



## Research paper

Genetic characterization of *Cryptosporidium* in animal and human isolates from JordanNawal Hijjawi<sup>a,1</sup>, Rami Mukbel<sup>b,\*,1</sup>, Rongchang Yang<sup>c</sup>, Una Ryan<sup>c,\*</sup><sup>a</sup> Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, The Hashemite University, PO Box 150459, Zarqa, 13115, Jordan<sup>b</sup> Department of Basic Veterinary Medical Sciences, Faculty of Veterinary Medicine, Jordan University of Science and Technology, Irbid, Jordan<sup>c</sup> Division of Veterinary and Life Sciences, Murdoch University, Murdoch, WA, 6150, Australia

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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 IIaA20G1 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.

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## 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	<b>332</b>	<b>37</b>				

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, 2016; Geospiza, 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% 95% CI) in autumn, 8.6% (3.2–13.9% 95% CI) in winter and 11.1% (3.9–28.4% 95% CI) in spring. There were no statistical differences in the prevalence in different hosts

**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	–	<i>C. baileyi</i>	–
Sheep	A123	Autumn	–	<i>C. xiaoi</i>	–
Sheep	A128	Autumn	<i>C. parvum</i>	<i>C. parvum</i>	IlaA19G2R1
Sheep	A131	Autumn	–	<i>C. xiaoi</i>	–
Sheep	W244	Winter	–	<i>C. xiaoi</i>	–
Sheep	W245	Winter	–	<i>C. andersoni</i>	–
Sheep	SP130	Spring	–	<i>C. xiaoi</i>	–
Sheep	SP131	Spring	<i>C. parvum</i>	<i>C. parvum</i>	IlaA19G2R1
Sheep	SP132	Spring	–	NS	–
Sheep	SP134	Spring	–	<i>C. xiaoi</i>	–
Sheep	SP138	Spring	<i>C. parvum</i>	<i>C. parvum</i>	IlaA16G1R1
Horse	A48	Autumn	–	Novel	–
Horse	A52	Autumn	–	Novel	–
Horse	A59	Autumn	–	<i>C. parvum</i>	–
Horse	W124	Winter	–	NS	–
Horse	W126	Winter	–	NS	–
Horse	W138	Winter	–	NS	–
Cattle	A160	Autumn	–	<i>C. andersoni</i>	–
Cattle	A161	Autumn	–	<i>C. andersoni</i>	–
Cattle	A170	Autumn	–	<i>C. andersoni</i>	–
Cattle	A172	Autumn	–	<i>C. andersoni</i>	–
Cattle	A173	Autumn	–	<i>C. xiaoi</i>	–
Cattle	A179	Autumn	–	<i>C. ryanae</i>	–
Cattle	A180	Autumn	–	<i>C. ryanae</i>	–
Cattle	W01	Winter	–	<i>C. xiaoi</i>	–
Cattle	W05	Winter	–	<i>C. ryanae</i>	–
Cattle	W07	Winter	–	<i>C. ryanae</i>	–
Cattle	W19	Winter	<i>C. parvum</i>	<i>C. parvum</i>	IlaA16G1R1
Cattle	SP60	Spring	–	<i>C. andersoni</i>	–
Cattle	SP63	Spring	–	<i>C. ryanae</i>	–
Cattle	SP79	Spring	–	<i>C. andersoni</i>	–
Goat	A240	Autumn	–	<i>C. xiaoi</i>	–
Goat	A250	Autumn	–	<i>C. xiaoi</i>	–
Human	H15	–	<i>C. parvum</i>	<i>C. parvum</i>	IIdA20G1
Human	H27	–	<i>C. hominis</i>	<i>C. hominis</i>	1bA9G3
Human	H39	–	<i>C. parvum</i>	<i>C. parvum</i>	IlaA15G2R1
Human	H40	–	<i>C. hominis</i>	<i>C. hominis</i>	IbA10G2R2

NS = positive by PCR but no sequence obtained.

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.

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-

tified in the remaining two positives from horses (A48 and A52) (Table 2). These two sequences were identical to each other and exhibited 22 single nucleotide polymorphisms (SNP's) and only 92% similarity to a novel isolate from a calf from Turkey (JN245625). The 18S sequence for the novel genotype has been submitted to GenBank under accession number KX685592. Analysis of the four human positives identified *C. parvum* (n=2) and *C. hominis* (n=2). The *C. parvum*/*C. hominis* specific qPCR correctly identified the *C. parvum* and *C. hominis* samples identified by 18S sequencing but no mixed infections were detected (Table 1).

### 3.2. gp60 sub-typing

Of the 5C. *parvum* positives from animals, sub-type analysis at the gp60 locus was successful for 4 isolates (3 from sheep and 1 from cattle) and subtypes IlaA19G2R1 (n=2) and IlaA16GR1 (n=2) were identified (Table 2). Analysis of the four human positives identified *C. parvum* subtypes IIdA20G1 and IlaA15G2R1 and *C. hominis* subtypes 1bA9G3 and 1bA10G2R2 (Table 2).

