Prevalence, Clinical Manifestations and Genotyping of Cryptosporidium Spp. in Patients with Gastrointestinal Illnesses in Western Iran

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Abstract

Background: Cryptosporidium species are recognized as important gastrointestinal pathogens. This study was conducted to identify the prevalence, clinical manifestations and genotyping of Cryptosporidium spp. in patients with gastrointestinal illnesses (GIs) in western Iran.

Methods: Overall, 1301 fecal samples were collected from patients with GIs referred to the 12 clinical laboratories in Nahavand County, west of Iran. Modified Ziehl-Neelsen staining method was used to identify the oocysts. DNA was extracted from positive samples and Cryptosporidium spp. were characterized by Nested PCR and sequence analysis of the 60-kDa glycoprotein (gp60) gene. Data analysis was performed using SPSS ver. 16.

Results: Prevalence of cryptosporidiosis was 1.3% (17/1301). Cryptosporidium infection was significantly associated with vomiting and nausea (P=0.001, OR=0.013; CI 95%=0.004–0.044), abdominal pain (P=0.018, OR=0.073; CI 95%=0.008–0.633) and diarrhea (P=0.001, OR=0.092; CI 95%=0.023–0.362). Of the 17 isolates typed, 11 belonged to the C. parvum IId subtype family (subtypes IIdA26G1 and IIdA20G1) and six belonged to the C. parvum IIa subtype family (subtypes IIaA15G2R1 and IIaA16G3R1). There was no significant difference between subtype families IIa and IId in occurrence of clinical symptoms (P=0.75).

Conclusion: Improved hygiene and avoidance of contact with animals and contaminated soil should be advocated to reduce the occurrence of Cryptosporidium infections, especially in children.

Keywords: Cryptosporidiosis, Clinical manifestations, Genotyping, Gastrointestinal illnesses, Iran
Introduction

Acute gastrointestinal illnesses (AGIs) are major causes of hospitalization throughout the world. In developing countries, AGIs are one of the leading causes of morbidity and mortality (1). The most common symptoms of gastrointestinal illnesses (GI) are diarrhea, abdominal pain, and vomiting. Diarrhea is the second leading cause of deaths among children less than five years of age, especially in low and middle-income countries (2). Intestinal protozoan and helminthic infections are among leading causes of gastrointestinal disorders (3).

Protozoa of the genus Cryptosporidium are recognized as important gastrointestinal pathogens that infect a wide range of vertebrates including humans. Cryptosporidium spp. are well adapted to zoonotic, waterborne and foodborne transmission, and transmitted to hosts by the fecal-oral route (5). Cryptosporidium spp. can cause a wide spectrum of symptoms, from severe life-threatening diarrhea or vomiting in immunocompromised patients to asymptomatic and self-limiting infection in immunocompetent individuals (4, 6).

C. hominis and C. parvum are the most common etiologic agents of human cryptosporidiosis worldwide, and the latter is commonly responsible for zoonotic infections (7). Other reported zoonotic Cryptosporidium species include C. meleagridis, C. felis, C. muris, C. canis, and C. ubiquitum (8, 9).

A variety of molecular methods has been used for differentiation of Cryptosporidium species/genotypes and C. parvum and C. hominis subtypes. Subtyping tools have been used extensively in studies of the transmission of C. hominis in humans and C. parvum in humans and ruminants (9). The DNA sequence analysis of 60-kDa glycoprotein gene (gp60) is currently the most widely used genetic marker in studies of the host adaptation, genetic diversity, transmission dynamics and infection sources of Cryptosporidium spp. (8, 9). The gp60 subtyping showed that C. parvum had 12 subtype families (Ila–III) and subtype families Ila and IId are considered major zoonotic ones, whilst Ile subtype family considered the major anthropoanotic one. C. hominis has been polymorphic and has at least seven subtype families (Ia–Ig) (9, 10). Several molecular and epidemiological studies in Iran have demonstrated moderate prevalence of Cryptosporidium spp. in different populations and have shown that C. parvum is the predominant species in human and livestock (4, 11-13).

