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Original Article

Identification and Subtyping of *Cryptosporidium parvum* and Cryptosporidium *hominis* in Cancer Patients, Isfahan Province, Central Iran

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Abstract

Background: *Cryptosporidium* spp. are protozoan parasites that cause diarrhea in humans and animals. Subtyping data about *Cryptosporidium* spp. in Isfahan, Iran is limited; therefore, we aimed to study the prevalence rate of *Cryptosporidium* spp. in cancer patients, associated risk factors, and subtypes of *Cryptosporidium* spp.

Methods: Fecal samples were collected from 187 cancer patients from the Oncology Department of Seyed-al-Shohada Hospital, Isfahan University of Medical Sciences during 2014–2020 and screened for *Cryptosporidium* spp. using microscopical techniques. Nested PCR amplifying 18S rRNA gene was used to detect *Cryptosporidium* spp. in samples, followed by subtyping using nested PCR amplifying gp60 sequences.

Results: Overall, the rate of infection with *Cryptosporidium* spp. was 4.3% (n=8). Five samples out of eight samples were identified as *Cryptosporidium* spp. using a nested PCR for the 18S rRNA gene, two subtypes of *C. parvum* named IIaA18G3R1 (n = 2) and IIaA17G2R1 (n = 2), and one subtype of *C. hominis* named IbA6G3 were identified by sequencing of the gp60. The IbA6G3 subtype has rarely been detected in other investigations.

Conclusion: This is the first survey on the subtyping of *Cryptosporidium* spp. in this region. The results of the present survey show both zoonotic and anthroponotic transmission routes in the region.

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Introduction

Cryptosporidium spp. are important zoonotic pathogens and infect the microvillus borders of the gastrointestinal and respiratory epithelium of vertebrate hosts, including reptiles, amphibians, birds, ruminants, rodents and humans. The prevalence rates of cryptosporidiosis in humans are reported 0.1%–25.9% worldwide, based on the previous reports in Iran, 0.3% of children and 26.7% of immunocompromised patients are infected with *Cryptosporidium* spp., In a review article (27 studies), *Cryptosporidium* were found in 3.8% children and 8% among immunosuppressed patients in Iran (1), therefore, it should be noted that various prevalence rates are reported (1-4).

About 38 species and 40 genotypes of *Cryptosporidium* have been identified until now; *C. hominis* and *C. parvum* are the main pathogenic species in humans and account for about 90% of infections. *C. parvum* is considered the first cause of infection and *C. hominis* is the second cause of infection (5). The major hosts of *C. parvum* are cattle, which means that zoonotic transmission between humans and animals occurs in infection with *C. parvum*, while *C. hominis* is transmitted between humans and is the major cause of anthroponotic transmission (6).

Infection with *Cryptosporidium* spp. shows a range of symptoms, including watery diarrhea, nausea, vomiting, abdominal pain, and fever. *Cryptosporidium* infection could be one of the most causes of diarrhea in humans. It was reported that 1-3% and 10-15% of diarrhea cases in the US and Europe and developing countries accounted for *Cryptosporidium* infections, respectively (7). The infection in immunocompetent hosts is asymptomatic or transient and watery diarrhea may occur, which resolves without treatment, but it may cause severe and persistent watery diarrhea in immunodeficient hosts. *Cryptosporidium* spp. infection in patients with a suppressed immune system, including AIDS, organ transplants, hemodialysis patients, and cancer patients, is one of the common causes of death (3, 8). Infections have been suggested to be associated with some cancers, severe infections occur more frequently in cancer patients. The results of a study demonstrated that the possibility of *Cryptosporidium* infection was 2.8 times higher among cancer patients in comparison with healthy individuals. Besides, a study reported an association between cryptosporidiosis and cancer (9).

To identify *Cryptosporidium* species, a nested PCR was performed using the 18S rRNA gene, which contains conserved regions interspersed with highly polymorphic regions (10).

Currently, analysis of the gp60 gene has been performed on subtyping *Cryptosporidium* spp. including *C. parvum* and *C. hominis*. Sequence analysis of the gp60 gene has identified the *C. parvum* and *C. hominis* subtype families (11).

