First genetic characterisation of *Giardia* in human isolates from Jordan

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Abstract

Little is known about the epidemiology of *Giardia* in Jordan and to date, no genotyping studies have been conducted on *Giardia* isolates from Jordanians. In the present study, a total of 49 microscopy-positive faecal samples from Jordanian patients suffering from giardiasis were analysed at two loci: the triose phosphate isomerase (*tpi*) gene and the glutamate dehydrogenase (*gdh*) gene. At the *tpi* locus, a total of 28 samples amplified and assemblage A was identified in 46.4 % (13/28) samples, while assemblage B was identified in 50 % (14/28) samples and a mixed assemblage A and B was identified in one sample (3.6 %) (Table 1). At the *gdh* locus 48 isolates amplified and of these assemblages A was identified in 43.7 % (21/48) of isolates and assemblage B in 56.3 % (27/48) of isolates. No mixed infections were detected at the *gdh* locus. Subtyping at the *gdh* locus identified sub-assemblage AII in 43.7 % (21/48) of isolates and sub-assemblages BIII and BIV in 25 % (12/48) and 31.2 % (15/48) of isolates, respectively, with more genetic diversity in AII isolates than BIII or BIV isolates. Novel sub-types within each sub-assemblage were identified suggesting unique endemicity and anthroponotic transmission of *Giardia* in Jordanian patients suffering from giardiasis.

Further studies are required to better understand the epidemiology and transmission of *Giardia* in Jordan.

Keywords: *Giardia;* Humans; Jordan; Glutamate dehydrogenase; Triose phosphate isomerase

Introduction

Giardia duodenalis (also known as *Giardia intestinalis* and *Giardia lamblia*), is a protozoan parasite that causes hundreds of millions of annual cases of diarrhoeal disease worldwide and is a common cause of outbreaks associated with untreated surface and groundwater (Karanis et al. 2007; Baldursson and Karanis 2011; Miyamoto and Eckmann 2015; Painter et al. 2015). In the USA, an estimated 1.2 million cases occur annually and hospitalizations resulting from giardiasis cost approximately US$34 million (Painter et al. 2015). Giardiasis is generally a self-limited illness typically characterised by diarrhoea, abdominal cramps, bloating, weight loss and malabsorption and asymptomatic infection also occurs frequently (Rodriguez-Hernandez et al. 1996; Hellard et al. 2000; Painter et al. 2015).

Giardia infection is transmitted by the faecal-oral route and results from the ingestion of *Giardia* cysts through the consumption of faecally contaminated food or water or through person-to-person (or animal-to-person) transmission (Xiao and Fayer 2008). The cysts are environmentally hardy, moderately chlorine tolerant and infectious immediately upon being excreted in faeces (Rendtorff 1954). To date, eight major genetic groups (assemblages) have been identified, two of which (A and B) are found in both humans and animals, whereas the remaining six (C to H) are host-specific and do not infect humans (Ryan and Cacciò 2013). Despite many investigations, several important epidemiological aspects remain unclear, including the role of animals in zoonotic transmission (Ryan and Cacciò 2013) and the clinical significance of single and mixed infections with assemblages A and B (Kohli et al. 2008).

Currently nothing is known about molecular epidemiology of giardiasis amongst humans in Jordan. The aim of the present study was to characterise the assemblages and sub-assemblages of *Giardia* in Jordanian patients suffering from giardiasis.

Material and methods

***Giardia* isolates** A total of 49 microscopy-positive *Giardia* faecal samples were obtained between early November 2014 and until the end of October 2015, from clinical laboratories of major hospitals in five regions of Jordan: Amman (22 samples), Irbid (13 samples), Jordan Valley (10 samples), Zarqa (3 samples) and Maan (1 sample) (Table 1). Human samples were collected under human ethics permit number 1401254/32.

Demographic data regarding age, gender, residency, medical history, duration of symptoms and common life style habits were obtained upon filling a designed questionnaire by the patient themselves or their guardian in case of children. The age groups of giardiasis patients ranged between 4 months and 57 years and they were categorised into four age groups: 4 months − 14 years (34 samples), 15–29 years (7 samples), 30–44 years (6 samples) and 45–60 years (3 samples) (Table 1).