The main aim of the present study was to evaluate the occurrence, clinical manifestations and subtypes of Cryptosporidium spp. in patients with acute gastrointestinal illnesses in Nahavand County, western Iran.

Materials and Methods

Study area and population

This cross-sectional study was conducted from Apr to Sept 2014 in 1301 patients with GIs referred to the 12 clinical laboratories in Nahavand County, west of Iran. Patients not given any anti-parasitic drugs in the week prior to the study were included in this study. A questionnaire survey was administered to each participant focusing on demography (age, gender, and location), gastrointestinal symptoms (abdominal pain, cramping, bloating, vomiting & nausea, diarrhea, dysentery, and constipation), living condition and water usage.

Microscopy of stool specimens

After completing the questionnaire, all participants were given a clean and dry plastic container pre-labeled with their identification numbers. The fecal specimens were examined microscopically to determine the consistency, presence of blood and mucus and any other abnormalities. To identify oocysts of Cryptosporidium spp., a permanent slide was prepared for each sample after oocyst concentration with the formaldehyde-diethyl ether centrifugation method, and stained with the modified
Ziehl–Neelsen acid-fast technique, as described previously (14). Samples with excessive mucus were smeared directly and stained without concentration technique. The stained smears were examined under a microscope (Zeiss, Germany, 100× magnification). All positive Cryptosporidium specimens were stored in 70% ethanol for DNA extraction.

**DNA extraction**
Extraction of genomic DNA was performed using 100 mg of stool specimens and the DNA isolation stool mini kit (Yekta Tajhiz Azma Co., Iran) according to the manufacturer’s instructions, after washing of specimens three times with phosphate buffered saline (PBS) by centrifugation at 14000 rpm for 4 min. The extracted DNA was stored at -20°C until PCR analysis.

**PCR Amplification**
A ~400-bp fragment of the gp60 gene was amplified by nested PCR using the primer sets 5’-ATAGTCTCCGCTGTATT-3’ and 5’-GCA GAGGAACCAGCATC-3’ in the primary PCR and 5’-TGGCTGTATTCTCAGCC-3’ and 5’-GAGATATATCTTGGTGCG-3’ in the secondary PCR, as described previously (13). The PCR was performed using the Taq DNA Polymerase Master Mix Red (Amplicon, Denmark). The reaction mixture contained 5 µl distilled water, 7.5 µl master mix, 20 pmol forward and reverse primers and about 25-100 ng/µl of extracted DNA in a final volume of 15 µl. DNA from a known Cryptosporidium species and a blank containing all PCR reagents but no DNA were included in each set of PCR as positive and negative controls, respectively. PCR products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide.

**DNA sequence analysis**
Products of the secondary PCR were sequenced in using Applied Biosystems 3730/3730xl DNA Analyzers (Bioneer, Korea). All sequences were assembled and edited manually using the Chromas program version 1.0.0.1. Basic Local Alignment Search Tool (BLAST) was used to analyze sequences obtained from this study against data in GenBank. The established subtype nomenclature was used in naming C. parvum subtypes (9).

**Statistical analysis**
Data from the study were analyzed using the SPSS software version 16 (SPSS, Chicago, IL, USA). Categorical variables are presented as frequencies and percentage. Logistic regression analysis was used to identify potential risk factors for cryptosporidiosis occurrence. Associations were tested using odds ratios (OR) and 95% confidence intervals (CI) after adjustments. P values <0.05 were considered statistically significant.

**Ethical Considerations**
All procedures in this study were approved by the Ethics Committee of the Shahid Beheshti University of Medical Science, before the beginning of the study (Grant. No. 13/1285). All study participants were informed about the study procedures and written informed consents were obtained from all of them prior to sample collection.

**Results**

**Occurrence of cryptosporidiosis**
Overall, 1301 GIs patients, 619 (47.6%) were female, 682 (52.4%) male. The median age of the study participants was 26 yr (range: 22 d to 90 yr). The prevalence of cryptosporidiosis among patients was 1.3% (17/1301). The prevalence of cryptosporidiosis among patients was 1.3% (17/1301).

