Molecular characterization of Cryptosporidium isolates from humans by nested polymerase chain reaction – restriction fragment length polymorphism (nPCR-RFLP) analysis in Egypt

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Abstract. Cryptosporidium species is a group of protozoan parasites recognized as a cause of diarrhea with significant morbidity and mortality in industrialized and developing countries. Cryptosporidium hominis and Cryptosporidium parvum seem to be the most common causes of human cryptosporidiosis, however, other species are also found. Hence, the present study aimed to determine Cryptosporidium spp. infecting humans by nested PCR-RFLP targeting 18 sRNA gene in Minia Governorate, Egypt. A total of 300 diarrheic stool samples have been collected from inpatient and outpatient clinics of University Hospitals and Tropical Hospital, Minia Governorate, Egypt. One hundred twelve positive samples for Cryptosporidium infection (37.3%) were detected by nPCR while, 60 positive samples (20%) were detected by routine microscopy (p-value < 0.0004). The one hundred twelve positive samples detected by nested PCR were processed to RFLP. The RFLP yielded a typical restriction patterns for C. hominis in 73 (65.2%) cases, C. parvum in 25 (22.3%) cases, and C. meleagridis in 14 (12.5%) cases. C. hominis was more prevalent among cases of urban areas with negative history of animal contact in comparison to cases of rural areas (95.5% vs. 21.7%; p-value < 0.001). Moreover, there was a statistically significant association between C. hominis infection and drinking tap water. C. parvum was more prevalent among cases of rural areas than among those of urban areas (47.8% vs. 4.5%; p-value < 0.001) While C. meleagridis was only present among cases live in rural areas (30.4%; p-value < 0.001).

INTRODUCTION

Cryptosporidium species (spp.) infect many different hosts including humans. In humans, this protozoan parasite causes mainly diarrhea, which is self-limiting in immunocompetent individuals, or severe diarrhea that may be lethal in immunosuppressed individuals (Chen et al., 2002). Cryptosporidium spp. has a direct transmission route, since, the infective oocysts have already passed in feces and few oocysts are enough to cause infection. Because of a wide range of hosts, multiple transmission routes and the ability of oocysts to stay viable in the environment, the epidemiology of human cryptosporidiosis is complex. Genotyping of Cryptosporidium spp. figured out its epidemiology in different geographical areas, seasonal and socio-economic conditions (Chappell et al., 2006, Caccio’ & Putignani, 2014). Thirty Cryptosporidium spp. as well as more than forty genotypes have been reported. However only, 20 Cryptosporidium spp. / genotypes are detected in humans (Holubova et al., 2016). Cryptosporidium hominis (previously known as the C. parvum human genotype), C. parvum (bovine genotype), C. meleagridis, C. canis and C. felis are the main species that infect humans (Xiao et al., 2001, Xiao & Feng, 2008).
Common methods used for genotyping of Cryptosporidium spp. are genotype-specific PCR and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Sulaiman et al., 1999; Feng et al., 2013).

Identification of genetic polymorphism within the human Cryptosporidium spp. isolates will be helpful in determining the sources of oocyst contamination, and ensuring appropriate preventive measures (Blears et al., 2000). Accordingly, this study aimed to investigate the genotypes of Cryptosporidium spp. that mostly affect humans in Minia Governorate, Egypt.

MATERIALS AND METHODS

Ethical consideration
All participants provided verbal approval before participating in the study. All procedures were conducted according to the ethical standards approved by the Institutional Human Ethics Committee, Faculty of Medicine, Minia University, Egypt.

Collection of samples
Three hundred diarrheic stool samples were collected from cases from the inpatient and outpatient clinics of University Hospitals and Tropical hospital, Minia Governorate, Egypt during the period from June 2016 to May 2017. Information on the potential risk factors for infections was gathered by using structured questionnaires. Risk factors included sources of water, and the presence of animals (dogs, chicken, ducks, guinea pigs, rabbits, parrots, and sheep).

