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Prevalence of *Cryptosporidium* species isolated from diarrheic children in Makkah, Saudi Arabia

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Abstract. Cryptosporidiosis has been identified as a significant underlying cause of morbidity and mortality worldwide. Studies in high and low income countries have recognized the importance of *Cryptosporidium* as a cause of diarrhea. The objectives of the current study were to determine the prevalence rate and genotypes of *Cryptosporidium*in in diarrheic children in Makkah Region. A total of 1,380 fecal samples were collected from children aged up to 14 years attending 3 major hospitals of Makkah between March 2015 and March 2016. Stool collected were subjected to direct microscopic examination and crypto antigen detection using ImmunoCard STAT, *Cryptosporidium/Giardia* rapid test. Part of each positive stool sample was kept frozen at -20°C for molecular characterization. Initial screening by immunochromatographic detection kit revealed 23 positive cases. PCR was performed for positive cases by amplification of a piece of the gene encoding the small (18S) subunit of rRNA producing a 435-438 bp product. *Cryptosporidium* genotyping was performed by RFLP analysis of PCR products. Genotyping revealed 18 cases *C. hominis* genotype, 4 cases *C. parvaum* genotype and one sample failed to be amplified. The data revealed a higher incidence of the common human species *C. hominis* (81.8%). The detection of both *C. hominis* and *C. parvaum* genotypes point to the possibility of both anthroponotic and zoonotic transmission routes occurring in Makkah region. Further studies are needed to verify the subgenotypes of *Cryptosporidium* to elucidate the real transmission modes and hence plan for effective control strategies.

INTRODUCTION

Cryptosporidium is a protozoan parasite infecting a variety of hosts including humans and animals. "Cryptosporidiosis is identified as an important cause of morbidity and mortality worldwide. Studies in low and high-income countries have confirmed the importance of *Cryptosporidium* as a cause of diarrhea and childhood malnutrition" (Checkley *et al.*, 2015). Cryptosporidiosis diarrhea is usually self-limiting in immunocompetent people, but may be chronic and life-threatening to immunocompromized persons (Hunter *et al.*, 2000).

The oocyst is the infective stage which remains viable and infective for months outside the host. Cryptosporidiosis can appear as an anthroponosis or as a zoonosis. *Cryptosporidium* thas the potential to infect many species of animals due to the presence of parasitic' oocysts everywhere in the

environment, and the susceptibility of humans to infections through many routes, such as ingestion of contaminated food, drink or direct contact with infected persons or animals. The incubation period ranges from 2–10 days and infected persons can excrete the pathogens for several months after symptoms have stopped (Fayer, 2004).

Cryptosporidium is an important enteric parasite to health authorities due to its ability to resist routine disinfection and filtration procedures. It has been recognized as a cause of many outbreaks of diarrhea in many countries all over the world (Samie *et al.*, 2006 and Ng *et al.*, 2010).

To date, about 30 species of *Cryptosporidium* are identified. *C. hominis* is the predominant species affecting man, while *C. parvum* infects a wide range of mammals (Slapeta, 2013). Understanding the epidemiology of Cryptosporidiosis and its transmission routes is essential for planning an effective prevention and control strategies (Xiao, 2010).

For better understanding of the epidemiology of cryptosporidiosis, it is necessary to recognize the species and genotypes in the studied region (Neira-Otero *et al.*, 2005).

Most species of *Cryptosporidium* are morphologically identical: therefore additional molecular tools such as PCR, RFLP and sequence analysis are needed to accurately identify different species and genotypes. The 60-KDa glycoprotein gene (gp60) and the 18S ribosomal RNA gene (rRNA) have been extensively studied to identify species and subtypes (Ng *et al.*, 2011).

In Saudi Arabia, there are only few published reports on the prevalence of *Cryptosporidium* infection in various populations (Khan *et al.*, 1988 and Al-Harthi & Jamjoom, 2007). The objectives of the current study were to determine the prevalence rates and genotypes of *Cryptosporidium* in in diarrheic children in Makkha region of Saudi Arabia.

MATERIALS AND METHODS

Subjects

A total of 1,380 fecal samples were collected from children up to 14 years attending 3 of the major hospitals of Makkah, Saudi Arabia between September 2015 and September 2016. Samples were collected from either inpatients or outpatients requesting stool analysis due to diarrhea. Informed consents were obtained from parents of those children. The study protocol was approved by Ethics Review Board for Human Studies at Faculty of Medicine, Umm Al-Qura University.

Samples collection, preservation and transport

Collected stools were subjected to direct microscopic examination and crypto antigen detection using immunochromatographic test. Part of each positive stool sample was kept frozen at -20°C for further molecular characterization.