Now, it is obvious that immunosuppressed patients like cancer patients are at higher risk for *Cryptosporidium* infections. Therefore, we aimed to identify *Cryptosporidium* species, associated risk factors with infection, and characterize the subtypes of *Cryptosporidium* spp. in cancer patients in Isfahan, Iran, to introduce possible transmission routes. The findings of the current study could be helpful for the management and prevention and knowing the pathogenicity of cryptosporidiosis among immunocompromised patients.

Materials and Methods

Ethical considerations

The current study was approved by the Ethics Committee of Isfahan University of Medical Sciences (Isfahan, Iran) (Ethical approval number: 192055). Written informed consent

was obtained from all the participants in the study, including participants above 16 years old and legal guardians of children who participated in the study before the investigation. All the participants filled out a questionnaire containing information about gender, age, marital status, residence, animal contact, education level, and clinical symptoms.

Study design

One hundred and eighty-seven fecal samples were obtained from cancer patients who were referred to the Oncology Department of Seyed-al-Shohada Hospital, Isfahan University of Medical Sciences during 2014–2020. All samples were delivered to the lab of the Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

Stool examinations

For every sample, a direct microscopic examination of prepared smears applying normal saline and formalin ether concentration techniques was carried out. In addition, all slides were stained with a modified ZiehlNeelsen acid-fast method for detecting *Cryptosporidium* as previously described (12). To investigate the presence of *Cryptosporidium* oocysts, the stained slides were examined at a 100x oil immersion lens under a light microscope. The positive *Cryptosporidium* samples identified by microscopic examination were stored in 2.5% potassium di-chromate at 4 °C for DNA extraction.

DNA extraction

The eight positive samples with microscopical techniques were subjected to DNA extraction. *Cryptosporidium* oocysts destruction were performed using glass beads. Finally, phenol chloroform method was used for DNA purification (13).

PCR amplification

The presence of *Cryptosporidium* spp. in the samples was detected via a nested PCR for the 18S rRNA gene using the following primers:

The outer forward primer: 5 ' -GGA AGG GTT GTA TTT ATT AGA TAA AG-3 ', the common reverse primer: Cr550 5 ' -TGA AGG AGT AAG GAA CAA CCT CC-3 '; which amplified an 835-bp fragment. The second step of nested-PCR was performed with an amplicon of 530 bp with the inner forward primer Cr250 5 ' -GGA ATG AGT KRA GTA TAA ACC CC-3 ' (12).

Briefly, the mixture for both reactions consisted of **12.**5 µL of 2x Taq DNA Polymerase Master Mix RED **(**AMPLIQON®**)**, 2 µL of DNA template, and 5 pmol/µL of each primer. Thermal cycling for the first and second nested PCR was performed according to the previous study (13).

Subtyping

The positive samples were further characterized for the subtypes using nested PCR amplifying gp60 sequences (800 to 850 bp), with the primary and secondary primers mentioned in a previous study (14). PCR products were purified and sequenced in both directions. The obtained nucleotide sequences were subjected to the BLAST website (HTTPS://blast.ncbi.nlm.nih.gov/Blast.cgi) in comparison with the reference sequences in GenBank to determine the species and subtypes of *Cryptosporidium* spp. isolates.

Statistical analysis

Data analysis of all variables was performed using SPSS software version 24 (IBM Corp., Armonk, NY, USA). Descriptive statistics were conducted. A *P* value less than 0.05 was considered statistically significant.

Results

In total, *Cryptosporidium* spp. were microscopically detected in 8 out of 187 patients with cancer (4.3%). The sociodemographic characteristics of cancer patients with *Cryptosporidium* spp. infection are presented in Table 1.