Molecular typing and phylogenetic analysis

Total DNA was extracted using a Qiagen QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). Isolates were genotyped using a nested PCR of the triose phosphate isomerase (*tpi*) gene. The primary PCR was performed as described by Sulaiman et al. (2003). Assemblage A-specific primers (Af: CGC CGT ACA CCT GTC A and Ar: AGC AAT GAC AAC CTC CTT CC) and assemblage B-specific primers (Bf: GTT GTT GTT GCT CCC TCC TTT and Br: CCG GCT CAT AGG CAA TTA CA) were used for the second round as described by Geurden et al. (2008) and by Geurden et al. (2009). Typing was also conducted using sequence analysis of a nested PCR at the glutamate dehydrogenase (*gdh*) gene. The primers GDHeF—TCA ACG TYA AYC GYG GYT TCC GT (Read et al. 2004) and GDH2—ACC TCG TTC TGR GTG GCG CA (Cacciò et al. 2008) were used for the external amplification, followed by the primers GDHiF—CAG TAC AAC TCY GCT

CTC GG (Read et al. 2004) and GDH4—GTG GCG CAR GGC ATG ATG CA (Cacciò et al. 2008) for the internal reaction.

The PCR reactions for both the *tpi* and *gdh* genes consisted of 1 μL of DNA (~50 ng), 1 μL each of dNTP (10 mM), 2.5 μL of 10× Kapa Taq DNA polymerase buffer (Geneworks, Adelaide, South Australia), 2.0 μL of 25 mM $MgCl_2$, 1.0 U of Taq polymerase (Geneworks, Adelaide, South Australia), and 100 nM of each primer in a 25-μL PCR reaction. For the *tpi* locus, the primary PCR reactions were performed for 35 cycles (94 °C for 45 s, 50 °C for 45 s and 72 °C for 60 s) with an initial cycle at 94 °C for 3 min and a final extension at 72 °C for 5 min in an Applied Biosystems GeneAmp PCR 2400 thermocycler. For the secondary assemblage-specific PCR, the conditions were identical to the primary reaction except the annealing temperature was 64 °C for the assemblage A-specific primers and 62 °C for the assemblage B-specific primers. For the *gdh* locus, the primary and secondary PCR reactions were identical; 35 cycles (94 °C for 45 s, 60 °C for 30 s, and 72 °C for 60 s) with an initial at 94 °C for 3 min and a final extension at 72 °C for 5 min.

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 on an Applied Biosystem 3730 DNA Analyzer. 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, Inc.; Seattle, WA, USA; http://www.geospiza.com) and aligned with reference *Giardia* sequences from GenBank using Clustal W (http://www.clustalw.genome.jp). Distance, parsimony and maximum likelihood trees were constructed from *gdh* sequences using MEGA version 6 (Tamura et al. 2013). *Giardia ardeae* (AF069060) was used as an outgroup.

Results

In the present study, the highest numbers of giardiasis cases (34/49) were identified in the younger age group (4 months to 14 years). No difference was observed between males and females regarding susceptibility to giardiasis (24 males vs 26 females).

At the *tpi* locus, a total of 28 samples amplified and assemblage A was identified in 46.4 % (13/28) samples, while assemblage B was identified in 50 % (14/28) samples and a mixed assemblage A and B was identified in one sample (3.6 %) (Table 1). At the *gdh* locus, a total of 48 isolates amplified and of these, assemblage AII was identified in 43.7 % (21/48) of isolates and assemblage B in 56.3 % (27/48) of isolates. No mixed infections were detected at the *gdh*locus. For the 27 isolates for which assemblage information was available for both loci, there was 100 % agreement between the loci, with the exception of isolate 83 which was a mixed assemblage A and B at the *tpi* locus but was typed as AII at the *gdh* locus.

