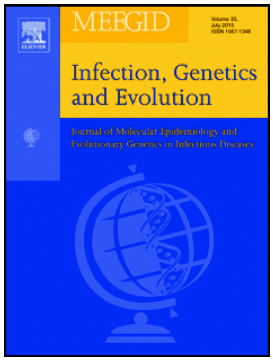

Prevalence of Cryptosporidium species and subtypes in paediatric oncology and non-oncology patients with diarrhoea in Jordan

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Prevalence of *Cryptosporidium* species and subtypes in paediatric oncology and non-oncology patients with diarrhoea in Jordan

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Abstract

Cryptosporidiosis is a protozoan parasitic disease which affects human and animals worldwide. In adult immunocompetent individuals, cryptosporidiosis usually results in acute and self-limited diarrhea; however, it can cause life threatening diarrhea in children and immunocompromised individuals. In the present study, we compared the prevalence of *Cryptosporidium* species and *gp60* subtypes among paediatric oncology patients with diarrhoea (n=160) from King Hussein Medical Centre for Cancer in Jordan, and non-oncology paediatric patients with diarrhea (n=137) from Al-Mafraq paediatric hospital. Microscopy results using modified acid fast staining identified a significantly (p=<0.05) higher prevalence of *Cryptosporidium* in paediatric oncology patients with diarrhoea (14.4% - 23/160), compared to non-oncology paediatric patients with diarrhea only (5.1% - 7/137). With the exception of one sample, all microscopy-positive samples (n=29) and an additional 3/30 microscopy-negative controls were typed to species and subtype level at the 18S and *gp60* loci, respectively. All *Cryptosporidium* positives were typed as *C. parvum*. Of the 22 typed *Cryptosporidium* positives from the paediatric oncology patients, 21 were subtyped as IIaA17G2R1 and one as IIaA16G2R1 *C. parvum* subtypes. The 7 typed positives from the paediatric patients from Al-Mafraq hospital were subtyped as IIaA17G2R1 (n=5) and IIaA16G2R1 (n=2). The 3 additional positives from the 30 microscopy negative control samples were subtyped as IIaA17G2R1. The high prevalence of the IIaA17G2R1 subtype, particularly amongst oncology patients, suggests that an outbreak of cryptosporidiosis may have been occurring in oncology patients during the collection period (April to December, 2016). New therapies for cryptosporidiosis in immunocompromised patients are urgently required.

Keywords: Cryptosporidium; oncology; diarrhea; *C. parvum*; *gp60*; immunocompromised.

1. Introduction

Cryptosporidium species are intracellular protozoan parasites that infect a wide range of hosts including humans, domestic and wild animals (Ryan et al., 2014; Zahedi et al., 2016). World-wide, human cryptosporidiosis is mainly caused by two species of *Cryptosporidium* (*C. hominis* and *C. parvum*); although numerous species of *Cryptosporidium* have been reported in humans, including *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum*, *C. viatorum*, *C. suis*, *C. scrofarom*, *C. viatorum*, *C. tyzerri*, *C. xiaoi*, *C. fayeri*, *C. muris*, and *C. andersoni* (Xiao, 2010; Ryan et al., 2016).

Cryptosporidium infection can result in acute diarrhea, nausea, vomiting and weight loss, which is usually self-limiting (Ryan et al., 2016). In infants, cryptosporidiosis can be more serious and can lead to malnutrition, growth retardation and impairment in cognitive function (Shrivastava et al., 2017). Similarly, patients with some type of immunocompromised condition have an increased probability of acquiring cryptosporidiosis, which can manifest as severe protracted diarrhoea, chronic malabsorption, failure to thrive, malnutrition and increased mortality (Assefa et al., 2009; Idris et al., 2010; Domenech et al., 2011; Kurniawan et al., 2013; Marcos and Gotuzzo, 2013; Valenzuela et al., 2014; Nsagha et al., 2016).