Table 1: Sociodemographic characteristics of cancer patients with *Cryptosporidium* spp. infection in Isfahan province, the center of Iran

Characteristic		*Total*	*Infected (%)*	*Non-infected (%)*	*P-value*
Gender	Male	52	5 (9.6)	47 (90.4)	0.025
	Female	135	3 (2.2)	132 (97.8)	
Age group (yr)	≤ 40	48	2 (4.2)	46 (95.8)	0.965
	>40	139	6 (4.3)	133 (95.7)	
Marital status	Single	11	2 (18.2)	9 (81.8)	0.019
	Married	176	6 (3.4)	170 (96.6)	
Residence	Rural	18	2 (11.1)	16 (88.9)	0.132
	Urban	169	6 (3.6)	163 (96.4)	
Education level	diploma or under	156	6 (3.8)	150 (96.2)	0.513
	upper than diploma	31	2 (6.5)	29 (93.5)	
Animal contact	Yes	46	4 (8.7)	42 (91.3)	0.088
	No	141	4 (2.8)	137 (97.2)	

A *P-value less than 0.05* is statistically *significant*

Although females and males represented 135 and 52 out of 187 cases, respectively, infection with *Cryptosporidium* spp. was higher in males (9.6%) than in females (2.2%) , which was statistically significant ($P = 0.025$, Table 1). Forty-eight out of 187 participants were ≤ 40 years old, of which two cases (4.2%) were diagnosed with *Cryptosporidium* infection. 139 out of 187 cases were > 40 years old, of which six cases (4.3%) had *Cryptosporidium* infection. However, there was not a significant difference ($P = 0.965$) in the age groups. No significant difference was observed between the rate of *Cryptosporidium* infection and other Characteristics of the study (Table 1).

Clinical manifestations including abdominal pain ($P = 0.005$) and watery diarrhea ($P = 0.001$) occurred significantly higher in cancer patients with positive *Cryptosporidium* infection compared to cancer patients without *Cryptosporidium* infection.

To determine the presence of *Cryptosporidium* spp., a nested PCR using the 18S rRNA gene was performed on eight positive samples. Of those eight samples, five samples were successfully amplified and were identified as *Cryptosporidium* spp.

Finally, for the identification of Subtypes of *Cryptosporidium* spp. in cancer patients, the PCR positive products of the gp60 gene (Fig. 1) of five specimens were sequenced.

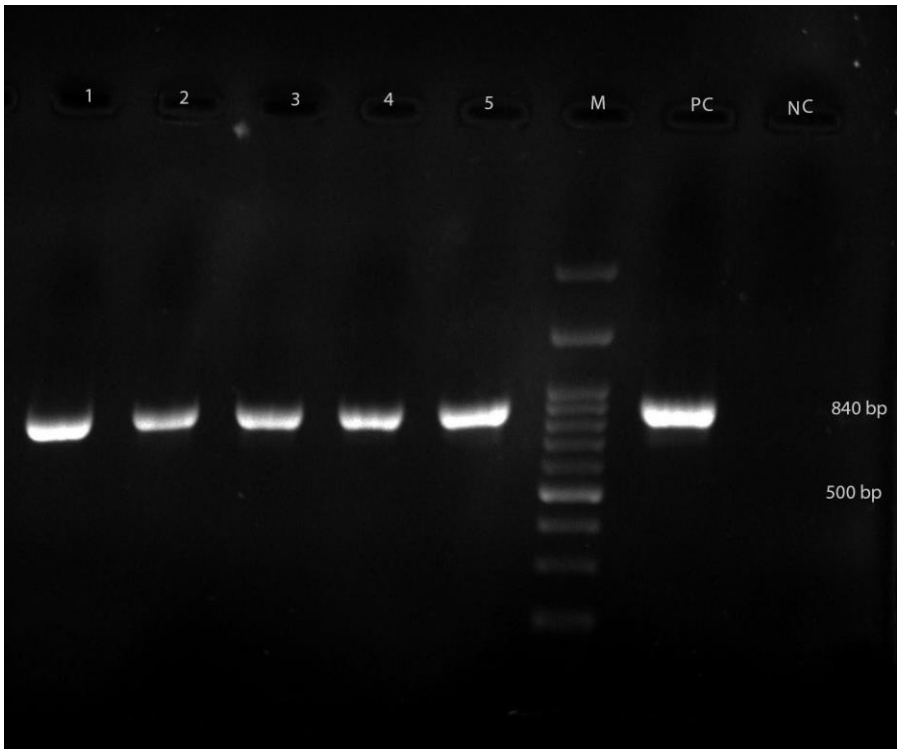

Fig. 1: PCR of isolates from cancer patients based on gp60 gene. Lanes 1-5: *Cryptosporidium* isolates (840 bp), M: DNA size marker (100 bp), PC: Positive control, NC: Negative control

All five *Cryptosporidium*-positive specimens were successfully sequenced at the gp60 gene. All cases of *C. parvum* infection were attributed to a subtype family named IIa while regarding *C. hominis*, Ib was the only subtype family, out of five specimens, four were identified as *C. parvum* (IIaA18G3R1, IIaA17G2R1) and the other species was *C. hominis* (IbA6G3), Table 2.

