First molecular characterization of *Cryptosporidium* in Yemen

N. A. ALYOUSEFI[1], M. A. K. MAHDY[1,2]*, Y. A. L. LIM[1], L. XIAO[3] *and* R. MAHMUD[1]

[1] *Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia*
[2] *Department of Parasitology, Faculty of Medicine, Sana'a University, Sana'a, Yemen*
[3] *Division of Foodborne, Waterborne and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*

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SUMMARY

Cryptosporidium is a protozoan parasite of humans and animals and has a worldwide distribution. The parasite has a unique epidemiology in Middle Eastern countries where the IId subtype family of *Cryptosporidium parvum* dominates. However, there has been no information on *Cryptosporidium* species in Yemen. Thus, this study was conducted in Yemen to examine the distribution of *Cryptosporidium* species and subtype families. Fecal samples were collected from 335 patients who attended hospitals in Sana'a city. *Cryptosporidium* species were determined by PCR and sequence analysis of the 18 s rRNA gene. *Cryptosporidium parvum* and *C. hominis* subtypes were identified based on sequence analysis of the 60 kDa glycoprotein (*gp60*) gene. Out of 335 samples, 33 (9·9%) were positive for *Cryptosporidium*. Of them, 97% were identified as *C. parvum* whilst 1 case (3%) was caused by *C. hominis*. All 7 *C. parvum* isolates subtyped belonged to the IIaA15G2R1 subtype. The common occurrence of the zoonotic IIa subtype family of *C. parvum* highlights the potential occurrence of zoonotic transmission of cryptosporidiosis in Yemen. However, this postulation needs confirmation with future molecular epidemiological studies of cryptosporidiosis in both humans and animals in Yemen.

Key words: *Cryptosporidium*, genotyping, subtyping, Yemen.

INTRODUCTION

Cryptosporidium is a protozoan parasite in humans and animals and has a worldwide distribution. *Cryptosporidium* infection has been implicated as a cause of multiple waterborne and foodborne outbreaks of diarrhoea (MacKenzie *et al*. 1994; Fayer *et al*. 2000; Slifko *et al*. 2000). Humans can also acquire the infection through direct contact with humans (anthroponotic transmission) or animals (zoonotic transmission). Although cryptosporidiosis causes self-limiting diarrhoea in immunocompetent persons, it may lead to life-threatening diarrhoea in immunocompromised patients (DuPont *et al*. 1995; Kjos *et al*. 2005). The disease has also been considered as a predictor of malnutrition in early childhood (Huang and White, 2006).

The genus *Cryptosporidium* is a multispecies complex. To date, approximately 27 species and more than 60 genotypes have been identified (Fayer and Santin, 2009; Fayer, 2010; Traversa, 2010; Elwin *et al*. 2012). Most of the species or genotypes infect a particular host with a few notable exceptions. *Cryptosporidium parvum* infects humans and several species of animals, and is the major species responsible for zoonotic cryptosporidiosis in many developed countries. Another species, *C. hominis*, commonly infects humans and is responsible for anthroponotic transmission, with high prevalence in developing countries compared with industrial nations (Raccurt, 2007; Xiao, 2009). Subtyping of *C. parvum* based on 60 kDa glycoprotein (*gp60*) gene identified at least 12 subtype families (IIa – IIl). The zoonotic IIa subtype is predominant in developed nations and rarely isolated from humans in developing nations where the anthroponotic IIc subtype family is the most common cause of human *C. parvum* infections. The *C. parvum* IIa subtype family has a high genetic diversity with IIaA15G2R1 being predominant in calves and the major subtype infecting human and cattle in some countries (Xiao, 2009, 2010).

The epidemiology of *Cryptosporidium* in Middle Eastern countries is different from other developing countries as earlier studies reported that *C. parvum* had higher prevalence than *C. hominis* in Saudi Arabia (Al-Brikan *et al*. 2008), Kuwait (Sulaiman *et al*. 2005), Jordan (Hijjawi *et al*. 2010), Turkey (Tamer *et al*. 2007), and Iran (Nazemalhosseini-Mojarad *et al*. 2011). However, in nearby Egypt, *C. hominis* was more prevalent than *C. parvum* (Eida *et al*. 2009; Abd El Kader *et al*. 2011). Subtype analysis based on *gp60* has shown that the IId subtype family is the predominant subtype in Middle Eastern countries whereas the IIc subtype family is the dominant *C. parvum* in developing countries (Xiao, 2010).