Phylogenetic analysis using distance, parsimony and maximum likelihood produced trees with very similar topologies (data not shown). Subtyping at the *gdh* locus identified sub-assemblage AII in 21 isolates (01, 02, 11, 12, 17, 19, 20, 21, 35, 39, 46, 48, 50, 54, 60, 68, 77, 83, 90, 94 and 95) (Table 1). Of these, three isolates (12, 19 and 68) exhibited 1 single nucleotide polymorphism (SNP's) from reference sub-assemblage AII (AY178737). A further six isolates; 39, 46, 50, 54, 90 and 95, exhibited between 5 and 7 SNP's from AII (AY178737). Sub-assemblage BIII was identified in 12 samples (05, 13, 18, 26, 30, 56, 63, 66, 79, 85, 91, 96) and exhibited between 1–3 single nucleotide polymorphisms (SNPs) from reference BIII subtype AY178756 (Supplementary Table S1). Sub-assemblage BIV was identified in 15 isolates (06, 09, 14, 23, 25, 33, 37, 41, 42, 49, 67, 69, 70, 78 and 93) and exhibited between 1 and 3 SNPs from reference BIV subtype EF507654 (Supplementary Table S1). Representative AII, BIII and BIV *gdh* sequences have been submitted to GenBank under the following GenBank accession numbers: KX228236-KX228245.

Combined molecular typing from both the *tpi* and *gdh* loci indicated that assemblage B ($n = 28$) was more prevalent amongst giardiasis patients than assemblage A ($n = 22$) (including isolate 83 which was identified as a mixed assemblage A and B at the *tpi* locus) (Table 1). Assemblage A ($n = 16$) and B ($n = 17$) were detected in equal frequency in the younger age group (4 months to 14 years). Of the assemblage B isolates detected in this age group, 10 were BIII and 7 were BIV. All 49 patients experienced diarrhoea and abdominal pain was identified in 37 patients (Table 1). Blood and mucus was identified in one stool sample (isolate 69) that was characterised as BIV. Vomiting was identified in 4 patients; isolate 18 (BIII), isolate 19 (AII), isolate 23 (BIV), isolate 69 (BIV). However, there were insufficient numbers of samples to determine if these differences were statistically significant.

Discussion

In the present study, genetic characterisation of *Giardia* isolates from Jordanian patients suffering from giardiasis was conducted for the first time. Previous studies in Jordan, have identified *Giardia* prevalences by microscopy ranging from 0.8 to 36 %, with the highest prevalence (44.8 %) amongst children aged 6 < 9 (Nimri 1993; Nimri 1994; Youssef et al. 2000; Nimri 2003; Nimri and Meqdam 2004; Abdel-Dayem et al. 2014). In the present study, *Giardia* assemblages A and B were identified in Jordanian patients ranging in age from 4 months to 53 years, with assemblage A and assemblage B accounting for 44.9 and 57.1 % of infections respectively using combined analysis of the *tpi* and *gdh* loci. The highest incidence of giardiasis, 69.4 % (34/49), occurred in children aged 4 months to 14 years with assemblage A and B occurring in equal frequency in this age group. Mixed infections were detected in one isolate (2.0 %) at the *tpi* locus using assemblage-specific primers. Analysis at the *gdh* locus (48 positives) was more sensitive than the *tpi* locus (28 positives). It is unclear why this was the case as both genes are single copy (Yee and Dennis 1992; Mowatt et al. 1994). In addition, the *tpi* amplicon size (332 bp for assemblage A and 400 bp for assemblage B) was smaller than the *gdh* amplicon size (743 bp) and therefore it would be expected that the *tpi* PCR would be more sensitive. Most studies worldwide have reported that assemblage B (~58 %) has a higher prevalence than assemblage A (~37 %) in both developed and

developing countries and that the prevalence of mixed infections is higher (5.2 %) in developing countries than in developed ones (3.2 %) (Ryan and Cacciò 2013).

In middle-eastern countries, the prevalence of assemblage A and B varies widely from 0–85.3 % for assemblage A and 9.2–100 % for assemblage B (Table 2). However, as not all these studies used sequencing analysis to confirm the assemblage, the assignment to assemblage may not always be reliable (Table 2). The prevalence of mixed assemblage A and B infections also varies widely in middle-eastern countries (Table 2), with one study reporting that 54 % of symptomatic patients and 64 % of asymptomatic patients, had mixed A and B infections in contrast to all other studies (Rafiei et al. 2013). However, this study relied on restriction fragment length polymorphism (RFLP) analysis to identify assemblages and sequencing analysis is required to confirm this. It is also likely that in many studies, the true prevalence of mixed infections is unknown.

