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Identification of rare and novel *Cryptosporidium* GP60 subtypes in human isolates from Jordan

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ABSTRACT

Little is known about the epidemiology of *Cryptosporidium* in Jordan and no genotyping studies have been conducted on *Cryptosporidium* isolates from humans or animals from Jordan. Genotyping of 44 *Cryptosporidium* isolates from Jordanian children at the 18S rRNA locus and a unique diagnostic locus identified four *Cryptosporidium* species; *C. parvum* (22), *C. hominis* (20), *C. meleagridis* (1) and *C. canis* (1). Sub-genotype analysis of 29 isolates at the 60-kDa glycoprotein (GP60) locus identified three *C. parvum*, two *C. hominis* subtype families and one *C. meleagridis* subtype. Several rare and novel subtypes were identified indicating unique endemicity and transmission of *Cryptosporidium* in Jordan.

1. Introduction

Cryptosporidium is an enteric parasite that infects a wide range of hosts including humans, domestic and wild animals. In humans, *Cryptosporidium* infection can result in severe diarrhoea, which is usually self-limiting in immunocompetent individuals, but may be chronic and life-threatening to those that are immunocompromised (Xiao, 2010). *Cryptosporidium* is prevalent in Jordan (Youssef et al., 2000; Nimri, 2003; Abo-Shehada et al., 2004; Mahgoub et al., 2004), but to date no genotyping studies have been conducted on *Cryptosporidium* isolates from humans. The aim of the present study, therefore, was to examine samples from sporadic cases of cryptosporidiosis in Jordanian children over a 2-year period (2007–2008) to identify the species and subtypes of *Cryptosporidium* involved to better understand the transmission dynamics and distribution of the parasite in Jordan.

2. Materials and methods

2.1. Cryptosporidium isolates

A total of 1585 diarrheic stool specimens were collected from May 2007 to November 2008 from children from birth to 12 years, admitted to the Princess Rahma Teaching Hospital in Irbid, suffering from gastrointestinal illness. A total of 104 microscopy negative samples were also screened directly by PCR to determine the percentage of samples not detected by microscopy. During this period an outbreak of cryptosporidiosis occurred in Jordan and samples were also collected from four sick children admitted to King Abdulla Hospital in Irbid and screened by PCR.

2.2. Microscopy and DNA typing

Microscopy was performed using malachite green negative staining as previously described (Elliot et al., 1999). Briefly, 1 g aliquots of faeces were emulsified in 8 ml of distilled water and then spun at 800g for 5 min and the supernatant removed. A drop of 5% malachite green in distilled water was then placed on a slide and mixed with one drop of the fecal pellet collected from the surface of the fecal pellet. Slides were examined for the presence of *Cryptosporidium* oocysts under 40× and 100× objectives. Total DNA was extracted using a QIAmp DNA Stool Kit (Qiagen, Germany). All samples were initially screened using a *C. parvum* and *C. hominis* specific qPCR at a unique *Cryptosporidium* specific protein-coding locus previously described (Yang et al., 2009). Isolates were further genotyped using a two-step nested PCR and sequencing of a ~540 bp product at the 18S rRNA locus (Ryan et al., 2003). Positive samples were subtyped at the GP60 gene locus using a two-step nested PCR that amplifies a ~830 bp fragment (Strong et al., 2000; Sulaiman et al., 2005). In preparation for DNA sequencing, secondary PCR products were purified and sequenced as previously described (Ng et al., 2006).

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Nucleotide sequences were analyzed using ChromasPro version 2.3 (http://www.technelysium.com.au) and aligned with reference genotypes from GenBank using ClustalW (http://clustalw.genome.jp).

3. Results

3.1. Cryptosporidium species

A total of 28 samples from Rahma Hospital were positive by microscopy for the presence of *Cryptosporidium* oocysts giving a microscopy prevalence of 1.8% (28/1585). qPCR screening of 104 microscopy negative samples detected an additional 18 positives however, indicating that the true prevalence was much higher (>19%). All four samples collected from children at the King Abdulla Hospital were positive by PCR.