**Cryptosporidium genotypes and subtypes**
Species identification by nested PCR was successful for all 17 Cryptosporidium-positive specimens (Fig. 1).
Sequence analysis of the gp60 locus revealed that all 17 positive isolates were from C. parvum. Representative sequences from each identified subtype in this study were deposited
in GenBank/EMBL/DDBJ under accession no. KR982672–KR982688. Two *C. parvum* subtype families, IId (11/17) and IIA (6/17), were identified. Within these two *C. parvum* subtype families, two subtypes were each found in each subtype family: IIdA20G1 (7/17) and IIdA26G1 (4/17) in IId and IIAA15G2R1 (5/17) and IIAA16G3R1 (1/17) in IIA.

**Cryptosporidium subtypes and risk factors**

The results of the logistic regression analysis of risk factors associated with cryptosporidiosis are shown in Table 1. In the outcome of this model only contact with domestic animals or soil (*P*=0.007, OR = 0.128; CI 95%=0.029-0.565) and age (*P*=0.001) were identified as the major socio-demographic determinants of *Cryptosporidium* infection.

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**Fig. 1:** Identification of *Cryptosporidium* species using Nested PCR. Lan 1, DNA Marker (100 bp); Lan 2-15, DNA samples amplified with 60-kDa glycoprotein (gp60) gene amplimer pairs (400 bp); Lan 16, positive control sample; Lan 17, negative control

**Table 1:** Univariate analysis of risk factors associated with frequency of cryptosporidiosis among patients with gastrointestinal disorders from western Iran (*n* = 1301)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Positive n (%)</th>
<th>Negative n (%)</th>
<th>Total n (%)</th>
<th>OR</th>
<th>CI 95% Lower</th>
<th>CI 95% Upper</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>11 (1.6)</td>
<td>671 (98.4)</td>
<td>682 (100)</td>
<td>Reference</td>
<td></td>
<td></td>
<td>0.315</td>
</tr>
<tr>
<td>Female</td>
<td>6 (0.96)</td>
<td>613 (99)</td>
<td>619 (100)</td>
<td>1.698</td>
<td>0.604</td>
<td>4.773</td>
<td></td>
</tr>
<tr>
<td>Age (Year)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6</td>
<td>7 (1.7)</td>
<td>404 (98.3)</td>
<td>411 (100)</td>
<td>Reference</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>7-12</td>
<td>6 (6.7)</td>
<td>83 (93.3)</td>
<td>89 (100)</td>
<td>3.508</td>
<td>1.092</td>
<td>11.261</td>
<td>0.035</td>
</tr>
<tr>
<td>&gt; 12</td>
<td>4 (0.5)</td>
<td>797 (99.5)</td>
<td>801 (100)</td>
<td>0.194</td>
<td>0.055</td>
<td>0.680</td>
<td>0.010</td>
</tr>
<tr>
<td>Residence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>13 (1.9)</td>
<td>670 (98.1)</td>
<td>683 (100)</td>
<td>Reference</td>
<td></td>
<td></td>
<td>0.057</td>
</tr>
<tr>
<td>Urban</td>
<td>4 (0.6)</td>
<td>614 (99.4)</td>
<td>618 (100)</td>
<td>0.336</td>
<td>0.109</td>
<td>1.035</td>
<td></td>
</tr>
<tr>
<td>Contact with domestic animal &amp; soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td>Yes</td>
<td>14 (2.9)</td>
<td>471 (97.1)</td>
<td>485 (100)</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>3 (0.4)</td>
<td>813 (99.6)</td>
<td>816 (100)</td>
<td>0.128</td>
<td>0.029</td>
<td>0.565</td>
<td></td>
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<tr>
<td>Water supply status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.057</td>
</tr>
<tr>
<td>Untreated (river, well, rain water)</td>
<td>13 (1.9)</td>
<td>670 (98.1)</td>
<td>683 (100)</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated pipe water</td>
<td>4 (0.6)</td>
<td>614 (99.4)</td>
<td>618 (100)</td>
<td>0.336</td>
<td>0.109</td>
<td>1.035</td>
<td></td>
</tr>
<tr>
<td>Seasons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.082</td>
</tr>
<tr>
<td>Spring</td>
<td>3 (0.55)</td>
<td>535 (99.4)</td>
<td>538 (100)</td>
<td>Reference</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>14 (1.8)</td>
<td>749 (98.2)</td>
<td>763 (100)</td>
<td>3.151</td>
<td>0.865</td>
<td>11.479</td>
<td></td>
</tr>
</tbody>
</table>