Immunosuppression and diarrhoea are well-recognised side-effects of cancer treatment, yet relatively few studies have been conducted examining the prevalence of *Cryptosporidium* in cancer patients (Botero et al., 2003; Tamer et al., 2008; Al-Qobati et al., 2012; Hassanein et al., 2012; Sulżyc-Bielicka et al., 2012; García-Elorriaga et al., 2013). In Jordan, the molecular epidemiology of cryptosporidiosis is poorly understood and to date, only two genotyping studies have been conducted (Hijjawi et al., 2010; Hijjawi et al., 2016). The aim of the present study was to investigate the prevalence of *Cryptosporidium* species and subtypes in paediatric oncology patients with diarrhoea and paediatric patients with diarrhoea only, to better understand the epidemiology

and management of cryptosporidiosis in these patients.

2. Materials and methods

2.1. Cryptosporidium isolates

A total of 297 fresh diarrheic stool specimens were collected from two groups of children (aged 1-5 years old) from April until December, 2016; including paediatric oncology patients with diarrhoea at the King Hussein Medical Centre for Cancer (n=160) and paediatric patients with diarrhoea only at the Al-Mafraq paediatric hospital (n=137). The samples from King Hussein Medical Centre for Cancer were collected from children undergoing chemotherapy for different types of cancer (mainly leukemia) and who resided in different parts of Jordan but regularly visited the centre for treatment. The children who were referred to the Al-Mafraq paediatric hospital, were mainly from Al-Mafraq city and nearby villages. In addition, a further 30 samples were collected from the same two hospitals (18 samples from the King Hussein Medical Centre for Cancer and 12 from Al-Mafraq hospital), from children with diarrhoea, but which were negative by microscopy for *Cryptosporidium* oocysts, in order to serve as a negative control to compare the sensitivity of microscopy and PCR.

Ethical approval to conduct the study was obtained from the King Hussein Medical Centre for Cancer and Al-Mafraq paediatric hospital and issued by the Institutional Review Board at Hashemite University (Ethics permit number 150/1313/18). A signed consent form was obtained from parents or guardians of each child, who voluntarily participated after a clear explanation of the research objectives. Demographic data regarding age, gender, residency, medical history and duration of symptoms were obtained via questionnaire from the guardian of each child (Ethics permit number 150/1313/18).

2.2. Microscopy, DNA extraction and typing

Microscopy was performed on all faecal samples using a routine modified acid fast staining procedure. Briefly, a smear was prepared using 1 to 2 drops of the collected stool specimen, fixed with absolute methanol for 30 seconds, stained with carbol fuchsin for 3 minutes, rinsed briefly with tap water before being stained with methylene blue. The stained dried slides were labelled and examined at 100x oil immersion lens under a light microscope (Olympus CH40/RF200, Japan) for the presence of *Cryptosporidium* oocysts.

For subsequent DNA extraction, 1 to 2 gm of the individually collected fresh stool specimen from each child were fixed in 2.5% potassium dichromate and left at room temperature for 2-6 months. Before DNA extraction, the stool samples were washed three times in 10% PBS buffer and centrifuged at 2000 x g for 2-3 minutes in order to remove the potassium dichromate. Total DNA was extracted using a QIAmp DNA Stool Kit (Qiagen, Germany), following the recommendation of the supplier after 5 cycles of freezing and thawing. The extracted DNA was stored at -80ºC until further molecular characterization. All samples which were positive for *Cryptosporidium* by microscopy (n=30) and the 30 samples that were *Cryptosporidium* negative by microscopy (negative controls) were initially screened at the 18S locus and identified to species level using nested PCR amplification and Sanger sequencing as previously described (Xiao et al., 1999). Samples were then subtyped at the glycoprotein 60 (*gp60*) locus using a nested PCR to amplify a ~400 bp product using the primers AL3531 (5'-ATAGTCTCCGCTGTATTC-3') and AL3533 (5-GAGATATATCTTGGTGCG-3) for the primary PCR, and AL3532 (5'-TCCGCTGTATTCTCAGCC-3') and LX0029 (5'-CGAACCACATTACAAATGAAGT-3') for the secondary PCR (Sulaiman et al., 2005).

2.3 Statistical analysis

The prevalence of *Cryptosporidium* in faecal samples collected from each group was expressed as the percentage of samples positive by microscopy, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). Fisher's exact test was performed using SPSS 22 for Windows (SPSS Inc. Chicago, USA), to determine if there was a statistical difference in the prevalence of *Cryptosporidium* in paediatric oncology patients with diarrhoea and paediatric patients with diarrhoea only.

