Tropical Biomedicine 36(1): 1–10 (2019)

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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Received 3 April 2018; received in revised form 25 November 2018; accepted 3 December 2018

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 cryptosporiodosis, 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-Neelsen 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-Neelsen 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'-CTGGTTGATCCT GCCAGTAG-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'-CAGTTATAGTTTACTTGATAATC-3') and reverse primer SCR2 (5'-CAATA CCCTACCGTCTAAAG-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, Lithuonia, #ER0671, 3000U, Lot: 00399406, concentration: 10 U/µl, supplied with; 1ml of 10X buffer *Taq*I). 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 ethanol; the pellet was washed 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 *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 (Thermo Scientific, Thermo Fisher Scientific, EU, Lithuonia).

Statistical analysis

Statistical significance was determined using "t" tests and one-way analysis of variance. Data are presented as means±SD using Statistical SPSS for Windows, version 22. All data were qualitative data and were presented by frequency distribution. The Chi-square test was used to compare between proportions. The probability of less than 0.05 was used as a cut off point for all significant tests and all statistical tests which were 2 tailed.

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).

Restriction fragment length polymorphism (RFLP)

The results of RFLP are shown in Figs. 1b-1d. Out of 112 positive nPCR samples, 98 (87.5%) samples were digested into 2 bands of different molecular size 77 bp and 137 bp (Fig. 1b). This obtained pattern belongs to either *C. parvum* or *C. hominis* (Fig. 1b). Out of the 98 *TaqI* positive samples, 73 (65.2%) samples were digested by *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 n PCR-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

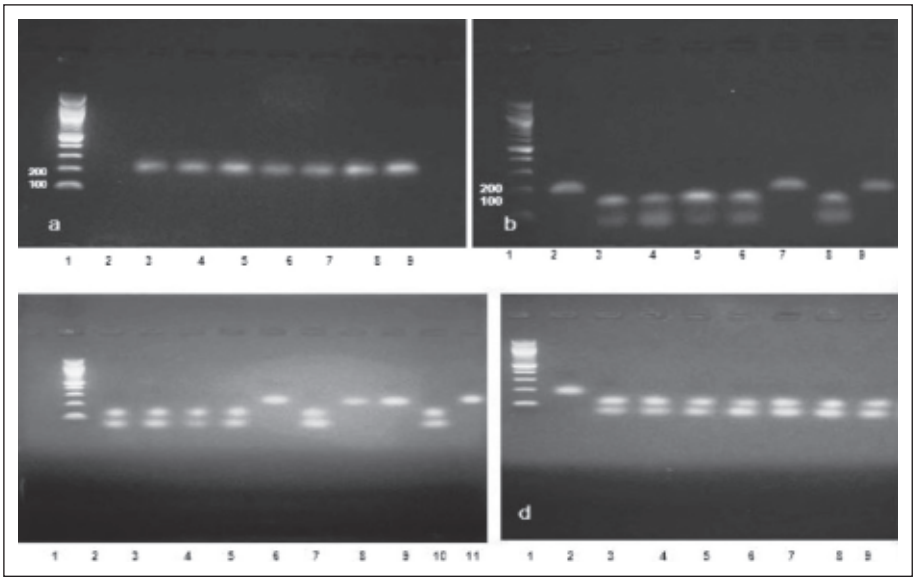

Figure 1a. An ethidium bromide stained agarose gel electrophoresis showing the secondary PCR products of the *Cryptosporidium* 18S rRNA gene (214 bp).
1: DNA molecular weight marker (100 bp).
2: negative control.
3-8: Positive samples.

Figure 1b. An ethidium bromide stained agarose gel electrophoresis showing the digested product of *TaqI*
1: DNA molecular weight marker (100 bp).
2: Positive control.
3-6, 8: *TaqI* positive samples (77bp, 137bp).
7, 9: *TaqI* negative samples.

Figure 1c. An ethidium bromide stained agarose gel electrophoresis showing the digested product of *AseI* (*C. hominis* and *C. parvum*)
1: DNA molecular weight marker (100 bp).
2-5,7,10: AseI +ve product (*C. hominis* , 155 bp and 59bp).
6,8,9: AseI –ve product (*C. parvum*).
11: +ve control.

Figure 1d. An ethidium bromide stained agarose gel electrophoresis showing the digested product of *MseI* (*C. meleagridis*)
1: DNA molecular weight marker (100 bp).
2: positive control
3-9: *MseI* positive samples (*C. meleagridis*, 132bp and 82bp)

present among cases live in rural areas (30.4%; p-value $\leq$ 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 $\leq$ 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 $\leq$ 0.001, $\leq$ 0.001, 0.005 respectively) as shown in Table 3.

Table 1. Distribution of *Cryptosporidium* spp. in relation to residence

Results of nPCR-RFLP	Urban N=66	Rural N=46	p-value
C. hominis			
• Positive	63 (95.5%)	10 (21.7%)	<0.001*
• Negative	3 (4.5%)	36 (78.3%)	
C. parvum			
• Positive	3 (4.5%)	22 (47.8%)	<0.001*
• Negative	63 (95.5%)	24 (52.2%)	
C. meleagridis			
• Positive	0 (0%)	14 (30.4%)	<0.001*
• Negative	66 (100%)	32 (69.6%)	

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

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

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

Results of nPCR-RFLP	Animal contact N=39	No animal contact N=73	p-value
C. hominis			
• Positive	6 (15.4%)	67 (91.8%)	<0.001*
• Negative	33 (84.6%)	6 (8.2%)	
C. parvum			
• Positive	23 (59%)	2 (2.7%)	<0.001*
• Negative	16 (41%)	71(97.3%)	
C. meleagridis			
• Positive	10 (25.6%)	4 (5.5%)	0.005*
• Negative	29 (74.4%)	69 (94.5%)	

DISCUSSION

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 $\leq$ 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. meleagridis* 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. melegridies* (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 $\leq$ 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 $\leq$ 0.001). *C. meleagridis* was only present among patients in rural areas (30.4%; p-value $\leq$ 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 cryptospordiosis (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 $\leq$ 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 $\leq$ 0.001, $\leq$ 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).

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