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Research paper

Genetic characterization of *Cryptosporidium* in animal and human isolates from Jordan

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ABSTRACT

Little is known about the epidemiology of *Cryptosporidium* in Jordan and to date, only one genotyping study has been conducted on *Cryptosporidium* isolates from Jordanian children. In the present study, a total of 284 faecal samples from Jordanian cattle, sheep, goats and chicken and 48 human faecal samples were screened for the presence of *Cryptosporidium* using an 18S quantitative PCR (qPCR) and a *C. parvum/C. hominis* specific qPCR at a lectin locus. Of these, 37 of 284 animal faecal samples were positive by qPCR at the 18S locus giving an overall prevalence of 11.6%. The point prevalence of *Cryptosporidium* in chickens, sheep, horses, cattle and goats ranged from 4.8% (chickens) to 18.7% (cattle). A total of six species were detected; *C. xiaoi* (n = 9), *C. andersoni* (n = 7), *C. ryanae* (n = 5), *C. parvum* (n = 4), *C. baileyi* (n = 1) and a genetically distinct and potentially novel species in two isolates from horses. Sub-genotype analysis of the 4 *C. parvum* isolates at the 60-kDa glycoprotein (gp60) locus identified subtype IIaA19G2R1 (n = 2) and IIaA16GR1 (n = 2). For the human samples, 4 positives (8.3% prevalence) were detected. Of these, two were *C. parvum* (subtypes IIdA20G1 and IIaA15G2R1) and two were *C. hominis* (subtypes 1bA9G3 and 1bA10G2R2). Further studies are required to better understand the epidemiology 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 (Zahedi et al., 2016). Human cryptosporidiosis is frequently accompanied by abdominal pain, fever, vomiting, malabsorption and diarrhoea that may sometimes be profuse and prolonged (Chalmers and Davies, 2010). Little is known about the epidemiology of *Cryptosporidium* in Jordan and to date, only one genotyping study has been conducted on *Cryptosporidium* isolates from Jordanian children (Hijjawi et al., 2010). In that study, four *Cryptosporidium* species; *C. parvum* (n = 22), *C. hominis* (n = 20), *C. meleagridis* (n = 1) and *C. canis* (n = 1) were identified in 44 *Cryptosporidium*-positive faecal samples from children at the Princess Rahma Teaching Hospital in Irbid (Hijjawi et al., 2010). Sub-genotype analysis of 29 isolates at the 60-kDa glycoprotein (gp60) locus identified several rare and novel subtypes indicating unique endemicity and transmission of *Cryptosporidium* in Jordan (Hijjawi et al., 2010). The aim of the present study therefore, was to follow on from the previous genotyping study and determine the prevalence, species and subtypes of *Cryptosporidium* in animals and humans from different regions of Jordan over different seasons, to better understand the transmission dynamics and distribution of the parasite in Jordan.

2. Materials and methods

2.1. Cryptosporidium isolates

A total of 284 faecal samples were collected from farmed chickens, sheep, horses, cattle and goats, more than one year old, with no clinical signs of diarrhoea. Animal faecal samples were collected from regions of Jordan (Irbid, Amman, Mafraq and Jordan Valley) over three seasons; autumn, winter and spring from October 2014 to May 2015 (Table 1). Animals were sampled either directly from rectum when possible or from freshly deposited faeces on the ground, using procedures approved by the Animal Ethics Committee at Jordan University of Sciences and Technology. Cattle and

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Table 1
Prevalence of *Cryptosporidium* in animal and human hosts in Jordan.

Host	Number screened	Number positive	Prevalence% (95% CI)	No. collected in Autumn (prevalence)	No. collected in Winter (prevalence)	No. collected in Spring (prevalence)
Chickens	21	1	4.8 (0.0–13.9)	7 (14.3%)	14 (0%)	0 (0%)
Sheep	63	10	15.9 (6.8–24.9)	19 (15.8%)	20 (10%)	24 (20.8%)
Horses	74	6	8.1 (1.9–14.3)	25 (12.0%)	25 (12.0%)	24 (0%)
Cattle	75	14	18.7 (9.8–27.5)	26 (26.9%)	25 (16.0%)	24 (12.5%)
Goats	51	2	3.9 (0.0–9.2)	30 (6.7%)	21 (0%)	0 (0%)
Humans	48	4	8.3 (0.5–16.2)	–	–	–
Totals	**332**	**37**				

horses were mostly kept in barns. Sheep and goats were housed at night-time, but were allowed on pastures during the day.

A total of 48 human faecal samples were collected from clinical laboratories at two hospitals; Rahma hospital in Irbid (Northern Jordan) and from a hospital in Amman (central Jordan), from November 2014 to March 2015. All were from hospitalised patients with history of diarrhoea, abdominal pain and gastroenteritis, that were admitted for stool analysis. The age of the humans sampled ranged from 10 months to 56 years. All patients were immunocompetent and did not suffer from any underlying diseases. Information about contact with animals was not recorded. Human samples were collected under human ethics permit number 1401254/32.