3. Results

3.1. Prevalence by microscopy and demographic data

Microscopic screening using modified acid fast staining of the 297 stool specimens from the recruited children in the present study identified a prevalence of 14.4% (23/160) – 95% CI 9.3-20.8, in paediatric oncology patients with diarrhoea and 5.1% (7/137) – 95% CI, 2.1-10.2 for non-oncology paediatric patients with diarrhoea. This difference was significant ($p<0.05$). The overall prevalence of *Cryptosporidium* across the two groups was 10.1% (30/297) 95% CI 6.9-14.1.

The majority of *Cryptosporidium*-positive paediatric oncology patients (82.6% - 19/23) were experiencing diarrhoea, whereas the remaining 4 had soft stools (Table 1). Of the paediatric patients from Al-Mafraq hospital, 57.1% (4/7) had diarrhoea, while the remaining 3 had soft stools (Table 2). Leukemia was the most common type of cancer experienced by the patients that were

positive for *Cryptosporidium* (n=12), followed by lymphoma (n=2). Data on the type of cancer was unavailable for 6 patients (Table 1).

3.2. Cryptosporidium species and gp60 subtypes

Sequences were obtained for 29/30 positives from the two groups. In addition, screening of the additional 30 faecal samples from paediatric patients from both hospitals, which were negative for *Cryptosporidium* by microscopy, identified another 3 positives. A total of 32 samples typed. All were identified as *C. parvum.* Of the 22 typed *Cryptosporidium* positives from the paediatric oncology patients, from King Hussein Medical Centre for Cancer, 21 were typed as IIaA17G2R1 and one as IIaA16G2R1 (Table 1). The 7 typed positives from the paediatric patients from Al-Mafraq hospital were typed as IIaA17G2R1 (n=5) and IIaA16G2R1 (n=2). The 3 additional positives from the 30 microscopy negative control samples were typed as IIaA17G2R1 (Supplementary Table 1). There was no heterogeneity within individual subtypes. Representative *gp60* sequences from *C. parvum* subtypes IIaA17G2R1 and IIaA16G2R1 were submitted to GenBank under accession numbers MF770731 – MF770734.

4. Discussion

The present study compared the prevalence of *Cryptosporidium* species and *gp60* subtypes among paediatric oncology and non-oncology patients with diarrhoea. As expected, the prevalence of *Cryptosporidium* was significantly higher amongst paediatric oncology patients with diarrhoea (14.4%), compared to non-oncology paediatric patients with diarrhea (5.1%), with 2.8 times more cryptosporidiosis cases amongst the former. This result is similar to a previous study in Egypt,

which detected *Cryptosporidium* in 24% of children with acute lymphoblastic leukemia (ALL) on maintenance chemotherapy, compared to 3% in control patients with diarrhoea (Hassanein et al., 2012). In addition to being a cause of diarrhoea in oncology patients, *Cryptosporidium* has been associated with colon cancer (Sulżyc-Bielicka et al., 2013) and has been shown to induce low-to-high grade intestinal dysplasia in immunocompromised mice (Abdou et al., 2013).

Previous studies in Jordan (for which the immune status is unknown) have reported prevalences ranging from 1.5 – 37.7% in humans and 3.9 – 18.7% in production animals using both microscopy and molecular tools (Youssef et al., 2000; Nimri, 2003; Mahgoub et al., 2004; Hijjawi et al., 2010; Hijjawi et al., 2016). These differences likely reflect differences in detection methods, sample size, area of collection and microscopist skill etc.