## 4. Discussion

In the present study, using qPCR the overall prevalence of *Cryptosporidium* in various animals was 11.6% (33/284) and in humans was 8.3% (4/48). Previous studies based on microscopy or direct immunofluorescence in Jordan, have reported a prevalence of 1.5% to 37.3% in human patients (Youssef et al., 2000; Nimri, 2003; Abo-Shehada et al., 2004; Mahgoub et al., 2004; Areeshi et al., 2007). In the only previous molecular analysis conducted on Jordanian human faecal samples, the prevalence was 1.8% (28/1585) by microscopy and >19% by qPCR at the lectin locus (Hijjawi et al., 2010). Previous studies mainly using microscopy, in neighboring countries to Jordan, have reported *Cryptosporidium* prevalence in humans ranging from 1 to 32% in Saudi Arabia, 1.4–1.6 in Kuwait, 5–9.7% in Iraq and 6% by microscopy and 11% by PCR in Lebanon (cf. Areeshi et al., 2007; Hawash et al., 2014; Osman et al., 2015).

This is the first study to determine the prevalence and genotypes of *Cryptosporidium* in animal hosts in Jordan and in this preliminary study on a relatively small number of samples, five different *Cryptosporidium* species were detected; *C. parvum*, *C. xiaoi*, *C. andersoni*, *C. ryanae*, *C. baileyi* and a potentially novel species in horses. Previous studies in Middle Eastern countries identified a prevalence of 37.5% in calves, 21% in goat kids and 12.5% in lambs (12.5%) in Kuwait using microscopy and immunological methods (Majeed and Alazemi, 2014), 0.3% in chickens in Iran using microscopy and PCR (Hamidinejat et al., 2014) and 11.3% (198/1749) in sheep in Iran using microscopy (Gharekhanian et al., 2014). Amongst sheep, *C. xiaoi* and *C. parvum* accounted for 88.8% (55.5% and 33.3% respectively) of isolates typed. *C. xiaoi* is commonly reported in older lambs and sheep and is often apparently asymptomatic (Robertson et al., 2014). To date there have been no reports of *C. xiaoi* in humans. *C. parvum* is the most commonly reported zoonotic species of *Cryptosporidium* infecting humans. Two subtypes (IlaA19G2R1 and IlaA16G1R1) belonging to the Ila subtype family were identified in sheep. The Ila subtype family has a worldwide distribution in mammals including humans, with cattle and sheep commonly infected with this subtype family (Xiao, 2010). However, the IlaA19G2R1 subtype is a relatively rare subtype and has previously been described by Xiao et al. (2007), Wielinga et al. (2008), Ng et al. (2012), Rieux et al. (2013) and Couto et al. (2014) infecting cattle in the North America, The Netherlands, Australia, France and Brazil, respectively and by Nolan et al. (2013) in deer in Australia. To the best of our knowledge, this is the first report of this subtype in sheep. Subtype IlaA16G1R1 has a wide geographic distribution (Xiao, 2010; Robertson et al., 2014) and has

recently been detected in calves and humans in Sweden (Insulander et al., 2013; Silverlås et al., 2010, 2013), in humans in Canada (Iqbal et al., 2015), Australia (Koehler et al., 2014), Estonia (Lassen et al., 2014) and Mexico (Valenzuela et al., 2014), dairy cattle in Argentina (Del Coco et al., 2014) and sheep and cattle in Romania (Imre et al., 2011; Imre et al., 2013). This subtype of *C. parvum* also caused an outbreak in Sweden through direct contact between calves and humans (Kinross et al., 2015).