Microscopic examination by modified Ziehl-Neelseen stain
Fresh stool specimens were examined for Cryptosporidium spp. oocysts. Water-ether concentration technique was carried out for all fecal samples (Smith, 2007) then, the samples were examined microscopically by the modified Ziehl-Neelseen stain (Casemore et al., 1985).

Extraction of DNA
DNA was extracted from samples using PureLink™ Microbiome DNA Purification Kit (Cat. no. 9200B, Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer’s recommendations using about 200 µL of fresh stools for the first buffer step. The extracted DNA was measured by a spectrophotometer (Genova Plus, Jenway, ST1505A, UK) at a wavelength of 260 nm and 280 nm, and then was frozen at -20°C until analyzed. The concentration of DNA in each sample was determined according to the equation: ds DNA concentration = 50µg/ ml x OD_{260} x dilution factor.

Nested polymerase chain reaction (nPCR)
A Cryptosporidium genus specific nested PCR was used to amplify a 214-base pair fragment of the Cryptosporidium 18S ribosomal RNA gene encompassing the polymorphic region between nucleotides 179 and 271 (Coupe et al., 2005). The first PCR amplification was performed with forward primer SCL1 (5'-CTGGTTGTACCTGCCAGTAG-3’) and reverse primer CBP-DIAGR (5'-TAAGGTGCTGAAGGAGTAAGG-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’-CAGTTATAGTTTACTTAGGATAAG-3’) and reverse primer SCR2 (5’-CAATA CCTACCTTCAAGG-3’) complementary to nucleotides 106–128 and 299–318 respectively (Coupe et al., 2005).

The first PCR amplification was performed in a 25 µL volume containing 200ng in 5 µL of DNA template, 12.5 µL of master mix (Thermo scientific, U.K, Lot no. #K 1081), 0.2 µM of each primer and 3.5 µL nuclease free water. Cycling conditions were an initial denaturation at 94°C for 7 minutes, followed by 30 cycles of three steps (denaturation at 94°C for 30 sec, annealing at 60°C for 34 sec, and extension at 72°C for 90 sec) then a final extension at 72°C for 10 minutes (Coupe et al., 2005).
The optimized conditions for second-round PCR were the same as those in the first round, except that final volume was 50 µl, the primer concentrations were 0.4 µM, 5 µl of the first amplification product was used as the template, and annealing lasted for 45 sec at 58°C and extension for 60 sec at 72°C. Amplified products were separated by electrophoresis on a 2% agarose gel and were visualized after being stained with ethidium bromide.

**Restriction fragment length polymorphism (RFLP) (Essid et al., 2008)**

The amplified product was initially digested with the restriction endonuclease TaqI (Thermo Scientific, EU, Lithuania, #ER0671, 3000U, Lot: 00399406, concentration: 10 U/µl, supplied with; 1ml of 10X buffer TaqI). The reaction contained 10 µL PCR product, 2 µL of the recommended 10× buffer, 1 unit of the enzyme, and distilled water to give a volume of 30 µL. Reactions were incubated for 2 hours at 65°C. The enzyme was inactivated by phenol/chloroform extraction, precipitated with 75% cold ethanol and dried in the air (Green and Sambrook, 2012). The DNA was dissolved in nuclease free water and the concentration of DNA was checked. Fragments were separated by electrophoresis on a 3% agarose gel in 1× Tris-borate-EDTA buffer, and the fragments were visualized after staining with ethidium bromide. The products that were digested by TaqI were digested with AseI enzyme giving two bands (at 155 bp and 59 bp), which is the pattern of C. hominis, while 25 (22.3%) were not digested by AseI enzyme (C. parvum pattern) (Fig. 1c). All the 14 (12.5%) TaqI negative samples were digested by MseI into two bands 132 bp and 82bp which is the pattern of C. meleagridis (Fig. 1d).