Previous analysis of genetic variability within assemblages has shown that isolates of assemblage A can be divided into four sub-assemblages (AI, AII, AIII and AIV) by protein polymorphisms of 23 loci (Monis et al. 1996; Monis et al. 2003), with human isolates belonging to AI and AII and animal isolates belonged to AI, AIII and AIV (Monis et al. 2003; van der Giessen et al. 2006; Robertson et al. 2007; Cacciò et al. 2008; Sprong et al. 2009; Ryan and Cacciò 2013). Within assemblage B, sub-assemblages BI, BII, BIII and BIV have been described by enzyme electrophoresis, with human isolates forming two clusters (BIII and BIV) and animal isolates (monkeys and a dog) belonging to sub-assemblages BI and BII (Monis et al. 2003). However, BIII and BIV sub-assemblages identified by allozyme electrophoresis are not always supported by DNA sequence analysis as subtyping analyses of field isolates produced inconsistent sub-assemblages amongst different loci (Feng and Xiao 2011).

Isolates that belong to sub-assemblages are genetically close, but not identical: i.e. sub-assemblages are clusters of closely related isolates (sub-types) (Ryan and Cacciò 2013), and a single point mutation has been considered sufficient to describe a new subtype (Cacciò et al. 2008; Sprong et al. 2009). Isolates are first assigned to the sub-assemblage level (AI or AII, etc.) and then particularly for multi-locus typing, are further identified by an Arabic numeral suffix (AI-1, AI-2, AII-1, AII-2

etc.) (Ryan and Cacciò 2013). Whether all these sub-types correspond to new genotypes or whether some of them will turn out to be (sequence) artefacts is unclear. In the present study, sub-assemblages AII, BIII and BIV were detected in Jordanian patients suffering from giardiasis, similar to the majority of studies in middle-eastern countries (Table 2), suggesting anthroponotic and not zoonotic transmission. Sub-assemblage AI was not detected but has been detected in humans in Egypt and Saudi Arabia (Table 2).

A higher genetic diversity was observed within sub-assemblage AII isolates (1–7 SNPs) compared to BIII and BIV (1 and 3 SNPs each). The occurrence of heterogeneous sequences may be due to the presence in *Giardia* of two nuclei, which are thought to accumulate mutations and evolve separately leading to allele sequence heterozygosity (e.g. the appearance of nucleotidic differences in the sequence between alleles of the same gene) (Ryan and Cacciò 2013). In addition, novel sub-types within each sub-assemblage were identified suggesting unique endemicity and transmission of *Giardia* in Jordan, which has previously been reported for *Cryptosporidium* (Hijjawi et al. 2010). Further studies on a larger number of isolates from humans and animals and using multiple loci are required to better understand the transmission dynamics and epidemiology of giardiasis in Jordan.

Compliance with ethical standards

Human samples were collected under human ethics permit number 1401254/32.

Conflict of interest

The authors declare that they have no competing interests. The authors certify that they have no affiliation with or financial involvement in any organisation or entity with a direct financial interest in the subject matter or materials discussed in the manuscript.

References

Abdel-Dayem M, Al Zou'bi R, Hani RB, Amr ZS (2014) Microbiological and parasitological investigation among food handlers in hotels in the Dead Sea area, Jordan. J Microbiol Immunol Infect 47(5):377–380

Al-Mohammed HI (2011) Genotypes of *Giardia intestinalis* clinical isolates of gastrointestinal symptomatic and asymptomatic Saudi children. Parasitol Res 108:1375–1381

Alyousefi NA, Mahdy MA, Xiao L, Mahmud R, Lim YA (2013) Molecular characterization of *Giardia duodenalis* in Yemen. Exp Parasitol 134(2):141–147

Amer SE (2013) Genotypic and phylogenetic characterization of *Giardia intestinalis* from human and dairy cattle in Kafr El Sheikh Governorate, Egypt. J Egypt Soc Parasitol 43(1):133–146

Baldursson S, Karanis P (2011) Waterborne transmission of protozoan parasites: review of worldwide outbreaks—an update 2004–2010. Water Res 45:6603–6614

Cacciò SM, Beck R, Lalle M, Marinculic A, Pozio E (2008) Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. Int J Parasitol 38(13):1523–1531

Çiçek C, Şakru N (2015) Genotyping of *Giardia intestinalis* isolates in the Thrace Region, Turkey. Mikrobiyol Bul 49(4):576–585