During the present study, only one species, *C. parvum* and two *gp60* subtypes (IIaA16G2R1, IIaA16G2R1), were detected in all the typed isolates. This was an unexpected finding as in two previous genotyping studies in Jordan, up to 4 *Cryptosporidium* species (*C. parvum, C. hominis, C. meleagridis,* and *C. canis*) and six subtype families (IIa, IIc, IId, 1b, 1d and IIIa) were detected in human isolates (Hijjawi et al., 2010; Hijjawi et al., 2016). In the previous two studies, *C. parvum* and *C. hominis* were the dominant species and were detected in almost equal frequencies (Hijjawi et al., 2010; Hijjawi et al., 2016). The lack of identification of the *C. parvum* IId subtype and also *C. hominis* is surprising, as previous studies have shown that both are common in humans in Middle Eastern countries including Jordan, although most studies have reported that *C. parvum* is the dominant *Cryptosporidium* species (Sulaiman et al., 2005; Meamar et al., 2007; Al-Brikan et al., 2008; Pirestani et al., 2008; Hijjawi et al., 2010; Iqbal et al., 2011; Nazemalhosseini-Mojarad et al., 2011; Taghipour et al., 2011; Alyousefi et al., 2013; Sharbatkhori et al., 2015), which may explain the lack of detection of *C. hominis*. The IIa subtype family has been previously detected in Jordan children, however, this is the first report of the IIaA17G2R1 and IIaA16G2R1 subtypes in humans

in Jordan. The IIaA16G2R1 subtype has also been previously reported from one cattle isolate from Jordan (Hijjawi et al., 2016).

The dominant subtype, IIaA17G2R1, was detected in 91.3% (95% CI,72 – 98.9) of paediatric oncology patients with diarrhoea and 71.4% (95% CI, 29 – 96.3) of paediatric patients with diarrhoea only. The high prevalence of this subtype, particularly amongst oncology patients, suggests that an outbreak may have been occurring in oncology patients at the King Hussein Medical Centre for Cancer and Al-Mafraq hospital during the collection period (summer 2016). This may also explain the lack of detection of the *C. parvum* IId subtype and *C. hominis*. However, Multi-Locus Sequence Typing (MLST) is required to confirm this. Alternatively, the low heterogeneity observed at the subtype level, may reflect intensive and stable transmission of *C. parvum* in this region. For example, a study of several human populations in Tunisia reported that the *C. hominis* subtype IaA26G1R1, was the most dominant subtype (50%), suggesting stable anthroponotic cryptosporidiosis transmission (Essid et al., 2017). IIaA17G2R1 is a relatively common subtype that has been reported in livestock (Alves et al., 2006; Xiao et al., 2007; Mi et al., 2014; Kaupke and Rzezutka, 2015) and has also been responsible for an outbreak of cryptosporidiosis in a youth summer camp in North Carolina in 2009 (CDC, 2011). In the latter study, IIaA17G2R1 was identified in faecal samples from livestock and humans at the camp, indicating that zoonotic transmission may have occurred. In the present study, the source of the *Cryptosporidium* infection is unknown but may have been due to the consumption of contaminated food or water. In order to identify the risk factors involved in the acquisition of *Cryptosporidium* infections in children and immunocompromised individuals in Jordan, a well-designed case control study, with detailed collection of data on water and food sources, animal and human contact and immune status is required. As only one molecular study has been conducted on species and subtypes of *Cryptosporidium* in livestock in Jordan (Hijjawi et al., 2016), further studies on larger

numbers of animal and human samples, as well as water samples are essential to determine the transmission dynamics of cryptosporidiosis in Jordanian children

A limitation of the present study is that while a compromised immune system can be assumed for the paediatric oncology patients, the immune status of non-oncology paediatric patients with diarrhoea is unknown, as this is not part of routine monitoring for children in Jordan hospitalised for diarrhoea. In addition, in the present study, the prevalence of *Cryptosporidium* species and subtypes in these children was accessed using microscopy only and therefore the true prevalence is likely underestimated. For example, one previous which compared microscopy and molecular analysis on Jordanian human patients identified a prevalence of 1.8% by microscopy and >19% by quantitative PCR (qPCR) confirming the superior sensitivity of PCR (Hijjawi et al., 2010). The finding of an additional 3 positives in the present study in the 30 microscopy negative samples, further supports this.