Amongst the 28 microscopy positive isolates, the most common clinical symptoms were diarrhoea and abdominal pain, reported in all 28 cases, vomiting and loss of appetite was the second most common symptom and was reported in 57.2% (16/28). The four children admitted to King Abdulla Hospital during the outbreak were critically ill suffering from fever, vomiting and general weakness. Amongst the 18 PCR positives identified from the microscopy negative samples, diarrhoea and abdominal pain were experienced by 83% (15/18) and the remaining 3 were asymptomatic. All the children were aged 2 months–12 years but the majority of *Cryptosporidium*-positive samples were from children less than 5 years old.

At the 18S locus, a total of 22 of the 28 microscopy positive isolates from the Princess Rahma Teaching Hospital were successfully sequenced. Two *Cryptosporidium* species were identified; *C. parvum* (12) *and C. hominis* (10) (Table 1). The 18 microscopy-negative but qPCR-positive isolates were typed as *C. parvum* (9), *C. hominis* (8) and *C. meleagridis* (1). The four isolates collected from the King Abdulla Hospital during the outbreak were genotyped as *C. hominis* (2), *C. parvum* (1) and *C. canis* (1).

3.2. GP60 sub-typing

A total of 29 isolates were successfully subtyped at the GP60 locus. Sequence analysis of the GP60 locus identified three *C. parvum,* two *C. hominis* subtype families and one *C. meleagridis* subtype (Table 2). There was 100% agreement between 18S and GP60 sub-typing i.e. isolates that typed as *C. parvum* at the 18S, also typed as *C. parvum* at the GP60 locus, etc. The three *C. parvum* subtype families were IIa, IIc and IId. Within these three *C. parvum* subtype families, seven different subtypes were identified; IIaA15G1R1 (2), IIaA20G3R1 (1), IIcA5G3a (2), IIdA14G1 (1), IIdA20G1 (5), IId A24G1 (1), IIdA29G1 (1). The two *C. hominis* subtypes families were Ib and Id and six different *C. hominis* subtypes were identified; IbA6G3 (3), IbA9G3 (3), IbA10G2 (1), IbA20G2 (1), IdA21 (2) and IdA24 (5). The *C. meleagridis* isolate was typed as IIIa. Only one of the four isolates collected from the King Abdulla Hospital was typed at the GP60 locus and this was identified as *C. hominis* IbA10G2. The novel *C. parvum* GP60 IIdA29G1 subtype identified in the present study has been submitted to GenBank under the Accession No. GU458803.

Table 1
Cryptosporidium species identified in children in Jordan.

Source of isolates	No. isolates (*n*)	*Cryptosporidium species*			
		C. parvum	*C. hominis*	*C. meleagridis*	*C. canis*
Rahma Hospital (microscopy positive)	28 (22 were typed)	12	10	0	0
Rahma Hospital (microscopy negative)	18	9	8	1	0
King Abdulla Hospital	4	1	2	0	1
Total	50	22	20	1	1

Table 2
Cryptosporidium GP60 subtypes identified in children in Jordan.

Cryptosporidium species	GP60 subtype family	GP60 subtype	No. subtypes
C. hominis	Ib	IbA6G3	3
		IbA9G3	3
		IbA10G2	1
		IbA20G2	1
	Id	IdA21	2
		IdA24	5
C. parvum	IIa	IIaA15G1R1	2
		IIaA20G3R1	1
	IIc	IIcA5G3a	2
	IId	IIdA14G1	1
		IIdA20G1	5
		IIdA24G1	1
		IIdA29G1	1
C. meleagridis	IIIa	IIIaA12G3R1	1

4. Discussion

In the present study, the prevalence of *Cryptosporidium* was 1.8% (28/1585) using microscopy. Previous studies in Jordan have reported a prevalence of 37.7% (112/300) in children from birth to 12 years attending Princess Rahma Teaching Hospital in Irbid, Jordan (Mahgoub et al., 2004). In that study, direct immunofluorescence was used. An earlier study reported a much lower prevalence (8%) in 200 stool samples from patients with gastroenteritis from four health centers in a rural area of Jordan (Nimri, 2003). In that study, isolates were screened using formalin-ethyl acetate concentration, wet mount preparations and modified acid-fast staining methods (Nimri, 2003). Another study, which examined stool samples from 265 children, under 5 years of age, admitted to the pediatric ward at Princess Rahma Hospital for Children, Irbid, Jordan, reported a prevalence of 1.5% for *Cryptosporidium* using thin smears prepared from un-concentrated suspensions of stool (Youssef et al., 2000).