El Basha NR, Zaki MM, Hassanin OM, Rehan MK, Omran D (2016) *Giardia* assemblages a and b in diarrheic patients: a comparative study in Egyptian children and adults. J Parasitol 102(1):69–74

Elbakri A, Samie A, Bessong P, Potgieter N, Odeh RA (2014) Detection and molecular characterisation of *Giardia lamblia* genotypes in Sharjah, United Arab Emirates. Trans R Soc Trop Med Hyg 108(8):466–473

Fahmy HM, El-Serougi AO, El Deeb HK, Hussein HM, Abou-Seri HM, Klotz C, Aebischer T, El Sayed Khalifa Mohamed K (2015) *Giardia duodenalis* assemblages in Egyptian children with diarrhea. Eur J Clin Microbiol Infect Dis 34(8):1573–1581

Feng Y, Xiao L (2011) Zoonotic potential and molecular epidemiology of Giardia species and giardiasis. Clin Microbiol Rev 24(1):110–40

Foronda P, Bargues MD, Abreu-Acosta N, Periago MV, Valero MA, Valladares B, Mas-Coma S (2008) Identification of genotypes of *Giardia intestinalis* of human isolates in Egypt. Parasitol Res 103:1177–1181

Geurden T, Geldhof P, Levecke B, Martens C, Berkvens D, Casaert S, Vercruysse J, Claerebout E (2008) Mixed *Giardia duodenalis* assemblage A and E infections in calves. Int J Parasitol 38(2):259–264

Geurden T, Levecke B, Cacció SM, Visser A, De Groote G, Casaert S, Vercruysse J, Claerebout E (2009) Multilocus genotyping of *Cryptosporidium* and *Giardia* in non-outbreak related cases of diarrhoea in human patients in Belgium. Parasitology 136(10):1161–1168

Hazrati Tappeh K, Manafi G, Asgharzadeh M, Manafi F (2014) Incidence of *Giardia lamblia* Subspecies by PCR-RFLP in stool specimens of hospitalized children at Urmia Mutahhari Hospital, West Azerbaijan Province, Iran. Iran J Parasitol 9(4):541–547

Hellard ME, Sinclair MI, Hogg GG, Fairley CK (2000) Prevalence of enteric pathogens among community based asymptomatic individuals. J Gastroenterol Hepatol 15:290–293

Helmy MMF, Abdel-Fattah HS, Rashed L (2009) Real-time PCR/RFLP assay to detect *Giardia intestinalis* genotypes in human isolates with diarrhea in Egypt. J Parasitol 95:1000–1004

Helmy YA, Klotz C, Wilking H, Krücken J, Nöckler K, Von Samson-Himmelstjerna G, Zessin KH, Aebischer T (2014) Epidemiology of *Giardia duodenalis* infection in ruminant livestock and children in the Ismailia province of Egypt: insights by genetic characterization. Parasit Vectors 7(1):321

Hijjawi N, Ng J, Yang R, Atoum MF, Ryan U (2010) Identification of rare and novel *Cryptosporidium* GP60 subtypes in human isolates from Jordan. Exp Parasitol 125(2):161–164

Hussein AI, Yamaguchi T, Nakamoto K, Iseki M, Tokoro M (2009) Multiple-subgenotype infections of *Giardia intestinalis* detected in Palestinian clinical cases using a subcloning approach. Parasitol Int 58(3):258–262

Hussein EM, Zaki WM, Ahmed SA, Almatary AM, Nemr NI, Hussein AM (2016) Predominance of *Giardia lamblia* assemblage A among iron deficiency anaemic pre-school Egyptian children. Parasitol Res

Karanis P, Kourenti C, Smith H (2007) Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. J Water Health 5(1):1–38

Kohli A, Bushen OY, Pinkerton RC, Houpt E, Newman RD, Sears CL, Lima AA, Guerrant RL (2008) *Giardia duodenalis* assemblage, clinical presentation and markers of intestinal inflammation in Brazilian children. Trans R Soc Trop Med Hyg 102(7):718–725

Miyamoto Y, Eckmann L (2015) Drug development against the major diarrhea-causing parasites of the small intestine, *Cryptosporidium* and *Giardia*. Front Microbiol 6:1208

Monis PT, Mayrhofer G, Andrews RH, Homan WL, Limper L, Ey PL (1996) Molecular genetic analysis of *Giardia intestinalis* isolates at the glutamate dehydrogenase locus. Parasitology 112(Pt 1):1–12

