Identification of Cryptosporidium Species Infecting Humans in Tunisia

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Abstract. Prevalence and species distribution of Cryptosporidium spp. were determined among 633 immunocompetent children less than five years of age and 75 patients hospitalized for immunodeficiency who lived in northern Tunisia. Microscopy was used for initial screening to detect positive samples and a nested polymerase chain reaction and restriction fragment length polymorphism analysis was used to determine the species. Cryptosporidium spp. was identified in 2.7% of cases (19 stool samples), and there was a significant difference between samples collected from immunocompromised patients and those collected from healthy children (10.7% versus 1.7%). Prevalence was also significantly higher in diarrheal specimens than in formed specimens (6.3% versus 1.6%). Cryptosporidium hominis and C. parvum were responsible for most Cryptosporidium spp. infections (78.9%). Cryptosporidium hominis was more prevalent in children from urban areas than those from rural areas, and C. parvum was found with similar prevalence rates in the two populations. Cryptosporidium meleagris was identified in four children on farms.

INTRODUCTION

Cryptosporidium is a genus of enteric protozoans commonly associated with persistent diarrhea in immunocompromised persons that involves mostly persons with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome and primary immunodeficiency. It is also known to be a common cause of a self-limited diarrheal disease in all age groups of immunologically healthy people. In developing countries, the disease probably exerts most of its impact on pediatric health. In developed countries, cryptosporidiosis is frequently associated with water-borne outbreaks. In recent years, the development of genetic tools as part of the taxonomic characterization of Cryptosporidium spp. has promoted our understanding of the transmission mechanism and epidemiology of human cryptosporidiosis. Thus, numerous studies have identified five species of Cryptosporidium (C. parvum, C. hominis, C. meleagris, C. felis, and C. canis) with different reservoir hosts as being responsible for most human Cryptosporidium spp. infections.

Modified acid-fast staining has been used in Tunisia to identify Cryptosporidium spp. as a prevalent parasite in HIV-infected patients and immunocompromised and apparently immunocompetent children. However, microscopic examination of oocysts in clinical specimens does not enable identification of species and the assessment of the public health significance of Cryptosporidium spp. in the environment. The aim of this study was to determine the prevalence of Cryptosporidium species in different human populations. This information will contribute to a better understanding of transmission routes in Tunisia. A better understanding of the epidemiology will be helpful in devising appropriate control measures.

MATERIALS AND METHODS

Samples. From 2006 through 2007, 708 stools samples were collected from 633 apparently immunocompetent children less than five years of age (477 living in farming areas in northeastern Tunisia and 156 originally from Tunis City) and from 75 patients hospitalized for immunodeficiency (39 HIV-infected patients and 36 children with primary immunodeficiency). Informed consent was obtained from parents and adult patients before stool collection.

Fresh stool specimens (549 formed and 159 diarrheal) were examined for Cryptosporidium spp. oocysts (Table 1). Microscopic examination was carried out on smears of fecal concentrates (formalin-ethyl acetate concentration) after staining with the modified Ziehl-Neelsen technique. When Cryptosporidium oocysts were identified microscopically, an aliquot of the stool specimen was diluted [v/v] in distilled water, placed into tubes, and stored frozen for molecular analysis.

Extraction of DNA. DNA was extracted from samples using the QIAamp DNA Stool MiniKit (Qiagen Inc., Hilden, Germany) according to the manufacturer’s recommendations using approximately 200 μL of diluted stools for the first buffer step. After quantification, extracted DNA was frozen at −20°C until analyzed.