2.2. Molecular typing

Total DNA was extracted using a QIAamp Fast DNA stool kit (Qiagen, Germany) with five freeze thaw cycles prior to DNA extraction. All samples were screened using an 18S qPCR as previously described (Yang et al., 2014) and a *C. parvum* and *C. hominis* specific qPCR at a unique *Cryptosporidium* specific gene (Clec) coding for a novel mucin-like glycoprotein that contains a C-type lectin domain (CTLD) previously described (Morgan et al., 1996; Bhalchandra et al., 2013; Yang et al., 2009; 2013). This was done to determine if there were any mixed infections with *C. parvum* and/or *C. hominis* in the samples. Isolates were further genotyped using a two-step nested PCR and sequencing of a fragment of the 18S rRNA locus (Xiao et al., 1999). Positive samples were subtyped at the 60-kDa glycoprotein (gp60) locus using a two-step nested PCR that amplifies a ~830 bp fragment (Strong et al., 2000; Sulaiman et al., 2005). The amplified DNA from secondary PCR products were separated by gel electrophoresis and purified for sequencing using an in house filter tip method (Yang et al., 2013). Amplicons were sequenced in both directions using an ABI Prism™ Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions. Where possible, sequences were obtained for two separate amplicons from each positive isolate at both loci. Nucleotide sequences were analysed using Finch TV Version 1.4.0 (Geospiza, 2016Geospiza, Inc.; Seattle, WA, USA). Prevalences were expressed as the percentage of samples positive by PCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000).

3. Results

3.1. Cryptosporidium species

A total of 33 of 284 animal faecal samples were positive by qPCR at the 18S locus giving an overall prevalence of 11.6% (7.9-15.3% 95% CI). Prevalence in the various hosts ranged from 4.8% (chickens) to 18.7% (cattle) (Table 1). The overall prevalence in all hosts over different seasons was 15% (8.2-21.7% 95CI) in autumn, 8.6% (3.2-13.9% 95CI) in winter and 11.1% (3.9-28.4% 95CI) in spring. There were no statistical differences in the prevalence in different hosts over different seasons. The prevalence in the human samples was 8.3% (0.5-16.2 95% CI) (4/48). The ages of humans positive for *Cryptosporidium* ranged from 10 months to 56 years. Three of the human positives came from individuals who lived in Irbid in Northern Jordan, which is regarded as a semi-rural area, with many villages near the city and where contact with livestock is frequent.

Table 2
Cryptosporidium species and subtypes identified in qPCR positives from animal and human isolates from Jordan.

Host	Isolate Code	Sampling season	Lectin qPCR	18S	*gp60*
Chicken	A266	Autumn	–	*C. baileyi*	–
Sheep	A123	Autumn	–	*C. xiaoi*	–
Sheep	A128	Autumn	*C. parvum*	*C. parvum*	IIaA19G2R1
Sheep	A131	Autumn	–	*C. xiaoi*	–
Sheep	W244	Winter	–	*C. xiaoi*	–
Sheep	W245	Winter	–	*C. andersoni*	–
Sheep	SP130	Spring	–	*C. xiaoi*	–
Sheep	SP131	Spring	*C. parvum*	*C. parvum*	IIaA19G2R1
Sheep	SP132	Spring	–	NS	–
Sheep	SP134	Spring	–	*C. xiaoi*	–
Sheep	SP138	Spring	*C. parvum*	*C. parvum*	IIaA16G1R1
Horse	A48	Autumn	–	Novel	–
Horse	A52	Autumn	–	Novel	–
Horse	A59	Autumn	–	*C. parvum*	–
Horse	W124	Winter	–	NS	–
Horse	W126	Winter	–	NS	–
Horse	W138	Winter	–	NS	–
Cattle	A160	Autumn	–	*C. andersoni*	–
Cattle	A161	Autumn	–	*C. andersoni*	–
Cattle	A170	Autumn	–	*C. andersoni*	–
Cattle	A172	Autumn	–	*C. andersoni*	–
Cattle	A173	Autumn	–	*C. xiaoi*	–
Cattle	A179	Autumn	–	*C. ryanae*	–
Cattle	A180	Autumn	–	*C. ryanae*	–
Cattle	W01	Winter	–	*C. xiaoi*	–
Cattle	W05	Winter	–	*C. ryanae*	–
Cattle	W07	Winter	–	*C. ryanae*	–
Cattle	W19	Winter	*C. parvum*	*C. parvum*	IIaA16G1R1
Cattle	SP60	Spring	–	*C. andersoni*	–
Cattle	SP63	Spring	–	*C. ryanae*	–
Cattle	SP79	Spring	–	*C. andersoni*	–
Goat	A240	Autumn	–	*C. xiaoi*	–
Goat	A250	Autumn	–	*C. xiaoi*	–
Human	H15	–	*C. parvum*	*C. parvum*	IIdA20G1
Human	H27	–	*C. hominis*	*C. hominis*	1bA9G3
Human	H39	–	*C. parvum*	*C. parvum*	IIaA15G2R1
Human	H40	–	*C. hominis*	*C. hominis*	IbA10G2R2

NS = positive by PCR but no sequence obtained.

Of the 33 qPCR positives from animals at the 18S locus, 18S sequences were obtained for 29 positives. The following species were identified; *C. xiaoi* (n = 9), *C. andersoni* (n = 7), *C. ryanae* (n = 5), *C. parvum* (n = 5), *C. baileyi* (n = 1) and a novel species (n = 2) (Table 2). In sheep, of the 9 positives that were typed, *C. xiaoi* was the most common species (5/9), followed by *C. parvum* (3/9) and *C. andersoni* (1/9). In cattle, *C. andersoni* was the most common species detected (6/14), followed by *C. ryanae* (5/14), *C. xiaoi* (2/14) and *C. parvum* (1/14). Two positives from goats were typed as *C. xiaoi*. *C. baileyi* was identified in the one positive from a chicken. *C. parvum* was identified in one horse isolate and novel sequences were iden-