Monis PT, Andrews RH, Mayrhofer G, Ey PL (2003) Genetic diversity within the morphological species *Giardia intestinalis* and its relationship to host origin. Infect Genet Evol 3:29–38

Mowatt MR, Weinbach EC, Howard TC, Nash TE (1994) Complementation of an Escherichia coli glycolysis mutant by Giardia lamblia triosephosphate isomerase. Exp Parasitol 78(1):85–92

Nimri LF (1993) Evidence of an epidemic of *Blastocystis hominis* infections in preschool children in northern Jordan. J Clin Microbiol 31(10):2706–2708

Nimri LF (1994) Prevalence of giardiasis among primary school children. Child Care Health Dev 20(4):231–237

Nimri LF (2003) *Cyclospora cayetanensis* and other intestinal parasites associated with diarrhea in a rural area of Jordan. Int Microbiol 6(2):131–135

Nimri LF, Meqdam M (2004) Enteropathogens associated with cases of gastroenteritis in a rural population in Jordan. Clin Microbiol Infect 10(7):634–639

Painter JE, Gargano JW, Collier SA, Yoder JS, Centers for Disease Control and Prevention (2015) Giardiasis surveillance—United States, 2011–2012. MMWR Surveill Summ 64(Suppl 3):15–25

Pestechian N, Rasekh H, Rostami-Nejad M, Yousofi HA, Hosseini-Safa A (2014) Molecular identification of *Giardia lamblia*; is there any correlation between diarrhea and genotyping in Iranian population? Gastroenterol Hepatol Bed Bench 7(3):168–172

Rafiei A, Roointan ES, Samarbafzadeh AR, Shayesteh AA, Shamsizadeh A, Pourmahdi Borujeni M (2013) Investigation of possible correlation between *Giardia duodenalis*genotypes and clinical symptoms in Southwest of Iran. Iran J Parasitol 8(3):389–395

Rayani M, Zasmy Unyah N, Hatam G (2014) Molecular Identification of *Giardia duodenalis* isolates from Fars Province, Iran. Iran J Parasitol 9(1):70–78

Read CM, Monis PT, Thompson RC (2004) Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase *locus* using PCR-RFLP. Infect Genet Evol 4:125–130

Rendtorff RC (1954) The experimental transmission of human intestinal protozoan parasites. II. *Giardia lamblia* cysts given in capsules. Am J Hyg 59:209–220

Robertson LJ, Forberg T, Hermansen L, Hamnes IS, Gjerde B (2007) *Giardia duodenalis*cysts isolated from wild moose and reindeer in Norway: genetic characterization by PCR-RFLP and sequence analysis at two genes. J Wildl Dis 43:576–585

Rodriguez-Hernandez J, Canut-Blasco A, Martin-Sanchez AM (1996) Seasonal prevalences of *Cryptosporidium* and *Giardia* infections in children attending day care centres in Salamanca (Spain) studied for a period of 15 months. Eur J Epidemiol 12:291–295

Ryan U, Cacciò SM (2013) Zoonotic potential of *Giardia*. Int J Parasitol 43(12–13):943–956

Sarkari B, Ashrafmansori A, Hatam GR, Motazedian MH, Asgari Q, Mohammadpour I (2012) Genotyping of *Giardia lamblia* isolates from human in southern Iran. Trop Biomed 29(3):366–371

Skhal D, Aboualchamat G, Al Nahhas S (2016) *Giardia duodenalis* in Damascus, Syria: Identification of *Giardia* genotypes in a sample of human fecal isolates using polymerase chain reaction and restriction fragment length polymorphism analyzing method. Acta Trop 154:1–5

Soliman RH, Fuentes I, Rubio JM (2011) Identification of a novel assemblage B subgenotype and a zoonotic assemblage C in human isolates of *Giardia intestinalis* in Egypt. Parasitol Int 60:507–511

Sprong H, Cacciò SM, van der Giessen JW, ZOOPNET network and partners (2009) Identification of zoonotic genotypes of *Giardia duodenalis*. PLoS Negl Trop Dis 3(12):e558

Sulaiman IM, Fayer R, Bern C, Gilman RH, Trout JM, Schantz PM, Das P, Lal AA, Xiao L (2003) Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerg Infect Dis 9:1444–1452