In Yemen, cryptosporidiosis is an endemic disease with prevalence ranging from 1% to 50% (Al-Shibani *et al*. 2009; Al-Shamiri *et al*. 2010; Alyousefi *et al*.

* Corresponding author: Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. E-mail: alsharaby9@yahoo.com

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2011). Currently, there are no data on *Cryptosporidium* species and subtype families in Yemen. This study aims at identifying *Cryptosporidium* species and subtype families prevalent in Yemen, a poorer Middle Eastern country. Findings generated from this study will improve our understanding of molecular epidemiology of cryptosporidiosis not only in a developing country like Yemen but also in the larger Middle Eastern region.

MATERIALS AND METHODS

Sample collection

Fecal samples were collected during December 2008 to March 2009 from 3 main hospitals in Sana'a City, the capital of Yemen, including the Al-Jomhury Hospital, Al-Kuwait Hospital and Alzahrawy Clinical Center. Participation in the study was voluntary and each participant was asked to provide informed consent after a brief explanation of the research objectives by an attending physician or nurse. If the patient was a child, written informed consent was obtained from his/her parents. Permission was also obtained from the authorities of each hospital. The study protocol was approved by the Research and Ethical Committee of the Faculty of Medicine, University of Malaya, Malaysia (MEC RF. No: 782.9). Fecal samples were collected from all those who attended the outpatients clinics in hospitals, referred to the laboratory for fecal examination, and consent to participation in the study. Fecal specimens were collected in wide-mouthed screw-capped containers, mixed with 2·5% potassium dichromate, and stored at 4 °C until DNA extraction. The minimum sample size required for this study was 246 individuals. The expected sample size was calculated according to the following parameters – expected prevalence of *Cryptosporidium* at 20% (Al-Shibani *et al*. 2009), confidence level at 95%, and absolute precision at 0·05 (Lwanga and Lemeshow, 1991). However, 335 individuals were eventually included.

Questionnaire

Collection of samples was done simultaneously with data gathering via a pre-tested questionnaire, which included socio-demographical information such as age, gender and general area of residence, health practices, behavioural habits (i.e. washing hands before eating, washing hands after defecation, and washing fruits and vegetables), and health conditions including the occurrence and history of symptoms (e.g. diarrhoea, nausea, vomiting and abdominal pain). Environmental factors such as water supply and direct contact with animals were also included. The age of participants was categorized into 2 groups: $\leqslant$12 years and >12 years. Detection of *Cryptosporidium* in this study was by PCR since this approach was shown to have higher sensitivity than modified Ziehl-Neelsens stain (Starke *et al*. 2011).

DNA extraction

Fecal samples were washed by centrifugation with distilled water to remove potassium dichromate used in fecal storage. DNA was extracted directly from approximately 0·25 g of fecal samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, USA) and the manufacturer's recommended procedures. DNA was eluted in 50 μl of elution buffer and kept in the freezer at −20 °C until use.

Cryptosporidium *genotyping*

A partial polymorphic region of 18 s rRNA gene was amplified according to Nichols *et al*. (2003). In the primary reaction, a 655 to 667 bp fragment was amplified depending on the species of *Cryptosporidium* or *C. parvum* genotype, using a 27-mer forward primer (N-DIAGF; 5′-CAA TTG GAG GGC AAG TCT GGT GCC AGC-3′) and a 26-mer reverse primer (N-DIAGR2; 5′-CCT TCC TAT GTC TGG ACC TGG TGA GT-3′). The secondary PCR amplified a 435 bp fragment using the forward primer (CPB-DIAGF; 5′-AAG CTC GTA GTT GGA TTT CTG-3′) and the reverse primer (CPB-DIAGR; 5′-TAA GGT GCT GAA GGA GTA AGG-3′) that were previously developed by Johnson *et al*. (1995). The primary and the secondary PCRs were run in a total volume of 50 μL containing 200 μM of each of the 4 deoxynucleoside triphosphates (dNTP) (Fermentas, Ontario, Canada), 0·2 μM of each primer, 400 μg mL^{-1} of non-acetylated bovine serum albumin (New England Biolabs, Ipswich, USA), 3·5 mM of $MgCl_2$ (Fermentas, Ontario, Canada), 2·5 U of Taq polymerase (New England Biolabs, Ipswich, USA) and 1× ThermoPol PCR buffer (New England Biolabs, Ipswich, USA). Two μL of DNA template were used in the primary PCR whereas 5 μL of the primary PCR product was used as template in the secondary PCR. The cycling conditions were as follows: hot start at 95 °C for 5 min followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 30 s, and a final extension was at 72 °C for 10 min. The secondary PCR had similar cycling conditions except that the annealing temperature in the secondary PCR was 68 °C instead of 60 °C. To minimize cross-contamination, DNA extraction, PCR reaction and post-PCR manipulation were performed in 3 separate laboratory areas using 3 separate sets of micropipettes and tips. Genomic DNA positive for *Cryptosporidium*

was used as the positive control in each run of PCR. Distilled water was used as the negative control.