Table 2: Subtypes of *Cryptosporidium parvum* and *Cryptosporidium hominis* in cancer patients, Isfahan province, the center of Iran

Species	***Subtype***	***GenBank ID***
C. parvum	IIaA18G3R1	ON184039
C. parvum	IIaA17G2R1	ON184040
C. parvum	IIaA17G2R1	ON184040
C. parvum	IIaA18G3R1	ON184039
C. hominis	IbA6G3	ON184041

Discussion

Many species of *Cryptosporidium* share indistinguishable morphological characteristic, hence subtyping is helpful for accurate diagnosis and identification of *Cryptosporidium* spp., also, knowing the predominant species and subtypes of *Cryptosporidium* spp. is crucial in describing the epidemiology, controlling cryptosporidiosis and decreasing the possibility of disease transmission to humans and animals (15). In the present study, identification of the *Cryptosporidium* spp., and associated risk factors were investigated.

Although several studies have been carried out on the prevalence of cryptosporidiosis in Isfahan (2, 3, 12, 16), this is the first study that has investigated the subtypes of the isolates. The overall rate of infection with *Cryptosporidium* spp. was 4.3% in cancer patients. Different rates of *Cryptosporidium* spp. infection among cancer patients varied from 1.3% to 80% is

reported (7). The prevalence rates of infection with *Cryptosporidium* spp. in cancer patients in the previous reports were as follows: 17.24% in China patients with digestive malignancies before chemotherapy and 13% in Poland in patients with colorectal cancer before beginning oncological treatment, which was much higher than the result of the current study (7, 17). In Iran, the prevalence rates of *Cryptosporidium* infection in immunocompromised patients were 4.7% from children under five years of age, immunocompromised patients, and high risk persons in Isfahan in 2007 (16), 35.9% from 89 children with lymphohematopoitic malignancies under chemotherapy, between the age of 1 and 18 years in Mashhad in 2013 (18), and 0.9% in human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) patients in Tehran in 2007 (19).

Based on the findings of the current study, men showed a significantly higher rate of infection with *Cryptosporidium* spp. than women did (7). A study in Poland, presented similar results to our survey; the male gender was associated with a higher prevalence rate of infection with *Cryptosporidium* spp., a possible explanation for this might be that men are considered to be more exposed to the source of infection due to their occupations (7). In contrast to our findings, a previous survey in Canada showed a higher prevalence rate of infection in females from 20 to 35 years old; this observation might be related to the fact that females between 20-35 are in reproductive years, during which time child-rearing could increase the risk of exposure (20).

Variations in the age distribution of *Cryptosporidium* patients were presented in the previous studies. The cryptosporidiosis prevalence in the two age groups of this survey showed similar results (4.2% vs. 4.3%). This finding is in contrast with the fact that *Cryptosporidium* infections frequently occur in children under five years old (6); the possible explanation is the type of the investigated population in the current study. The findings of a molecular study of *Cryptosporidium* patients in Ontario, Canada showed the differences in the age distribution of *Cryptosporidium* isolates, which was similar to the results of other previous studies (6, 20, 21).

Although the majority of cases in the current study lived in urban regions (136 out of 187), most of the cases with *Cryptosporidium* infection were from rural areas (11.1% vs. 3.6%), which supports the finding that the distribution of *Cryptosporidium* spp. in some regions differs, with higher *C.hominis* in urban centers and *C.parvum* in rural regions (20).

The epidemiology of cryptosporidiosis depends on the interaction between humans and animals (livestock, wildlife, domestic animals) (11). Although the findings of this study showed no statistically significant relationship between the prevalence rate of infection and animal contact, it is reported that exposure to animals is an important issue in cryptosporidiosis.