Molecular analysis of 104 microscopy negative isolates identified an additional 18 positives (17.3%), indicating that a significant number of *Cryptosporidium* positives are not being detected by microscopy and indicating that the true prevalence is much higher (>19%).

In the present study, four different *Cryptosporidium* species were detected; *C. parvum, C. hominis, C. canis* and *C. meleagridis*. This is the first genetic characterization of *Cryptosporidium* in human patients in Jordan and the first report of *C. canis* and *C. meleagridis* in Jordan. The immune status of the patients was not known. The patient infected with *C. canis* was critically ill in hospital and was suffering from additional parasitic (*Giardia*) and bacterial (*Shigella*) infections.

Of the 44 isolates that were typed, 50% were *C. parvum* and ~45% were *C. hominis*. Previous studies in humans in the Middle East have also reported that *C. parvum* is the predominant *Cryptosporidium* species. For example, in Iran, 15 microscopy positive human fecal samples analysed using molecular tools identified 11/15 as *C. parvum* and 4/15 as *C. hominis* (Meamar et al., 2007). In Kuwait, 94% (58/62) of children screened had *C. parvum*, 5% (3/62) had *C. hominis*, and 1% (1/62) had both *C. parvum* and *C. hominis* (Sulaiman et al., 2005). Another study in Shahriar (a suburb of Tehran, Iran), identified 18/24 isolates as *C. parvum* and the

remaining 6 isolates as *C. hominis* from patients with diarrhea (Pirestani et al., 2008). However, another study which examined *Cryptosporidium* samples from HIV patients in Iran identified *C. hominis* in 15/21 and *C. parvum* in 6/21 (Zavvar et al., 2008). In Turkey, *C. parvum* was identified in four samples from school children (Tamer et al., 2007). In Saudi Arabia, *C. parvum* was identified in 15/35 (42.9%) *Cryptosporidium*-positive samples collected from children in Jeddah city, *C. hominis* in 13/35 (37%), *C. meleagridis* in one sample and *C. muris* in one sample (Al-Brikan et al., 2008). One isolate was a mixed infection of *C. parvum* and *C. hominis* (Al-Brikan et al., 2008).

The GP60 gene is highly polymorphic and within *C. hominis, C. parvum*, and *C. meleagridis*, several subtype families have been identified; seven subtype families in *C. hominis* (Ia–Ig), 2 zoonotic (IIa, IId) and 10 non-zoonotic (IIb, IIc, IIe–IIl) subtype families in *C. parvum*, and six subtype families in *C. meleagridis* (IIIa–IIIf) (Plutzer and Karanis, 2009; Xiao, 2010). In the present study, sequence analysis of the GP60 locus identified three *C. parvum* and two *C. hominis* subtype families. The *C. parvum* subtype IIaA15G1R1 was identified in two children. This subtype was also identified in two children in Kuwait (Sulaiman et al., 2005). Subtype IIaA20G3R1 was identified in one child. This subtype has been identified in humans and cattle in Australia and in a drinking water outbreak in Northern Ireland (Glaberman et al., 2002; Chalmers et al., 2005; Ng et al., 2008; Waldron et al., 2009).

Four different subtypes (IIdA14G1, IIdA20G1, IId A24G1 and IIdA29G1) of the less common *C. parvum* subtype family IId were identified. The IId subtype has previously been reported in humans and cattle in Portugal (Alves et al., 2003, 2006), cattle in Hungary (Plutzer and Karanis, 2007), lambs and goats in Spain (Quílez et al., 2008) and humans in Ireland, Kuwait, The Netherlands and Australia (Sulaiman et al., 2005; Wielinga et al., 2008; Waldron et al., 2009; Zintl et al., 2009). Interestingly, *C. parvum* IId subtypes have never been found in humans or calves in the United States and Canada (Xiao, 2010). Two of the subtypes identified in the present study (IIdA14G1 and IIdA24G1) have previously been reported in lambs in Spain (Quílez et al., 2008). The IIdA20G1 subtype identified in the present study has previously been identified at a high prevalence in children in Kuwait (Sulaiman et al., 2005). In that study, the other IId subtype identified was IIdA18G1. Subtype IIdA24G1 has previously been reported in one human in Australia (Waldron et al., 2009). Subtype IIdA29G1 however appears to be new IId subtype indicating unique and possibly anthroponotic transmission of *C. parvum* in Jordan.