Cryptosporidium *subtyping*

A nested-PCR was used to amplify the partial *gp60* gene (Strong *et al*. 2000; Mallon *et al*. 2003). In the primary PCR, a fragment of ~980–1000 bp was amplified using primers gp15-ATG and gp15-STOP. Subsequently, a ~450 bp fragment was amplified in the secondary PCR using the primers gp15-15A and gp15-15E. The primary and the secondary PCRs were done in a total volume of 50 μL containing 200 μM of each of the 4 dNTP (Fermantas), 0·4 μM of each primer, 3 mM of $MgCl_2$ (Fermentas), 2·5 U of Taq polymerase (New England Biolabs), and 1× ThermoPol PCR buffer (New England Biolabs). Two μL of DNA template were used in the primary PCR whereas 5 μL of the primary PCR product was used as the template in the secondary PCR. The cycling conditions were as follows: hot start at 94 °C for 5 min, followed by 40 cycles of denaturing for 30 s at 94 °C, annealing for 45 s at 55 °C and extension for 1 min at 72 °C, and a final extension at 72 °C for 10 min. The secondary PCR had 35 cycle conditions with annealing and extension times for 30 s for each 1 instead of 45 s and 1 min, respectively. The PCR products were resolved on 2% agarose gel and stained with Sybr Safe (Invitrogen, USA) for both 18S rRNA and *gp60* PCR products.

DNA sequencing and subtype determination

PCR products were purified using the QIAquick Gel Extraction Kit (QIAgen, Germany), according to the manufacturer's instructions, and sequenced in both directions using the secondary PCR primers. The sequencing was carried out in an automated DNA sequencer (3130 x 1 Genetic Analyzer, Applied Biosystems, USA) using the BigDye Terminator v3.1 Cycle sequencing Kit. Sequences were edited and the consensus sequences were created using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The consensus sequences were aligned with reference sequences downloaded from the GenBank database using ClustalX (http://www.clustal.org/). *Cryptosporidium parvum* and *C. hominis gp60* subtypes were named by counting the number of the trinucleotide repeats of TCA (A), TCG (G) and TCT (T) and the ACATCA repeat (R) after the trinucleotide repeats as described previously (Sulaiman *et al*. 2005). DNA sequences from this study were deposited in the GenBank database under Accession numbers JX032678–JX032693.

Statistical analysis

Data were analysed using the SPSS program for Window version 11.5 (SPSS Inc. Chicago, IL, USA). Chi-square test was used to assess the association between dependent and independent variables and 95% confidence intervals were calculated. Fisher exact test was used where applicable. Significance of the difference was defined as $P<0·05$.

RESULTS

Among the 335 study participants, 42% were males and 58% were females. The age of patients ranged from 1 to 80 years with a median of 22 years. The 18 s rRNA-based nested PCR detected *Cryptosporidium* in 33 (9·9%) of the 335 patients. Females had a slightly higher infection rate of *Cryptosporidium* than males (10·4% *vs* 9·3%). A higher prevalence of cryptosporidiosis was seen in patients who had a bath at least twice a week (14%) compared with those who had a bath less than twice a week (8·5%). Patients who drank untreated water had a higher infection rate (10·7%) than patients who use treated drinking water (8·7%). However, these differences were not significant. The result of the statistical analysis is shown in Table 1.

The 18 s rRNA PCR products of all 33 positive samples were successfully sequenced. All except 1 (97%) were identified as *C. parvum*; the remaining 1 (3%) was identified as *C. hominis*. All samples positive for *Cryptosporidium parvum/hominis* (33 samples) were subjected to PCR amplification targeting the partial sequence of the *gp60* gene. Of these, 21 samples produced PCR amplicons, which were sequenced in both directions. The partial sequence of *gp60* gene was successfully sequenced for 7 isolates of *C. parvum* and the isolate of *C. hominis*. Sequence analysis of the *gp60* gene identified all 7 *C. parvum* isolates as the IIaA15G2R1 subtype of the IIa subtype family. Compared with sequences deposited in the GenBank database, the sequence of the IIa subtype family from this study matched the IIaA15G2R1 subtype from a human in Australia (GenBank Accession no. JF727795) (Waldron *et al*. 2011) the UK (GenBank accession no. HQ149037) (Hadfield *et al*. 2011) and Iran (GenBank Accession no. AB560747) (Nazemalhosseini-Mojarad *et al*. 2011). The *C. hominis* isolate was identified as the IeA11G3T3 subtype of the Ie subtype family which matched *gp60* gene sequence of the IeA11G3T3 subtype of the Ie subtype family isolated from humans in the UK (GenBank Accession no. GU214354) (Pangasa *et al*. 2010) and Canada (GeneBank Accession no. DQ192509) (Trotz-Williams *et al*. 2006).