Tamer GS, Kasap M, Er DK (2015) Genotyping and phylogenetic analysis of *Giardia duodenalis* isolates from Turkish children. Med Sci Monit 21:526–532

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30(12):2725–2729

van der Giessen JW, de Vries A, Roos M, Wielinga P, Kortbeek LM, Mank TG (2006) Genotyping of *Giardia* in Dutch patients and animals: a phylogenetic analysis of human and animal isolates. Int J Parasitol 36(7):849–858

Xiao L, Fayer R (2008) Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. Int J Parasitol 38:1239–1255

Yang R, Murphy C, Song Y, Ng-Hublin J, Estcourt A, Hijjawi N, Chalmers R, Hadfield S, Bath A, Gordon C, Ryan U (2013) Specific and quantitative detection and identification of *Cryptosporidium hominis* and *C. parvum* in clinical and environmental samples. Exp Parasitol 135:142–147

Yee J, Dennis PP (1992) Isolation and characterization of a NADP-dependent glutamate dehydrogenase gene from the primitive eucaryote *Giardia lamblia*. J Biol Chem 267:7539–7544

Youssef M, Shurman A, Bougnoux M, Rawashdeh M, Bretagne S, Strockbine N (2000) Bacterial, viral and parasitic enteric pathogens associated with acute diarrhea in hospitalized children from northern Jordan. FEMS Immunol Med Microbiol 28:257–263

Table 1

Giardia duodenalis assemblages and sub-assemblages in Jordanian individuals with giardiasis.

Sample ID/area	Assemblage identified at the *tpi* locus by Ass-specific PCR[a]	Assemblage identified at the *gdh* locus by sequencing[b]	Sex/age	Clinical symptoms
1(Irbid)	A	AII	M/8 years	Diarrhoea
2 (Amman)	na	AII	F/5 years	Diarrhoea
5 (Amman)	B	BIII	F/10 years	Diarrhoea
6 (Amman)	B	BIV	M/33 years	Diarrhoea
9 (Amman)	na	BIV	F/28 years	Diarrhoea
11 (Amman)	A	AII	M/41 years	Diarrhoea
12 (Amman)	A	AII	M/5 months	Diarrhoea
13 (Amman)	B	BIII	F/6 months	Diarrhoea
14 (Amman)	na	BIV	M/10 years	Diarrhoea
17 (Amman)	na	AII	F/5 years	Diarrhoea

Sample ID/area	Assemblage identified at the *tpi* locus by Ass-specific PCR[a]	Assemblage identified at the *gdh* locus by sequencing[b]	Sex/age	Clinical symptoms
18 (Irbid)	B	BIII	M/10 years	Diarrhoea, nausea and vomiting
19 (Irbid)	na	AII	F/21 months	Diarrhoea, vomiting
20 (Irbid)	na	AII	M/6 years	Diarrhoea + abdominal pain
21 (Irbid)	A	AII	M/2 years	Diarrhoea + abdominal pain
23 (Amman)	na	BIV	F/36 years	nausea + vomiting (mucus present)
25 (Amman)	na	BIV	M/7 years	Diarrhoea + abdominal pain
26 (Amman)	B	BIII	M/6 years	Diarrhoea + abdominal pain
30 (Amman)	B	BIII	F/51 years	Diarrhoea + abdominal pain
33 (Amman)	na	BIV	M/20 years	Diarrhoea + abdominal pain
35 (Amman)	A	AII	M/31 years	Diarrhoea + abdominal pain
37 (Amman)	na	BIV	F/51 years	Diarrhoea + abdominal pain
39 (Irbid)	A	AII	F/9 months	Diarrhoea + abdominal pain
41 (Irbid)	B	BIV	M/2 years	Diarrhoea + abdominal pain

