12, **108, 254, 449**	102, **104,628**

Numbers in bold are the sizes of bands visible on the electrophoresis gel

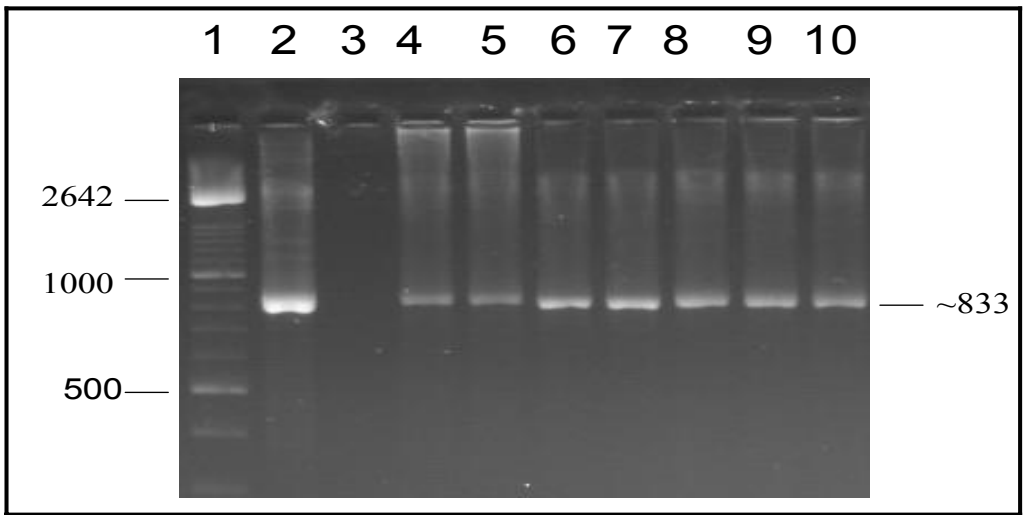

Fig.1: Molecular diagnosis of *Cryptosporidium* parasites by a nested PCR based on SSU-rRNA gene. Line 1: molecular weight marker XIV; Line 2: positive control; Line 3: negative control; Line 4, 5, 6, 7, 8, 9 and 10: patient isolates.

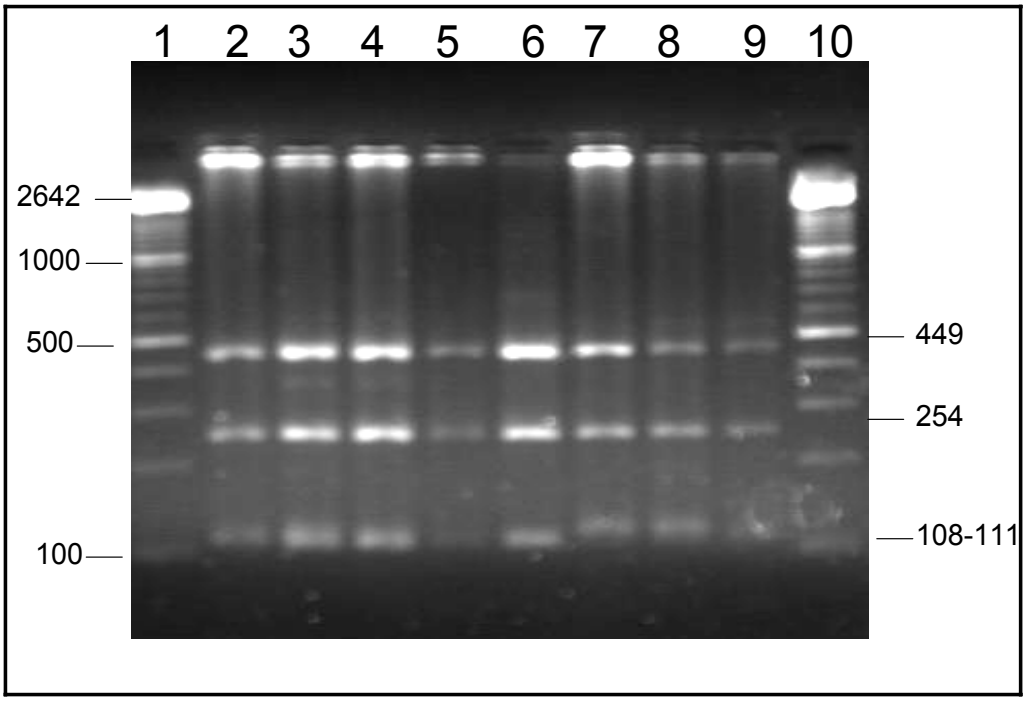

Fig. 2: Species diagnosis of *Cryptosporidium* parasites by a *Ssp*I digestion of the nested PCR product. Line 1 and 10: molecular weight marker XIII; Line 2 and 3: control samples (*C. hominis* and *C. parvum* bovine genotype, respectively); Line 4, 5 and 6: *C. parvum* bovine genotype; Line 7, 8 and 9: *C. parvum* human genotype (*C. hominis*).