Diagnostic procedures

1. Microscopic examination of wet mount and concentrated samples

Stool samples were examined for parasitic infection by saline and iodine wet mount using fecal parasite concentrator Kit (EvergGreen, Los Angeles, Ca) which is an efficient device to concentrate protozoan cysts following manufacturer's instructions.

2. Examination by rapid diagnostic tests

Examination of stool samples by the ImmunoCard STAT, *Cryptosporidium/ Giardia* rapid test (Meridian Bioscience Inc., Cincinnati, OH, USA) which has 100% specificity and 97.3% sensitivity. The test was performed according to the directions of the manufacturer.

3. Species identification by PCR-RFLP

Positive stool samples by the ImmunoCard STAT were subjected to DNA extraction using the MagaZorb DNA Mini-Prep Kit (Promega,

Madison, USA) according to manufacturer instructions with a slight modification. Briefly 100 mg of the stool concentrate was re-suspended in the provided lysis buffer and subjected to 5 consecutive cycles of freezing in liquid nitrogen for 1 min and thawing at 56°C for 2 min, with vortexing for 30 sec for every cycle to rupture the *Cryptosporidium* oocysts. Extracted DNA was quantified using Spectramax Plus 384 Microplate reader (Molecular Devices, Orleans, USA). Integrity of the DNA was determined by agarose gel electrophoresis and stored at -20°C until further examinations. PCR amplification of *Cryptosporidium* DNA using the 18S rRNA primers (CPB-DIAGF/R) of (Johnson *et al.*, 1995) yields products that vary in length from 428 bp to 455 bp. Primers used were, CPB-DIAGF (forward; 5'AAGCTCGTAG TTGGATTTCTG3') CPBDIAGR (reverse; 5'TAAGGTGCTGAAGGAGTAAGG3'). PCR programmed profile was, initial denaturation at 94°C for 30 s, followed by 40 cycles denaturation at 94°C for 30 s, annealing at 60°C for 1 min, elongation at 72°C for 30 s and a final extension at 72°C for 10 min.

4. Restriction Fragment Length Polymorphism (RFLP) analysis

Two microliters of amplified PCR products was restricted with *SspI* and *VspI*. Digestion was carried out under standard conditions according to (Xiao *et al.*, 2001).

A – *SspI* restriction enzyme digestion:

It was performed for the amplified PCR products. For each sample the following reagents were used in a sterile microfuge tube: 32.6 µl sterile deionized water, 4 µl RE Buffer *SspI* (Promega, Madison, USA), acetylated BSA 0.4 µl, 2 µl PCR product and 1 µl *SspI* restriction enzyme 10 U/µl (Promega, Madison, USA) in a total volume of 40 µl. After centrifugation for 10 seconds the samples were incubated at 37°C for 2 hours to complete the enzyme digestion.

B – *VspI* restriction enzyme digestion:

It was performed for the amplified PCR products. For each sample the following reagents were used in a sterile microfuge tube: 32.6 µl sterile deionized water, 4 µl RE Buffer *VspI* (Promega, Madison, USA), acetylated BSA 0.4 µl, 2 µl PCR product and 1 µl *VspI* restriction enzyme 10 U/µl (Promega, Madison, USA) in a total volume of 40 µl. After centrifugation for 10 seconds the samples were incubated at 37 °C for 2 hour to complete the enzyme digestion.

5. Detection of digested products by agarose gel electrophoresis

Digested DNA fragments were analyzed by horizontal electrophoresis using agarose 3.2% (Sigma, T9650, St. Louis, Missouri, USA).

RESULTS

In the current study, initial screening by immunochromatographic detection kit revealed 23 *Cryptosporidium* positive cases out of 1380 samples and one sample with combined infection of *Cryptosporidium* and *Giardia* which was excluded from further studies (Table 1). Direct microscopic examination detected parasitic infections rather than *Cryptosporidium* in 11 cases of the screened samples (unpublished data).

The DNA of positive samples by immunochromatographic detection kit was successfully amplified in 22 samples and one sample failed to be amplified (Table/Fig. 1). To determine the *Cryptosporidium* species, these 22 samples were subjected to RFLP analysis by digesting the PCR product with *Ssp*I and *Vsp*I restriction enzymes. Using *Vsp*I restriction enzymes 18 samples yielded (222, 104, 112) base pair fragments characteristic of *C. hominis* and 4 samples yielded (119, 104, 112) characteristic of *C. parvum.* Using *Ssp*I restriction enzymes 18 samples yielded (264, 111, 40, 12, 11) fragments characteristic of *C. hominis* and 4 samples yielded (264, 108, 40, 12, 11) characteristic of *C. parvum.*

DISCUSSION

Different types of molecular diagnostic tools have been used in the differentiation of *Cryptosporidium* genotypes and species. Currently there are no standard genetic loci

Table 1. Samples collected by age group, gender and percentage of positive samples