In conclusion, a high prevalence of cryptosporidiosis was detected amongst paediatric oncology patients. Routine screening for *Cryptosporidium* should be conducted for all oncology patients undergoing chemotherapy, preferably by PCR. The detection of cryptosporidiosis in these patients however presents specific challenges for the treatment, as the only FDA approved drug, nitazoxanide, is ineffective in immunocompromised individuals (Amadi et al., 2009). A previous study reported the eradication of *Cryptosporidium* in four children with acute lymphoblastic leukemia using paromomycin or azithromycin (Trad et al., 2003), and therefore these therapies should be considered for paediatric oncology patients. There is no vaccine for cryptosporidiosis and given the parasite's high infectivity, robustness, and resistance to disinfection (Ryan et al., 2016), improved therapeutics particularly for immunocompromised individuals are urgently required. The advent of whole genome sequencing has identified several promising drug targets including inosine-5'-monophosphate dehydrogenase (IMPDH) (essential for purine salvage) and acyl-coenzyme A

synthetases (LC-ACS) which are essential in fatty acid metabolism (Ryan and Hijjawi, 2015), which holds promise for the future.

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Table 1

Demographic data, *Cryptosporidium* species and subtypes for the 23 microscopy-positive faecal samples, which were collected from April until December, 2016 from paediatric oncology patients attending the King Hussein cancer centre.

Locality	Collection date (day/month)	Age (in years)	Sex	Clinical symptoms	Type of cancer	Species (18S)	*gp60* subtype
Amman	4/4	5	M	Diarrhoea/ watery	Leukemia	*C. parvum*	IIaA17G2R1
Zarqa	11/4	3	M	Diarrhoea/ mucus	Leukemia	*C. parvum*	IIaA17G2R1
Irbid	11/4	2	F	Diarrhoea/ watery	Leukemia	*C. parvum*	IIaA17G2R1
Al-Mafraq	24/4	5	M	Diarrhoea/ watery	NA	*C. parvum*	IIaA17G2R1
Amman	2/5	5	M	Diarrhoea/ watery	Neuroblastoma	*C. parvum*	IIaA17G2R1
Amman	16/5	2	F	Diarrhoea/ mucus	Lymphoma	*C. parvum*	IIaA17G2R1
Amman	30/5	5	M	Diarrhoea/ mucus	Leukemia	*C. parvum*	IIaA17G2R1
Amman	30/5	1	F	Soft stool	Brain cancer	*C. parvum*	IIaA16G2R1
Amman	6/6	2	F	Diarrhoea/ watery	Leukemia	*C. parvum*	IIaA17G2R1
Zarqa	20/6	3	M	Diarrhoea/ mucus	Bone cancer	*C. parvum*	IIaA17G2R1
Zarqa	4/7	4	F	Diarrhoea/ watery	Leukemia	*C. parvum*	IIaA17G2R1
Zarqa	18/7	4	F	Diarrhoea/ watery	Leukemia	*C. parvum*	IIaA17G2R1
Zarqa	1/8	3	F	Diarrhoea/ mucus	NA	*C. parvum*	IIaA17G2R1
Amman	15/8	2	M	Diarrhoea/ watery	Leukemia	*C. parvum*	IIaA17G2R1
Jarash	15/8	1.5	M	Diarrhoea/watery	NA	*C. parvum*	IIaA17G2R1
Jarash	29/8	2	M	Diarrhoea/ mucus	Leukemia	No amplification	-
Amman	5/9	1	M	Soft stool	NA	*C. parvum*	IIaA17G2R1
Zarqa	5/9	1	F	Diarrhoea/watery	NA	*C. parvum*	IIaA17G2R1
Amman	12/9	5	M	Diarrhoea/watery	Leukemia	*C. parvum*	IIaA17G2R1
Asalt	26/9	5	F	Soft stool	Lymphoma	*C. parvum*	IIaA17G2R1
Zarqa	3/10	4	M	Diarrhoea/watery	Leukemia	*C. parvum*	IIaA17G2R1
Zarqa	17/10	2	M	Soft stool	NA	*C. parvum*	IIaA17G2R1
Amman	17/10	1	M	Diarrhoea/ mucus	Leukemia	*C. parvum*	IIaA17G2R1

NA: not available.

Table 2

Demographic data, *Cryptosporidium* species and subtypes for the 7 microscopy-positive faecal samples from non-oncology paediatric patients with diarrhea attending the Al-Mafraq paediatric hospital (all were from the Al-Mafraq area and surrounding villages).