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All patients (5/5) who were infected with subtype family IIa had contact with domestic animals or soil. Children 7-12 yr were more commonly infected (6.7%) than other age groups (P=0.035, OR = 3.508; CI 95%= 1.902-11.261). All subtypes IIa were found in children younger than 10 yr, but IId subtypes were identified in all age groups. Although the majority of Cryptosporidium-positive patients were male and lived in rural areas, we did not find any significant association between Cryptosporidium infection and residence or gender (P>0.05). Moreover, there was no significant association between Cryptosporidium infection and season or water supply type (P>0.05).

### Clinical features of cryptosporidiosis

Overall, Cryptosporidium infection was significantly associated with diarrhea (P=0.001, OR=0.092; CI 95%=0.023-0.362), vomiting & nausea (P=0.001, OR=0.013; CI 95%=0.004-0.044) and abdominal pain (P=0.018, OR=0.073; CI 95%=0.008-0.633) in logistic regression analysis. No significant associations were found between cramping (P=0.052) or bloating (P=0.746) and Cryptosporidium infection (Table 2).

### Table 2: Clinical features associated with frequency of cryptosporidiosis among patients with gastrointestinal disorders from western Iran (n = 1301)

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Samples (n) Pos</th>
<th>Cryptosporidium</th>
<th>OR</th>
<th>CI 95%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal pain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>980</td>
<td>16 (1.6)</td>
<td>0.073</td>
<td>0.008-0.633</td>
<td>0.018</td>
</tr>
<tr>
<td>No</td>
<td>321</td>
<td>1 (0.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea or vomiting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>Yes</td>
<td>58</td>
<td>10 (17.24)</td>
<td>0.013</td>
<td>0.004-0.044</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1243</td>
<td>7 (0.56)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cramping</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.052</td>
</tr>
<tr>
<td>Yes</td>
<td>523</td>
<td>3 (0.6)</td>
<td>4.519</td>
<td>0.990-20.632</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>778</td>
<td>14 (1.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bloating</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.746</td>
</tr>
<tr>
<td>Yes</td>
<td>168</td>
<td>1 (0.6)</td>
<td>1.490</td>
<td>0.133-16.647</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1133</td>
<td>16 (1.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>Yes</td>
<td>585</td>
<td>13 (2.2)</td>
<td>0.092</td>
<td>0.023-0.362</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>716</td>
<td>4 (0.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Among patients infected with the IId subtype family, 90.1% (10/11) reported abdominal pain, 72.7% (8/11) reported diarrhea and 54.5% (6/11) of patients reported vomiting and nausea. Among those infected with the IIa subtype family, all (6/6) had abdominal pain, 83.3% (5/6) had diarrhea and 50% (3/6) of patients had vomiting and nausea. There was no significant difference between subtype families IIa and IId in the occurrence of clinical symptoms (P= 0.75).

### Discussion

The infection rate of Cryptosporidium spp. in our study (1.3%) was lower than rates (2.3%-11.5%) reported from previous studies in Iran (4, 11, 15, 16). This difference may be due to differences in geographical locations, study population, and detection methods. The rate of Cryptosporidium infection in our study was closer to that reported from children with gastrointestinal illness in Jordan (1.8%) and Philippines (1.9%), and far lower than the rate detected in diarrhea patients in Australia (78%), Ethiopia (20.8%) and Egypt (17%) (17-21).