In cattle, *C. andersoni* and *C. parvum* accounted for 50% of positives typed (42.8% and 7.2% respectively). *C. andersoni* is a gastric parasite which has been associated with reduced milk yield in dairy cattle and decreased weight gain in post weaned calves (Olson et al., 2004) and is occasionally detected in humans (Leoni et al., 2006; Morse et al., 2007; Waldron et al., 2009; Agholi et al., 2013; Jiang et al., 2014; Liu et al., 2014). Two studies in China by the same research group have reported that *C. andersoni* was the most prevalent *Cryptosporidium* species detected in humans (Jiang et al., 2014; Liu et al., 2014). However, further research is required to better understand the zoonotic importance of *C. andersoni* and it has not been identified in humans in Jordan in the one previous genotyping study (Hijjawi et al., 2010). *C. andersoni* is also frequently the dominant species in source and tap water in China and the UK (Feng et al., 2011; Nichols et al., 2010), suggesting that cattle may be the primary source of contamination. The *C. parvum* subtype identified in cattle (IlaA16G1R1) is a common subtype as discussed above. *C. ryanae* and *C. xiaoi* accounted for the remaining positives typed from cattle and neither are considered zoonotic. While *C. ryanae* is common (Xiao, 2010), *C. xiaoi* is not commonly reported in cattle but has previously been reported in yaks (*Bos grunniens*) in China (Ma et al., 2014). The sequences identified in cattle in the present study, were 100% identical over 822 bp to a *C. xiaoi* isolate from a goat (KM199750). As *C. xiaoi* is very common in sheep, the cattle may have acquired *C. xiaoi* from sheep, however there was no direct contact between the sheep and cattle in the present study, but it is possible that the cattle acquired *C. xiaoi* through contaminated water. Using samples collected from older cattle in the present study may have been a contributing factor to the low prevalence of *C. parvum*, as its prevalence decreases with increasing age of calves (Santín et al., 2004). A recent study in cattle in Iran identified *C. parvum* only in calves <six months (Mahami Oskouei et al., 2014), while another study, identified *C. andersoni* (based on microscopy) in cattle <1 year old in Iran (Mirzai et al., 2014). *Cryptosporidium baileyi* was identified in one chicken and has been identified in commercially reared poultry in Iran (Hamidinejat et al., 2014). In two horses, *C. parvum* and a genetically distinct and potentially novel species was identified, reflecting the unique endemicity of cryptosporidiosis in Jordan. A variety of *Cryptosporidium* species and genotypes have been reported in horses including *C. parvum*, *Cryptosporidium* horse genotype, *C. erinacei*, *C. muris*, *C. hominis*, *C. tyzzeri*, *C. felis*, *C. ubiquitum* and *C. andersoni* (Grinberg et al., 2003, 2009; Xiao, 2010; Guo et al., 2014; Liu et al., 2015; Qi et al., 2015; Jian et al., 2016; Wagnerová et al., 2016). In the present study, only 293 bp of clean sequence for the 18S locus was obtained and therefore further studies on a larger number of isolates at multiple loci are required to fully characterize the novel species from horses.

In humans, *C. parvum* (IIdA20G1 and IlaA15G2R1) and *C. hominis* (1bA9G3 and 1bA10G2R2) subtypes were detected. The *C. parvum* IIdA20G1 subtype has previously been reported in children in Jordan (Hijjawi et al., 2010) and has also been identified at a high prevalence in children in Kuwait (Sulaiman et al., 2005). The IlaA15G2R1 subtype is the most dominant *C. parvum* subtype infecting dairy cattle and humans in industrialised nations (Feng et al., 2013; Robertson et al., 2014; Ramo et al., 2015; Valenzuela et al., 2014). The *C. hominis* subtype 1bA9G3, is a common subtype and has also previously been reported in children in Jordan (Hijjawi et al., 2010). The 1bA10G2R2 subtype is common in humans

(Fuentes et al., 2015) and has been responsible for waterborne outbreaks of cryptosporidiosis (Glaberman et al., 2002; Cohen et al., 2006). This *C. hominis* subtype also infects cattle (Smith et al., 2005; Abeywardena et al., 2012) and the identification in a human patient in the present study, suggests possible zoonotic transmission. Based on its prevalence in humans and its association with waterborne outbreaks and transmission from animals to humans, it has been proposed that Iba10G2R2 merits attention as the most significant *C. hominis* gp60 subtype linked to human cryptosporidiosis globally with zoonotic potential (Jex and Gasser, 2010).

Previous studies in humans in the Middle East, have reported that *C. parvum* is the predominant *Cryptosporidium* species in humans (Sulaiman et al., 2005; Meamar et al., 2007; Al-Brikan et al., 2008; Pirestani et al., 2008; Hijawi et al., 2010; Nazemalhosseini-Mojarad et al., 2011; Taghipour et al., 2011; Sharbatkhori et al., 2015), however two studies reported that *C. hominis* was more prevalent than *C. parvum* (Ghaffari and Kalantari, 2014; Osman et al., 2015). Few studies have conducted subtyping, but studies in children from urban areas in Iran have reported a predominance of zoonotic *C. parvum* subtype families (IIa, IId) (Sulaiman et al., 2005; Sharbatkhori et al., 2015; Nazemalhosseini-Mojarad et al., 2011; Taghipour et al., 2011). Analysis of *C. parvum* and *C. hominis* isolates from river-water in Iran, showed that all belonged to the IId and Id subtype families, respectively (Mahmoudi et al., 2015). Analysis of *Cryptosporidium* in symptomatic hospitalized patients in Lebanon, identified the rare *C. hominis* subtype IIdA19 was predominant amongst the *C. hominis* isolates (Osman et al., 2015). In the previous study in Jordan, *C. hominis* accounted for ~45% of positives (Hijawi et al., 2010) and in the present study, although only four human isolates were typed, *C. hominis* accounted for 50% of positives, indicating that anthroponotic transmission is also important in the epidemiology of cryptosporidiosis in Jordan.

Future studies on larger numbers of cattle and sheep <6 months of age, and a larger study of human isolates, combined with clinical data are required to better understand the economic effects of *Cryptosporidium* and public health implications in Jordan.

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