DISCUSSION

In the current study the prevalence of *Cryptosporidium* was 9·9% by PCR, which is 10 times higher than our previous report based on the microscopical examination of fecal smears stained

Table 1. Prevalence and distribution of *Cryptosporidium* in patients seeking healthcare in Sana'a City

(The level of significance was defined as $P<0{\cdot}05$. The sum of some variables are not always 335 due to missing data.)

Variables		No. examined	No. positive (%)	OR (95%CI)	*P* value
Demographic					
Age (years)	>12	233	24 (10·3)	1	0·68
	⩽12	102	9 (8·80)	0·84 (0·38–1·88)	
Address	Urban	242	25 (10·3)	1	0·70
	Rural	90	8 (8·90)	0·85 (0·37–1·95)	
Gender	Male	151	14 (9·30)	1	0·74
	Female	183	19 (10·4)	1·13 (0·55–2·35)	
Household members	<5	94	9 (9·60)	1	0·92
	⩾5	241	24 (10·0)	1·05 (0·47–2·34)	
Personal hygiene					
Washing hands after defecation	Yes	151	15 (9·90)	1	0·85
	No	172	16 (9·30)	0·93 (0·44–1·95)	
Washing hands before eating	Yes	137	16 (11·7)	1	0·41
	No	191	17 (8·90)	0·74 (0·36–1·52)	
Washing fruits and vegetables	Yes	253	25 (9·90)	1	0·87
	No	76	8 (10·5)	1·07 (0·46–2·49)	
Type of drinking water	Treated	126	11 (8·7)	1	0·57
	Not treated	206	22 (10·7)	1·25 (0·58–2·67)	
Bathing twice or more weekly	Yes	248	21 (8·50)	1	0·13
	No	85	12 (14·1)	1·78 (0·83–3·79)	
Rearing animals[a]	No	238	23 (9·70)	1	0·84
	Yes	96	10 (10·4)	1·09 (0·49–2·38)	
Clinical symptoms					
Diarrhoea[#]	No	225	20 (8·90)	1	0·37
	Yes	108	13 (12·0)	1·40 (0·67–2·94)	
Other symptoms*	No	40	3 (7·5)	1	0·78
	Yes	294	30 (10·2)	1·40 (0·41–4·8)	

[a] Rearing animals was defined as the traditional rearing which involves sending animals to mountains and valleys for grazing and caring of animals in shelters within the house vicinity.
[#] Diarrhoea was defined as loose stools for 3 consecutive times/day.
* Other symptoms included vomiting, abdominal pain and/or nausea, Fisher exact test was used for calculating the significance in this variable.

with Ziehl–Neelsen stain (Alyousefi *et al*. 2011). This difference could be attributed to the higher sensitivity of PCR. The superiority of PCR compared with microscopy was previously reported (Johnson *et al*. 1995; Stark *et al*. 2011). In addition, it was demonstrated that the threshold of the 100% sensitivity of Ziehl–Neelsen staining technique is 500 000 oocysts per gram of feces (Weber *et al*. 1991).