Age	<2 years		2–5		6–10		11–14		Total		
Gender	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂+♀
Number of samples	288	201	247	301	177	79	53	34	765	615	1,380
Number of +ve samples	5	4	6	3	3	1	0	0	14	8	22
C. hominis /*C. parvum*	5/0	4/0	4/2	2/1	2/1	1/0	0	0	11/3	7/1	18/4
% +ve within the age group for each gender	1.7%	2%	2.4%	1%	1.7%	1.3%	0	0	1.8%	1.3%	
Combined (male and female) +ve %	1.8%		1.6%		1.5%		0		1.6%		

recommended for species identification, but RFLP or sequencing of 18S rRNA gene loci are widely used and has provided valuable information about *Cryptosporidium* species (Xiao *et al.*, 1999). For this reason (CPB-DIAGF/R) primers (Johnson *et al.*, 1995) targeting the small (18S) subunit of rRNA have been used.

"Cryptosporidiosis particularly affects children under 4 years of age. A high incidence of the disease in this age group has been reported in Canada, United States, New Zealand, England, Wales and France" (The ANOFEL Cryptosporidium National, 2010). The current study revealed that 18 out of the 22 positive cases were under the age of 5 years. The reason for the higher incidence in children under the age of 5 is uncertain, but could be due to "multiple transmission routes, including contact with infected animals, person-to-person transmission in households and care settings, consumption of contaminated food and drinks, consumption of water from private and public supplies, exposure to recreational water in swimming pools or water parks, and travel to endemic countries" (Nichols *et al.*, 2009). Furthermore, it may be related to less developed immunity in young children (Abd El Kader *et al.*, 2012).

The ability of *Cryptosporidium* to resist disinfection procedures applied in water purification system, leads to increased persistence of the viable infectious oocysts in treated drinking water (Smith *et al.*, 2006). In Al-Taif city which lies next to Makkha, *Cryptosporidium* was identified in 8.0% of desalinated water and in 7.0% of wells water (Hawash *et al.*, 2015). Sources of water supply in Makkha do not differ much from that of Al-Taif, therefore it could be also a potential source of infection in Makkha.

The prevalence of *Cryptosporidium* infection in the studied group of children complaining from diarrhea in Makkah was (1.7%); this is similar to previous report conducted in KSA. *Cryptosporidium* was detected in 1% of, routinely processed, fecal samples from children with diarrhea attending clinics at the Maternity and Children's Hospital in Dammam (Khan *et al.*, 1988), and similar results was reported in children investigated at King Abdul Aziz University Hospital in Riyadh (Bolbol, 1992). In the current study, the higher frequency reported in males (14/22) more than females (8/12) could be explained in a country like Saudi Arabia by more freedom given to male children moving in and outdoors and practice various activities compared with females. Therefore, they are more exposed to multiple transmission routes.

Knowledge concerning the genotypes of *Cryptosporidium* isolates from humans in Saudi Arabia is limited. In Jeddah city species identification of *Cryptosporidium* samples collected from children revealed 42.9% *C. parvum* and 37% *C. hominis* (Al-Brikan *et al.*, 2008). While in Taif city, all screened samples were *C. parvaum* (Shalaby *et al.*,

2014). Contrary to those reports, genotyping in the current study revealed a higher prevalence of the commonest human species *C. hominis* (81.8%).

In Cairo, Egypt, *Cryptosporidium hominis* was also isolated in 80.0% of the examined cases (Abd El Kader *et al*., 2012).

Studies in neighboring countries to Saudi Arabia have reported that *C. parvum* is the predominant species. For example, in Iran, molecular tools identified 73.3% *C. parvum* and 26.7 as *C. hominis* (Meamar *et al*., 2007). In Kuwait, 94% of screened children had *C. parvum*, 5% had *C. hominis*, and 1% had both *C. parvum* and *C. hominis* (Sulaiman *et al*., 2005). In 44 *Cryptosporidium* isolates from Jordanian children, four different species were recorded; 22 *C. parvum*, 20 *C. hominis*, 1 *C. canis* and 1 *C. meleagridis* (Hijjawi *et al*., 2010).

These genotypic variations recorded in the neighboring countries are acceptable and could be explained by the residents' culture and habits. The fact that both *C. hominis* and *C. parvum* were detected in the current study, suggests that both anthroponotic and zoonotic pathways are expected in Makkha region. Further studies are needed to verify the subgenotypes of *Cryptosporidium* to elucidate the transmission modes in our locality and hence plan effective control strategies.

CONCLUSION

The fact that both *C. hominis* and *C. parvum* were detected, suggests that both anthroponotic and zoonotic pathways are expected in Makkha region.

COMPETING INTEREST

The authors declared no potential conflict of interest with respect to the research, authorship or publication of this article.

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