Genetic characterization of Cryptosporidium spp. by nPCR-RFLP revealed that there is no significant association between different Cryptosporidium spp. to age or gender of patient.

* C. hominis was more prevalent among patients from urban areas than among those from rural areas (95.5% vs. 21.7%; p-value < 0.001), and *C. parvum* was more prevalent among patients from rural areas than among those from urban areas (47.8% vs. 4.5%; p-value < 0.001). *C. meleagridis* was only

**RESULTS**

Out of 300 diarrheic stool samples that were collected, 60 samples (20%) were positive for *Cryptosporidium* spp. oocysts by microscopy after staining by the modified Ziehl-Neelsen stain.

**Nested PCR amplification of 18S rRNA genes**

All samples were subjected for extraction of the genomic DNA. Nested PCR was done using 18S rRNA gene. Out of 300 cases, 112 cases (37.3%) were amplified giving a band 214 bp (Fig. 1a).
present among cases live in rural areas (30.4%; p-value ≤ 0.001) (Table 1).

Furthermore, this study showed statistically significant association between *C. hominis* infection and drinking tap water compared to insignificant association with drinking pumped water (74.4% vs. 34.6%; p-value ≤ 0.001) (Table 2). On the other hand, there was no relationship between the infections with *C. parvum* and *C. meleagridis* and the source of water used (Table 2).

Additionally, *C. hominis* infection appears to be more in patients with negative history of animal contacts, while *C. parvum* and *C. meleagridis* are more common in patients with history of animal contact (p-value ≤ 0.001, ≤ 0.001, 0.005 respectively) as shown in Table 3.
Table 1. Distribution of *Cryptosporidium* spp. in relation to residence

<table>
<thead>
<tr>
<th>Results of nPCR-RFLP</th>
<th>Urban N=66</th>
<th>Rural N=46</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. hominis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Positive</td>
<td>63 (95.5%)</td>
<td>10 (21.7%)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>• Negative</td>
<td>3 (4.5%)</td>
<td>36 (78.3%)</td>
<td></td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>• Positive</td>
<td>3 (4.5%)</td>
<td>22 (47.8%)</td>
<td></td>
</tr>
<tr>
<td>• Negative</td>
<td>63 (95.5%)</td>
<td>24 (52.2%)</td>
<td></td>
</tr>
<tr>
<td><em>C. meleagridis</em></td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>• Positive</td>
<td>0 (0%)</td>
<td>14 (30.4%)</td>
<td></td>
</tr>
<tr>
<td>• Negative</td>
<td>66 (100%)</td>
<td>32 (69.6%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. *Cryptosporidium* spp. with regard to source of water

<table>
<thead>
<tr>
<th>Results of nPCR-RFLP</th>
<th>Pumped N=26</th>
<th>Tap N=86</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. hominis</em></td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>• Positive</td>
<td>9 (34.6%)</td>
<td>64 (74.4%)</td>
<td></td>
</tr>
<tr>
<td>• Negative</td>
<td>17 (65.4%)</td>
<td>22 (25.6%)</td>
<td></td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>• Positive</td>
<td>8 (30.8%)</td>
<td>17 (19.8%)</td>
<td></td>
</tr>
<tr>
<td>• Negative</td>
<td>18 (69.2%)</td>
<td>69 (80.2%)</td>
<td></td>
</tr>
<tr>
<td><em>C. meleagridis</em></td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>• Positive</td>
<td>2 (8%)</td>
<td>12 (14%)</td>
<td></td>
</tr>
<tr>
<td>• Negative</td>
<td>24 (92%)</td>
<td>74 (86%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Occurrence of different *Cryptosporidium* spp. with regard to animal contact