Analysis by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). A Cryptosporidium genus-specific nested PCR was used to amplify a 214-basepair fragment of the Cryptosporidium 18S ribosomal RNA gene encompassing the polymorphic region between nucleotides 179 and 271 as described. The first PCR amplification was performed with forward primer SCL1 (5’-CTGGTTGATC-CTGCCAGTAGG-3’) and reverse primer CBP-DIAGR (5’-TAAGGTGTCAAGGTAAGG-3’) complementary to nucleotides 4–23 and 1016–1036, respectively (GenBank accession no. AF093489). The second-round PCR was carried out using forward primer SCL2 (5’-CAGTTATAGTT-TACCTGATAATC-3’) and reverse primer SCR2 (5’-CAATACCTACCGTCTAAAG-3’) complementary to nucleotides 106–128 and 299–318 respectively. The PCR amplification was performed in a 25-μL volume containing 5 μL of DNA template, 10X PCR buffer, 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1.25 units of AmpliTaq Gold Polymerase (Roche, Indianapolis, IN). Cycling conditions were an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of a three-step program (94°C for 30
The products that were digested by enzymes were visualized after staining with ethidium bromide. 3% agarose gel in 1 X Tris-borate-EDTA buffer, and the fragments were separated by electrophoresis on a 2% agarose gel and visualized after staining with ethidium bromide. Taq DNA polymerase and Taq DNA polymerase in the reaction contained 5 mM MgCl₂ and 0.8 U of enzyme were used to amplify a 100 bp fragment of the 18S rRNA gene. Amplification was performed in 25 μL aliquots that contained 200 μM each of the dNTPs, 2 μM of each primer, 10 mM Tris- HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, and 0.001% Tween 20. Reaction were performed using the following program: 94°C for 15 minutes, 35 cycles of 94°C for 1.5 minutes, 55°C for 1 minute, and 72°C for 1 minute, and a final extension at 72°C for 7 minutes. PCR products were visualized by ethidium bromide staining after separation by agarose gel electrophoresis. 

**Restriction assays.** Amplified product was initially digested with the restriction endonuclease Taq I as reported. The reaction contained 5 μL of the recommended 10X buffer, 2 units of enzyme, and distilled water to give a volume of 30 μL. Reactions were incubated for 2 hours at 65°C. Fragments were separated by electrophoresis on a 3% agarose gel in 1X Tris-borate-EDTA buffer, and the fragments were visualized after staining with ethidium bromide. The products that were digested by Taq I were digested with Ase I to differentiate C. hominis from C. parvum. In cases of negative results, the restriction enzymes Mse I, Bst UI, and Ssp I were used to identify other species. All restriction assays were conducted according to the manufacturer’s recommendations (New England Biolabs, Hitchin, United Kingdom).

**Sequencing.** Three second-round PCR products identified as C. hominis, C. parvum, and C. meleagridis by the restriction profile were purified using the Wizard Purification Kit (Promega, Charbonnières, France). The PCR products were then sequenced using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were compared with those available in the GenBank database with the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST).

**Statistical analysis.** Chi-square and Fisher exact tests were used to compare percentages (Epi-Info Software, version 6.04; Centers for Disease Control and Prevention, Atlanta, GA).

**RESULTS**

**Detection of Cryptosporidium spp.** Among 708 samples examined by microscopy, 19 (2.7%) were positive for Cryptosporidium spp. Oocysts were found in 10 (6.3%) diarrheal stools and 9 (1.6%) formed stools (P < 0.01) (Table 1). Positive samples were collected from 11 immunocompetent children (7 living in rural communities and 4 in urban communities) and from 8 immunocompromised patients (6 HIV-infected patients and 2 children with cellular immunodeficiency caused by defective expression of major histocompatibility complex II molecules). Prevalence was significantly higher in immunocompromised patients than in immunocompetent children (10.7% versus 1.7%; P < 0.01) (Table 1). In this latter pediatric population, there was no significant difference between urban and rural prevalence rates (2.5% versus 1.4%) (Table 2).