Findings from the present study revealed that zoonotic transmission of Cryptosporidium is common amongst humans in western Iran. Of the 17 isolates that were typed, all were C. parvum. This result is similar to recent reports from northern Iran (16). C. parvum was the...
predominant Cryptosporidium species in humans and animals (11, 22, 23). However, another study in Iran has identified C. hominis (15/21) as the most common species in HIV-positive patients (24). Elsewhere in Middle East countries, C. parvum is the predominant Cryptosporidium species in humans (19, 25-27).

In this study, sequence analysis of the gp60 locus identified two C. parvum subtype families (IIa, IIId) and four subtypes (IIaA15G2R1, IIaA16G3R1, IIIdA26G1, IIIdA20G1). The majority of Cryptosporidium infections were caused by IId subtypes (11/17). The IIId subtypes have previously been reported commonly in humans in Iran (13, 16) and Kuwait (26), but less frequently in Ethiopia (28), Australia (29) and United Kingdom (30). This subtype family has also been reported in sheep and goats in Spain (31) and calves in China, Egypt, and Sweden (32-34). In this study, 72% (8/11) of patients with IIId had contact with domestic animals. Two of the subtypes detected in this study (IIIdA26G1and IIIdA20G1) were previously reported in children in Iran (13). The IIIdA20G1 subtype was predominant subtype identified in our study and was previously reported in human in Kuwait and Jordan (19, 26). The subtype IIIdA26G1 was previously reported in lambs and goat kids in Spain (31). In addition to IIId, subtype family of IIa was also identified in six patients in this study (6/17). IIa is the most prevalent subtype family in animals and humans worldwide (32). The IIaA15G2R1 subtype identified in the present study (5/6) is a dominant C. parvum subtype in dairy calves around the world (9), supporting the role of zoonotic transmission in cryptosporidiosis in patients in our study. Consistent with this, all IIa patients in the present study had contact with domestic animals. The IIaA16G3R1 subtype was also reported in calves in United States, Ireland and Iran (13, 35-37). Moreover, it was found in humans in Canada and Denmark (35, 38). Our study is first to reporting the IIaA16G3R1 subtype in humans in Iran.

Different Cryptosporidium species and subtypes are associated with different clinical symptoms (28, 39). In the present study, Cryptosporidium infection was significantly associated with the occurrence of diarrhea, vomiting & nausea and abdominal pain. However, there was no significant difference between the two subtype families in clinical symptoms. In agreement with our results, C. parvum infection was associated with diarrhea and vomiting in HIV–infected persons, although in another study (40) they reported that C. parvum infection was associated only with diarrhea in children (39). Similar results were obtained from Ethiopia, where C. parvum especially IIa subtype, family was associated only with the occurrence of diarrhea (28). The role of parasite genetics in clinical manifestations of cryptosporidiosis is still not clear and further studies are needed to elucidate fully the characteristics of this association.

Results of the risk factor analysis support the role of zoonotic transmission in Cryptosporidium epidemiology in patients in western Iran. Among infected patients, 82.3% (14/17) reported contact with domestic animals. Another significant risk factor in our study was age. We found that all patients with IIa subtype family infection were younger than ten years, while those infected patients with IIId subtype family were in different age groups (8-45 yr). These results are consistent with previous studies in Iran and elsewhere (13, 16, 17, 28, 30, 41).

Conclusion

Cryptosporidiosis may be an important cause of gastrointestinal illnesses, especially among children. Moreover, C. parvum is the main species in Nahavan County, west of Iran, suggesting that zoonotic transmission is main route in the acquisition of cryptosporidiosis infection in this region. Therefore, improved hygiene and avoidance of contact with animals and contaminated soil should be advocated to reduce the occurrence of Cryptosporidium infec-

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tions, especially in children. Further investigations are needed to elucidate fully possible difference in clinical presentations among *Cryptosporidium* species and major subtypes.

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