Age (in years)	Collection date (day/month)	Sex	Clinical symptoms	Species (18S)	*gp60* subtype
4	6/4	F	Soft stool	*C. parvum*	IIaA17G2R1
3	20/4	F	Diarrhea/ mucus	*C. parvum*	IIaA17G2R1
3	11/5	M	Diarrhoea/watery	*C. parvum*	IIaA16G2R1
1	15/6	M	Diarrhoea/watery	*C. parvum*	IIaA16G2R1
5	29/6	M	Soft stool	*C. parvum*	IIaA17G2R1
4	6/7	F	Diarrhoea/watery	*C. parvum*	IIaA17G2R1
3	24/8	M	Soft stool	*C. parvum*	IIaA17G2R1

Supplementary Table 1

Demographic data, *Cryptosporidium* species and subtypes for the 30 microscopy-negative faecal samples from paediatric oncology patients with diarrhoea attending King Hussein Medical Centre for Cancer (1-18) and non-oncology paediatric patients with diarrhoea attending Al-Mafraq paediatric hospital (19-30).

Sample No	Collection date (day/month)	Location	Age (in Years)	Sex	Clinical symptoms	Type of cancer	Species (18S)	*gp60* subtype
1	24/4	Zarqa	4	M	Soft stool	NA	Negative	-
2	13/6	Amman	1	F	Diarrhoea/watery	Leukemia	Negative	-
3	20/6	Zarqa	1	M	Diarrhoea/watery	Leukemia	Negative	-
4	20/6	Al-Mafraq	2	M	Soft stool	NA	Negative	-
5	4/7	Al-Mafraq	2	M	Soft stool	Lymphoma	*C. parvum*	IIaA17G2R1
6	4/7	Zarqa	5	F	Diarrhoea/watery	NA	Negative	-
7	18/7	Amman	2	M	Diarrhoea/mucus	Leukemia	Negative	-
8	18/7	Amman	1	M	Soft stool	NA	Negative	-
9	1/8	Irbid	3	F	Diarrhoea/watery	Lymphoma	Negative	-
10	1/8	Amman	5	F	Diarrhoea/mucus	NA	Negative	-
11	15/8	Amman	5	F	Soft stool	Lymphoma	Negative	-
12	15/8	Amman	5	M	Diarrhoea/watery	NA	Negative	-
13	24/8	Jordan valley	3	M	Soft stool	NA	Negative	-
14	5/9	Jarash	2.5	M	Soft stool	NA	Negative	-
15	5/9	Irbid	1	F	Soft stool	Myeloma	Negative	-
16	5/9	Amman	3	F	Diarrhoea/mucus	NA	*C. parvum*	IIaA17G2R1
17	3/10	Zarqa	5	M	Diarrhoea/watery	Lymphoma	Negative	-
18	3/10	Amman	2	F	Diarrhoea/mucus	N/A	Negative	-
19	10/10	Al-Mafraq	1	F	Diarrhoea/watery	-	Negative	-
20	10/10	Zarqa	2	F	Soft stool	-	Negative	-
21	17/10	Al-Mafraq	2	M	Soft stool	-	Negative	-
22	17/10	Jarash	1.5	M	Diarrhoea/mucus	-	Negative	-
23	6/11	Al-Mafraq	1	M	Diarrhoea/mucus	-	Negative	-
24	6/11	Amman	2	M	Soft stool	-	Negative	-
25	13/11	Al-Mafraq	4	F	Soft stool	-	Negative	-
26	13/11	Al-Mafraq	2	F	Diarrhoea/watery	-	Negative	-
27	20/11	Al-Mafraq	4	F	Diarrhoea/mucus	-	Negative	-
28	27/11	Al-Mafraq	1	F	Diarrhoea/mucus	-	Negative	-
29	27/11	Al-Mafraq	1	M	Diarrhoea/watery	-	Negative	-
30	27/11	Al-Mafraq	1	F	Diarrhoea/watery	-	C. parvum	IIaA17G2R1

Highlights

- High prevalence of *Cryptosporidium* in paediatric oncology patients
- Comparison with non-oncology paediatric patients
- Subtyping identified a putative outbreak amongst oncology patients