**Species identification.** Nested PCR analysis of microscopy-positive samples confirmed the presence of Cryptosporidium species. The RFLP analysis yielded typical restriction patterns for C. parvum in 8 cases, C. hominis in 7 cases, and C. meleagridis in 4 cases (Figure 1). Sequences of 3 samples identified as C. parvum, C. hominis, and C. meleagridis have been stored in GenBank under the accession nos. EU675852, EU675853, and EU675854, respectively. These samples showed 100% similarity with C. parvum sequence (GenBank accession no. AF308600) and 99% similarity with C. hominis sequence (GenBank accession no. DQ388386) and C. meleagridis sequence (GenBank accession no. AF112574), respectively. Mutations were not detected around restriction enzyme sites (Figure 2).

Cryptosporidium parvum and C. hominis were responsible for 15 cases of Cryptosporidium spp. infections (78.9%). Their distribution was not different on the basis of stool consistency (five cases with C. parvum and four cases with C. hominis in formed stools versus three cases from each species in diarrheic specimens). Their distribution was not different by immune status (five and three cases of C. parvum and C. hominis, respectively, in immunocompetent children versus three and four cases, respectively, in immunocompromised persons). Cryptosporidium hominis was more prevalent in children from urban areas than in children from rural areas (1.9% versus 0%; P < 0.05), and C. parvum was found with similar prevalence rates in the two populations (0.6% versus 0.8%) (Table 2).

### Table 1

<table>
<thead>
<tr>
<th>Patients</th>
<th>Formed stools</th>
<th>Diarrheic stools</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>MZN⁺, no. (%)</td>
</tr>
<tr>
<td>Immunocompetent children</td>
<td>511</td>
<td>7 (1.36)</td>
</tr>
<tr>
<td>Immunocompromised children</td>
<td>9</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Immunocompromised adults</td>
<td>29</td>
<td>2 (6.8)</td>
</tr>
<tr>
<td>Total</td>
<td>549</td>
<td>9 (1.6%)</td>
</tr>
</tbody>
</table>

* MZN = Modified Ziel-Nelson technique.

### Table 2

<table>
<thead>
<tr>
<th>Community</th>
<th>C. hominis, no. cases (prevalence rate)</th>
<th>C. parvum, no. cases (prevalence rate)</th>
<th>C. meleagridis, no. cases (prevalence rate)</th>
<th>Cryptosporidium spp., no. cases (prevalence rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urban (n = 156)</td>
<td>3 (1.9%)</td>
<td>1 (0.6%)</td>
<td>0 (0%)</td>
<td>4 (2.5%)</td>
</tr>
<tr>
<td>Rural (n = 477)</td>
<td>0 (0%)</td>
<td>4 (0.8%)</td>
<td>3 (0.6%)</td>
<td>7 (1.4%)</td>
</tr>
<tr>
<td>Total (n = 633)</td>
<td>3 (0.45%)</td>
<td>5 (0.8%)</td>
<td>3 (0.45%)</td>
<td>11 (1.7%)</td>
</tr>
</tbody>
</table>
Cryptosporidium meleagridis was identified only in children from rural areas; three immunocompetent non-diarrheic children and a four-year-old diarrheic immunocompromised child. In rural areas, no significant difference between the prevalence rate of Cryptosporidium meleagridis and that of Cryptosporidium parvum was found (0.6% versus 0.8%) (Table 2).

**DISCUSSION**

As previously reported, this study has showed that Cryptosporidium spp. are frequently identified in diarrheic stools of immunocompetent children and immunocompromised patients in Tunisia.5,6 However, this study is the first in Tunisia and as far as we know in north Africa to attempt identification of Cryptosporidium species infecting humans.

Despite the relative small number (19) of isolates characterized, we identified three species: C. hominis, C. parvum, and C. meleagridis. Similar to another report, C. hominis and C. parvum were responsible for most infections (15 cases, 78.9%).4 These parasites were found with a similar frequency, 8 cases of C. parvum (53.3%) and 7 cases of C. hominis (46.7%). In agreement with previous reports, distribution of these species was similar in immunocompetent and immunocompromised persons and also in formed and diarrheic stools.10 Cryptosporidium meleagridis was more prevalent in our study (4 cases, 21.1%) than it had been previously reported.11 Immunosuppression is apparently not a prerequisite for infections with this species because it was found, similar to other reports,12 in immunocompetent children.