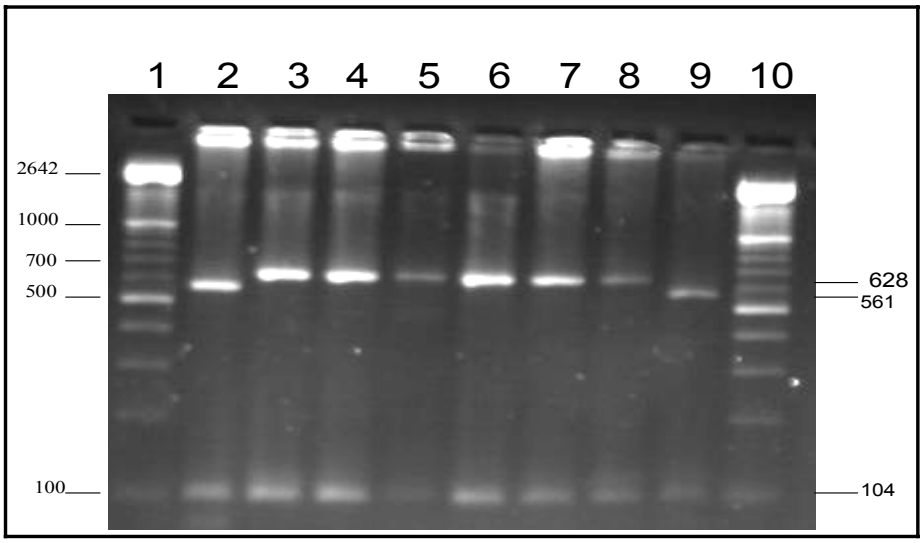

Fig. 3: Genotype diagnosis of Cryptosporidium parasites by *Vsp*I digestion of the nested PCR product. Line 1and10: molecular weight marker XIII; Line 2 and 3: control samples (*C. hominis* and *C. parvum* bovine genotype, respectively); Line 4, 5, 6, 7 and 8: *C. parvum bovine* genotype (628, 104bp); Line 9: *C. hominis* (561,104bp).

Discussion

Human cryptosporidiosis is mainly caused by *C. parvum* and *C. hominis* (13, 14). *C. hominis* is found almost exclusively in humans, whereas *C. parvum* is found in domestic livestock, wild animals, and humans, too (7, 15- 19). The occurrence of both *Cryptosporidium* species in humans has provided evidence that both anthroponotic and zoonotic cycles can occur in human infections (5, 20).

C. parvum bovine genotype parasites account for most cases of cryptosporidiosis in Iran in both HIV positive and HIV negative patients. Other researches have also pointed that *C. parvum* bovine genotype is more responsible for human infections than *C. hominis* in European countries including U.K. (21), Switzerland (22) and France (23). In contrast, in the United States (22), Thailand (16), Australia (24), Kenya (22), and South Africa (25), it has been stated that anthroponotic parasites are responsible for the majority cases of human cryptosporidiosis. Whether these results reflect the existence of some geographic variations in the distribution of *C. parvum* and *C. hominis* in humans is still unknown, as in most mentioned studies only a small number of isolates have been analyzed.

The results of current study indicate the possibility of co-existence of two distinct C*ryptosporidium* populations cycling in humans in Iran as suggested before (26). One population appears to involve zoonotic transmission from cattle to humans with subsequent human-to-human and human-to-cattle transmission; the other population seems to engage an anthroponotic transmission cycle, basically in humans.

Previous studies of the 18S-rRNA gene have shown that the ability to amplify this gene fragment from different species and genotypes of the organism with one set of primers makes this locus the most appropriate for screening, where the species and types of C*ryptosporidium* organisms' origin are unknown. This technique is quiet sensitive, as demonstrated by amplification of DNA from a single purified oocyst species (13). In the present study, the ability of this technique to differentiate extended members of the *C. parvum* group was evaluated.

The results of present study is coincident with a previous study, conducted in the Peruvian chil-

dren, both indicating on higher number of oocysts in stool specimens infected with *C. hominis* genotype, compared with those infected with *C. parvum* bovine genotype (7). Our data are in agreement with these findings. Adaptation to infect specific hosts is a common phenomenon in C*ryptosporidium* parasites (6, 13). It is not surprising that the human genotype C*ryptosporidium* parasites appear to be better adapted to infect humans than do zoonotic C*ryptosporidium* parasites.

In the present work, which is the first on molecular characterization of *Cryptosporidium* in Iran, the existence of both *C. parvum* and *C. hominis* among human cases of cryptosporidiosis in the country is emphasized. In this way, it was confirmed that zoonotic transmission is as common as anthroponotic transmission in infecting humans in Iran.

These outcomes have important epidemiological ramifications, as they enable the orientations of control strategy programs designed to prevent cryptosporidiosis in the country.

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