This study is the first report of *Cryptosporidium* species and subtypes in Yemen. The current results showed that all isolates were *C. parvum* except 1 isolate, which was identified as *C. hominis*. These findings are consistent with previous reports in other Middle Eastern countries (Sulaiman *et al*. 2005; Tamer *et al*. 2007; Al-Brikan *et al*. 2008; Hijjawi *et al*. 2010; Xiao, 2010; Nazemalhosseini-Mojarad *et al*. 2011). In Saudi Arabia, a neighbouring country of Yemen, of the 31 human fecal samples examined, Al-Brikan and his colleagues detected *C. parvum* and *C. hominis* in 15 and 13 samples, respectively (Al-Brikan *et al*. 2008). Subsequently, in the same country, another study found *C. parvum* in 43 of 53 samples examined (Nazemalhosseini-Mojarad *et al*. 2012). Similar results have also been reported in Kuwait where *C. parvum* was the predominant causative agent for cryptosporidiosis in children (Sulaiman *et al*. 2005; Iqbal *et al*. 2011). A molecular epidemiological study conducted in Jordan identified *C. parvum* and *C. hominis* in 22 and 20 specimens, respectively (Hijjawi *et al*. 2010). In Iran, collective data generated from 4 molecular epidemiological studies showed a predominance of *C. parvum* (Nazemalhosseini-Mojarad *et al*. 2012). In contrast, 2 molecular epidemiological studies reported the presence of *C. parvum* and *C. hominis* in Egypt with the predominance of *C. hominis* (Abd El Kader *et al*. 2012). It has been reported that *C. hominis* is the most common species in developing countries with the common occurrence of Ia, Ib, Id and Ie subtype families. In Europe and Australia, and North America, the IIa subtype family is the dominant *C. parvum*, suggesting zoonotic transmission of *Cryptosporidium* may be common in these countries. Although *C. parvum* is prevalent in some developing countries, the anthroponotic IIc subtype family is the most common subtype family of *C. parvum* in humans in these countries (Xiao, 2010).

It is our understanding that geographical distribution of *Cryptosporidium* species is not a conclusive indication of differences in the transmission of cryptosporidiosis. Although *C. parvum* has been known as a zoonotic species, most subtype families of *C. parvum* identified (IIb, IIc, IIe – IIl) in humans are probably not zoonotic in origin (Xiao, 2010). In this study, all *C. parvum* subtyped belonged to IIaA15G2R1 subtype of the IIa subtype family. The subtype IIaA15G2R1 is the major subtype of *C. parvum* in cattle in developed countries (Alves *et al*. 2003; Wu *et al*. 2003; Sulaiman *et al*. 2005; Abe *et al*. 2006; Cohen *et al*. 2006; Trotz-Williams *et al*. 2006; Feng *et al*. 2007; Thompson *et al*. 2007; Broglia *et al*. 2008; Wielinga *et al*. 2008). This subtype has also been found in humans in the USA (Santin *et al*. 2008), Canada (Budu-Amoako *et al*. 2012), the UK (Chalmers *et al*. 2009), the Netherlands (Wielinga *et al*. 2008), Slovenia (Soba and Logar, 2008), Portugal (Alves *et al*. 2006), Ireland (Thompson *et al*. 2007), Japan (Abe *et al*. 2006), Australia (Ng *et al*. 2008), and Malaysia (Iqbal *et al*. 2012). However, it has been rarely reported in humans in developing countries, where most *C. parvum* infections are caused by the anthroponotic IIc subtype family (Xiao, 2010).

In Middle Eastern countries, the subtype family IIa is responsible for some *C. parvum* infections in humans. It was reported in 7 of 22 *C. parvum*-infected patients from Iran (Nazemalhosseini Mojarad *et al*. 2010), 3 of 13 patients from Jordan (Hijjawi *et al*. 2010), and 1 of 37 patients from Saudi Arabia (Nazemalhosseini-Mojarad *et al*. 2012). Another zoonotic *C. parvum* subtype family, IId, is also common in humans in this region (Nazemalhosseini-Mojarad *et al*. 2012). The predominance of the zoonotic subtype families of *C. parvum* IIa and IId in the Middle Eastern regions suggests that animal-to-human transmission may be a common transmission route of *Cryptosporidium*. However, this postulation may be premature in view of findings in 2 studies conducted in Kuwait (Sulaiman *et al*. 2005; Iqbal *et al*. 2011) where there was no contact with farm animals by most of the study participants and desalinated seawater was the source of drinking water in the study area. As contact with animals in the context of illness course is a key requirement to document the occurrence of the zoonotic transmission, the authors suggested that the source of *C. parvum* infection in Kuwait was from humans.

In Yemen, the situation is different from other Middle Eastern countries whereby people in Yemen have very close contact with animals, especially in the rural and suburban areas. It is a very common practice to keep animals inside or next to houses rather than on farms. In addition, local veterinarian services are also limited, thus local or imported animals are not routinely screened for parasitic diseases. These circumstances provide a suitable environment for the transmission of zoonotic diseases. Thus, the fact that all *C. parvum* isolates successfully subtyped belong to the IIa subtype family may represent potential evidence for the possible zoonotic transmission of *Cryptosporidium* in Yemen. However, the small number of the *C. parvum* isolates subtyped has limited the strength of this conclusion, and more conclusive evidence should come from case-controlled studies and genotyping/subtyping of *Cryptosporidium* spp. from humans and animals in the same household or locality.

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