In immunocompetent children, although the prevalence was as high in urban communities (2.5%) as in rural communities (1.4%), variation in prevalence rates of species was noted within the 2 populations. Cryptosporidium hominis was absent in rural pediatric communities, which suggests that anthroponotic transmission is less common in this population. The presence of C. parvum and C. meleagridis is suggestive of zoonotic transmission and direct contact with farm animals.13,14 In urban pediatric communities, the high prevai-

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**FIGURE 1.** Polymerase chain reaction–restriction fragment length polymorphism analysis of the 18S ribosomal RNA gene of Cryptosporidium spp. A, Restriction patterns of C. parvum and C. hominis. Lanes 1a and 2a, undigested products; lanes 1b and 2b, products digested with Taq I; lanes 1c and 2c, products digested with Ase I; lane M, 100-basepair (bp) molecular mass markers. B, Restriction patterns of C. meleagridis. Lane 3a, undigested product; lanes 3b–d, products digested with Mse I, Ssp I, and Bst UI; Lane M, 100-bp molecular weight markers. *Partial digestion with Mse I.

**FIGURE 2.** Alignment of DNA samples with deposited sequences of Cryptosporidium parvum, C. hominis, and C. meleagridis small subunit ribosomal RNA gene. Restriction sites of enzymes and their positions are shown in **bold**.

<table>
<thead>
<tr>
<th>Speciation</th>
<th>Accession No.</th>
<th>+1 Sequence</th>
<th>Restriction Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parvum</td>
<td>AF308600</td>
<td>AAACACTGACATTATGGAA/CACATTAAATGTGC</td>
<td>Taq I 77</td>
</tr>
<tr>
<td>C. hominis</td>
<td>DQ388386</td>
<td>.....................................</td>
<td>Mse I 82</td>
</tr>
<tr>
<td>C. meleagridis</td>
<td>AF112574</td>
<td>.....................................</td>
<td>Ase I 155</td>
</tr>
</tbody>
</table>

**SAMPLE 1**

|           | EU675852      | AAACACTGACATTATGGAA/CACATTAAATGTGC +214
|-----------|---------------|--------------------------------------------------|

**SAMPLE 2**

|           | EU675863      | ..................................... | ATTAT.....       |

**SAMPLE 3**

|           | EU675864      | ..................................... | ATTAT.....       |
lence of *C. hominis* indicates that humans are a major source of infection and that person-to-person transmission probably plays a major role in the spread of *Cryptosporidium*. The concomitant presence of *C. parvum* could be the result of environmental transmission (contaminated water or food) or the result of an anthropopotic transmission. Caution should be used in interpreting the significance of finding parasites traditionally associated with animals in humans. There is good evidence that subpopulations of *C. parvum*, e.g., GP60 type IIc, have a strictly anthropopotic transmission cycle. Thus, further analyses, such as sequence analysis of the GP60 gene, are needed to confirm the origin of these strains.

Received January 28, 2008. Accepted for publication June 20, 2008.

Acknowledgments: We thank Dr. Ezzedine Brahem, Dr. Ridha Hamza, doctors and health agents from the Regional Directory of Public Health of Bizerte, doctors and nurses from PMI Cité Hlal, Tunis, and PMI Ibn Khaldoun, Tunis, for facilitated data and stool collection from healthy children. We also thank Professor Taoufik Ben Chaabane (Department of Infectious Diseases, La Rabta Hospital, Tunis, Tunisia), Ikram Rahmouni, and Nejla Chaibchoub (Department of Parasitology, Pasteur Institute of Tunis, Tunis, Tunisia) for cooperation and help.

Financial support: This study was supported by the Institut de Médecine et d’Épidémiologie Appliquée–Fondation Internationale Leon Mba, France, and the Ministry of Higher Education, Research and Technology, Tunisia in the setting of the Research Laboratory Parasitoses Emergentes (LR 05SP03).

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