<table>
<thead>
<tr>
<th>Results of nPCR-RFLP</th>
<th>Animal contact N=39</th>
<th>No animal contact N=73</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. hominis</em></td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>• Positive</td>
<td>6 (15.4%)</td>
<td>67 (91.8%)</td>
<td></td>
</tr>
<tr>
<td>• Negative</td>
<td>33 (84.6%)</td>
<td>6 (8.2%)</td>
<td></td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>• Positive</td>
<td>23 (59%)</td>
<td>2 (2.7%)</td>
<td></td>
</tr>
<tr>
<td>• Negative</td>
<td>16 (41%)</td>
<td>71 (97.3%)</td>
<td></td>
</tr>
<tr>
<td><em>C. meleagridis</em></td>
<td></td>
<td></td>
<td>0.005*</td>
</tr>
<tr>
<td>• Positive</td>
<td>10 (25.6%)</td>
<td>4 (5.5%)</td>
<td></td>
</tr>
<tr>
<td>• Negative</td>
<td>29 (74.4%)</td>
<td>69 (94.5%)</td>
<td></td>
</tr>
</tbody>
</table>
Laboratory diagnosis and immunoassays generally offer no information on determining the infecting Cryptosporidium species (Yu et al., 2009). Moreover, oocyst morphology and host specificity do not give enough information for Cryptosporidium spp. genotypes or sub genotypes (Jex and Gasser, 2010).

Molecular analysis is used to characterize the genetic structure of Cryptosporidium spp. and is proved to be a sensitive diagnostic method capable of determining Cryptosporidium spp. with high selectivity (Xiao, 2010). Moreover, 18S rRNA Nested PCR is proved to give better and more accurate results than primary PCR in Cryptosporidium spp. identification (Ruecker et al., 2011).

Microscopic examination of stool samples by Modified Zeel-Neelsen, which revealed 60 cases (20%), was found to be less sensitive than nPCR, which revealed 112 cases (37.3%) (p-value < 0.0004). However, microscopy is more commonly used to identify Cryptosporidium oocysts as it is cheaper and allow the detection of other parasites at the same time (Fayer et al., 2000; Aghamolaie et al., 2014; Karaman et al., 2015; Uppal et al., 2014).

PCR-RFLP procedure was done for the 112 positive Nested PCR samples. Amplified products were digested with Taq I in combination with AseI to identify C. parvum and C. hominis. The restriction enzymes Mse I, Bst UI, and Ssp I were used to identify other species.

The RFLP analysis yielded typical restriction patterns for C. hominis in 73 (65.2%) cases, C. parvum in 25 (22.3%) cases, and C. meleagridis in 14 (12.5) cases. In the present study, three species could be identified C. hominis, C. parvum and C. meleagridis. C. hominis was significantly more prevalent (65.2%) than C. parvum (22.3%) and C. meleagridis (12.5%). Mixed infections were not detected, indicating that the main source of Cryptosporidium infection in the study group was more of human than zoonotic source.

These findings were in accordance with the results of El-Badry et al. who reported that C. hominis was the most common species detected in diarrheic children (95.8%) (El-Badry et al., 2015). Moreover, the findings of this study were in line with Gatei et al. They showed that 87% of the Cryptosporidium isolates were C. hominis, 9% were C. parvum, and remaining 4% were C. canis, C. felis, C. meleagritidis and C. muris (Gatei et al., 2006). Morgan et al. reported that most of the human cases of cryptosporidiosis worldwide are caused by two species: C. hominis that causes infections in humans only (anthroponotic infection) and C. parvum that causes infection in humans and animals (zoonotic infection) (Morgan et al., 1999).

In contrast, Other studies stated that the most predominant Cryptosporidium spp. was C. parvum followed by C. hominis then C. meleagridies (Eida et al., 2009; Eraky et al., 2015; Essid et al., 2008).

The difference in the distribution of cryptosporidium genotypes may be attributed to the differences in the transmission routes and infection sources among communities (Essid et al., 2008). Infection by C. hominis is mainly anthroponotic and not zoonotic while C. parvum has long been considered zoonotic and the ability of this species to transmit from animals to humans is well documented (Widmer and Sullivan, 2012).