Cryptosporidium spp. are among the most common causes of watery diarrhea in humans, cryptosporidiosis causes dysentery, abdominal pain, vomiting, etc. in immunosuppressed patients and can be fatal (6). Similar to the previous data, diarrhea, and abdominal pains were observed significantly higher in cancer patients with *Cryptosporidium* in comparison with cancer patients without *Cryptosporidium* (22, 23).

The current study presented that *C. parvum* is the dominant *Cryptosporidium* spp. infecting cancer patients, similar to studies reported that *C. parvum* was more prevalent than *C. hominis* (11, 20), suggesting that contact with animals could be a route of transmission in our investigation. A survey was conducted about cryptosporidiosis in humans and animals in Isfahan, Iran, demonstrated that *C. parvum* was the most occurred species, generally (16). Therefore, it could be stated that cryptosporidiosis can occur due to close relationships with animals such as dogs, cattle, and sheep, especially in people regarding to their occupations as animal attendants or farmers. (24). Similar to our results, molecular investigation of collect-

ed stool samples in a study in Kurdistan Province, West of Iran, in 2020, identified the isolates as *C. parvum* and therefore suggested a zoonotic transmission in the area (25). In contrast, the results of other studies conducted in Isfahan, Iran (12) and in Australia (26) showed that *C. hominis* was more common than *C. parvum* (20). Most of the patients with *C. parvum* were from rural regions in our study, which is in agreement with the above-mentioned reports. Interestingly, it is stated that patients with *C. hominis* cryptosporidiosis were more likely to be reported from urban populations and daycare or childcare exposure than other species (27).

The results demonstrated that all the *C. parvum* subtypes characterized in this study belonged to the IIa family, a zoonotic subtype. Two *C. parvum* subtypes, IIaA18G3R1, and IIaA17G2R1 were described in the current study in cancer patients. As presented in the previous investigations, the *gp60* subtype IIaA15G2R1 is the most common *C. parvum* subtype in both humans and calves, worldwide, but this subtype was not observed in our survey (5, 6). On the other hand, IIaA18G3R1 was reported as the dominant in the previous investigations in Ireland (28), and Australia (29) which is in agreement with the results of our study. Analysis of the gp60 gene was helpful to define possible sources of the disease, for instance, in Australia, detection of the *C. parvum* IIaA18G3R1 subtype as the dominant subtype reflects that cattle are an important zoonotic source for infecting humans (11). In contrast to the results of our study, an investigation in Iran in 2012, demonstrated that the subtype family IId of *C. parvum* was the dominant cause of cryptosporidiosis in humans with a proposed zoonotic transmission route (30). Kiani et al. identified *C. parvum* subtypes IIdA26G1, IIdA20G1, IIaA15G2R1, and IIaA16G3R1 in gastrointestinal patients in Western Iran through sequence analysis of the gp60 gene (31).

Another subtype reported in the current study was IIaA17G2R1, which was represented in the previous studies worldwide (32-34), IIaA17G2R1 is frequently detected in calves and is related to contact with farm animals (6).

Although zoonotic subtypes predominated in the present survey, human-host adapted *C. hominis* was recognized. IbA6G3 was the only *C. hominis* subtype, which was described in this study. In the previous studies of cryptosporidiosis among humans, worldwide, the IbA10G2 *C. hominis* subtype contributed to the majority of cases of cryptosporidiosis in humans (5, 26, 35). IbA6G3 was a rare and unusual subtype within the *C. hominis* subtype families (36). In the United Kingdom, the subtype IbA6G3 was reported for the first time in a swimming pool survey (37). In addition, IbA10G2 was described as a subtype related to drinking water outbreaks in Canada (38, 39).

Conclusion

The subtyping of specimens from cancer patients suggests that *Cryptosporidium* spp. in Isfahan, Iran are *C. parvum* and *C. hominis*. The variation and introduction of a rare and unusual *C. hominis* subtype in the present survey are probably indicative of complicated transmission routes, both zoonotic and anthroponotic. Providing implementation of infectious disease prevention measures and hygiene education in high-risk groups in this region and developing an understanding of the patterns of transmission routes, including human-to-human or animal-to-human pathways seem to be important.

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Conflict of interest

None to declare.

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