The identification of the *C. parvum* subtype IIcA5G3 in two isolates provides further evidence for anthroponotic transmission of *C. parvum* in Jordan. The IIc subtype family has been found in humans in Portugal, Slovenia, The Netherlands, Australia, Japan (Alves et al., 2006; Jex et al., 2008; O'Brien et al., 2008; Soba and Logar, 2008; Waldron et al., 2009), but has not been identified in animals. IIc subtypes are also commonly found in humans in developing countries (Xiao, 2010). There are four major types of IIc sequences, which differ from each other significantly in the 3′ region of the gene (Xiao, 2010). The subtype seen in the present study was IIcA5G3a. This subtype was also identified in children in Kuwait (Sulaiman et al., 2005).

In the present study, four *C. hominis* 1b subtypes were identified; IbA6G3, IbA9G3, IbA10G2 and IbA20G2. The clinical significance of the various *C. hominis* subtypes in humans is not yet clear. However a recent study reported that the Ib subtype family appeared to be much more virulent than other subtype families and was significantly associated with diarrhea, nausea, vomiting and general malaise (Cama et al., 2008). Worldwide, IbA9G3 and IbA10G2 are the two common subtypes within the Ib subtype family. IbA9G3 is commonly seen in humans in Malawi, Kenya, India, and Australia, and IbA10G2 is commonly seen in South Africa, Botswana, Jamaica, Peru, USA, Canada, Australia, and European countries (Glaberman et al., 2002; Leav et al., 2002; Alves et al., 2003, 2006; Chalmers et al., 2005; Gatei et al., 2006, 2007). In addition, IbA10G2 is responsible for more than half of the waterborne outbreaks of gasterenteritis in the United States, United Kingdom, Canada and France (Xiao, 2010). One of the stool samples collected from four children from King Abdulla Hospital during the *Cryptosporidium* outbreak in Jordan was typed as IbA10G2. Subtype IbA20G2 is a rare subtype and has only been reported in wastewater samples in Shanghai (Feng et al., 2009). Subtype IbA6G3 is also a rare subtype and has only been reported previously in one human in Australia (Waldron et al., 2009).

In the present study, two Id subtypes were identified; subtype IdA21 in one child and IdA24 in five children. The *C. hominis* subtype family Id has been commonly reported in Australia, Canada, Kenya, Malawi, India, Peru, South Africa, and the USA (Strong et al., 2000; Peng et al., 2001; Leav et al., 2002; Alves et al., 2006; O'Brien et al., 2008; Ong et al., 2008; Xiao, 2010). In the present study, subtype IdA21 identified in one child, is a relatively rare subtype and has previously been reported in China and South Africa (Leav et al., 2002; Accession No. EF591785, EF591786, FJ707316). Subtype IdA24 has previously been identified in the US, in a waterborne outbreak of gastroenteritis in south Burgundy, France and in children in Kenya (Strong et al., 2000; Cohen et al., 2006; Gatei et al., 2006).

Results of our study show that anthroponotic transmission is important in the epidemiology of cryptosporidiosis in Jordan. This conclusion is based on our finding of *C. hominis* and anthroponotic *C. parvum* (IIc subtype family) as well as rare and novel IId subtypes that have not been reported in animals in 79% (23/29) of isolates subtyped. In addition, the identification of rare and novel *C. hominis* and *C. parvum* subtypes in children in Jordan suggest unique endemicity of cryptosporidiosis in Jordanian children. The majority of cases were associated with clinical symptoms but unfortunately, insufficient epidemiological data were collected to elucidate the source of *Cryptosporidium* infections and reasons responsible for the unique endemicity. A well-designed case control study, with detailed collection of data on water and food sources and animal contact, would be helpful in identifying risk factors involved in the acquisition of *Cryptosporidium* infection and in answering some of the above questions. Sampling of water and domestic animals would also be useful. In particular, sub-typing *C. parvum* from domestic animals in Jordan is needed in the determination of the extent of zoonotic transmission of cryptosporidiosis in Jordanian children.

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