The current study revealed a significant association between the infections with different Cryptosporidium spp. and the residence of patients. C. hominis was more prevalent among patients from urban areas than among patients from rural areas (95.5% vs. 21.7%; p-value < 0.001). On the other hand, C. parvum was more prevalent among patients from rural areas than among patients from urban areas (47.8% vs. 4.5%; p-value < 0.001). C. meleagridis was only present among patients in rural areas (30.4%; p-value < 0.001).

These findings are closely in agreement with many studies (Essid et al., 2008; Deshpande et al., 2014; Pollock et al., 2010).

Many other studies reported that differences also exist in the distribution of
C. parvum and C. hominis between urban and rural with the former more commonly detected in rural and the latter in urban areas (Learmonth et al., 2004; Llorente et al., 2007; Zintl et al., 2009; Chalmers et al., 2011). This difference in Cryptosporidium spp. distribution is probably due to differences in infection sources and transmission routes (Xiao, 2010).

In Contrast, Helmy and others reported that C. hominis was more frequently detected in rural areas (Helmy et al., 2013). Shalaby and Shalaby reported that Cryptosporidium spp. in rural areas were statistically significant than urban ones (Shalaby & Shalaby, 2015). Gatei et al. mentioned that there were no discernable differences in the distribution pattern of zoonotic species by region, with C. hominis being the most prevalent species in all areas (Gatei et al., 2006).

The presence of C. parvum and C. meleagridis in rural areas is suggestive of zoonotic transmission and direct contact with farm animals (Learmonth et al., 2004, Caccio, 2005). In urban communities, the high prevalence of C. hominis indicates that humans are the major source of infection and person-to-person transmission probably plays a major role in the spread of cryptosporidiosis (Essid et al., 2008).

Among the studied variables, type of water, and animal contact showed statistically significant association with cryptosporidiosis. C. hominis infection was more among patients who drink tap water compared to those who drink pumped water (74.4% vs. 34.6%; p-value < 0.001). On the other hand, there was no relationship between the infections with C. parvum or C. meleagridis and the source of drinking water.

Khalifa et al. recorded that the most common protozoa detected in different water was Cryptosporidium spp. (Khalifa et al., 2014). Studies reported that the significant majority of Cryptosporidium spp. infections were detected in diarrheic patients, who relied upon tap water sources (El-Badry et al., 2017; Al-Warid et al., 2012).

Further, research in England affirmed that the drinking of non boiled tap water from public drinking water supplies was a highly significant risk factor for sporadic human cryptosporidiosis (Goh et al., 2004).

Tap water from water treatment plants which use rapid sand filters and sequentially add chlorine-based disinfectants, is apparently not entirely free from Cryptosporidium spp. although of better hygienic quality (Abou-Eisha et al., 2000).

This research confirmed a significant association between animal contact and Cryptosporidium spp. infection. C. hominis infection appears more among patients with a negative history of animal contacts, while C. parvum and C. meleagridis are more common among patients with a history of animal contact (p-value < 0.001, < 0.001, 0.005 respectively).

This agreed with El-Badry et al. who reported that C. hominis is not associated with animal contact (El-Badry et al., 2017).

Also, Pollock et al. highlighted that C. parvum was more prevalent in rural areas with a higher density of livestock reflecting the zoonotic nature of the infection (Pollock et al., 2010).

Deshpande et al. illustrated that there is a strong evidence to highlight that contact with farm animals or their feces is associated with C. parvum infection (Deshpande et al., 2014).

Other studies have confirmed that the zoonotic route of infection of C. meleagridis, particularly in people who have close contact with animals, such as in rural areas, and suggests that cross-species transmission of C. meleagridis between birds or other animals and humans is possible (Silverlas et al., 2012; Widmer et al., 2015).

Contradictory, Al-Warid et al. mentioned that there was no significant relation between the presence of domestic animals and rate of infection (Al-Warid et al., 2012).

REFERENCE