Sample ID/area	Assemblage identified at the *tpi* locus by Ass-specific PCR[a]	Assemblage identified at the *gdh* locus by sequencing[b]	Sex/age	Clinical symptoms
42 (Irbid)	na	BIV	M/5 years	Diarrhoea + abdominal pain
46 (Irbid)	na	AII	M/4 months	Diarrhoea + abdominal pain
48 (Amman)	A	AII	M/23 years	Diarrhoea + abdominal pain
49 (Amman)	B	BIV	M/42 years	Diarrhoea + abdominal pain
50 (Zarqa)	A	AII	F/18 years	Diarrhoea + abdominal pain
51 (Amman)	A	na	F/25 years	Diarrhoea + abdominal pain
54 (Amman)	na	AII	F/8 years	Diarrhoea + abdominal pain
56 (Zarqa)	na	BIII	M/33 years	Diarrhoea + abdominal pain
60 (Amman)	A	AII	F/9 years	Diarrhoea + abdominal pain
63 (Amman)	na	BIII	F/6 years	Diarrhoea + abdominal pain
66 (Irbid)	na	BIII	M/5 years	Diarrhoea + abdominal pain
67 (Irbid)	B	BIV	M/17 months	Diarrhoea + abdominal pain
68 (Zarqa)	na	AII	F/45 years	Diarrhoea + abdominal pain

Sample ID/area	Assemblage identified at the *tpi* locus by Ass-specific PCR[a]	Assemblage identified at the *gdh* locus by sequencing[b]	Sex/age	Clinical symptoms
69 (Irbid)	na	BIV	M/20 months	Abdominal pain, nausea + vomiting (mucus + blood present)
70 (Maan)	na	BIV	F/25 years	Diarrhoea + abdominal pain
77 (Jordan Valley)	A	AII	F/2 years	Diarrhoea + abdominal pain
78 (Jordan Valley)	B	BIV	F/5 years	Diarrhoea + abdominal pain
79 (Jordan Valley)	B	BIII	F/3 years	Diarrhoea + abdominal pain
83 (Jordan Valley)	A + B	AII	F/13 years	Diarrhoea + abdominal pain
85 (Jordan Valley)	B	BIII	M/5 years	Diarrhoea + abdominal pain
90 (Jordan Valley)	na	AII	F/11 years	Diarrhoea + abdominal pain
91(Jordan Valley)	B	BIII	F/5 years	Diarrhoea + abdominal pain
93 (Jordan Valley)	na	BIV	F/15 years	Diarrhoea + abdominal pain

Sample ID/area	Assemblage identified at the *tpi* locus by Ass-specific PCR[a]	Assemblage identified at the *gdh* locus by sequencing[b]	Sex/age	Clinical symptoms
94 (Jordan Valley)	A	AII	F/3 years	Diarrhoea + abdominal pain
95 (Jordan Valley)	A	AII	M/10 years	Diarrhoea + abdominal pain
96 (Irbid)	B	BIII	F/6 years	Diarrhoea + abdominal pain
Total positives	28	48		

na no amplification

[a]A total of 28 assemblages were identified at the *tpi* locus: assemblage A ($n = 13$), assemblage B ($n = 14$) and mixed assemblage A + B ($n = 1$)

[b]A total number of 48 assemblages were identified at the *gdh* locus by sequencing: assemblage AII ($n = 21$), BIII ($n = 12$) and BIV ($n = 15$)

Table 2

Giardia assemblages and sub-assemblages identified in humans in middle-eastern countries

Country	Locus/loci used	Type of analysis	Assemblage A (%)	Assemblage B (%)	Mixed A and B/other	Assemblage sub-types	References
Egypt	*tpi*	Sequencing	5	80	E (15 %)	–	Foronda et al. 2008
Egypt	*tpi*		75.6	19.5	4.9 % mixed	A1, AII, B	Helmy et al. 2009
Egypt	*tpi*, *bg*, *gdh*	RFLP and sequencing	6.6	86.6	C (6.6 %[a])	AII, BIII, BIV	Soliman et al. 2011
Egypt	*tpi*	Sequencing	36	64		AII, B	Amer, 2013
Egypt	*tpi*, *bg*, *gdh*	Sequencing	11.7	64.7	5.8 % mixed, E/A (7.6 %)	AII, B	Helmy et al. 2014
Egypt	*tpi*, *bg*, *gdh*	Sequencing	27.3	70.1		AII, B	Fahmy et al. 2015
Egypt	*bg*	RFLP	36.6	63.3	–	–	El Basha et al. 2016

Country	Locus/loci used	Type of analysis	Assemblage A (%)	Assemblage B (%)	Mixed A and B/other	Assemblage sub-types	References
Egypt	IGS	HRMCA	55.8	40.9	2.3 % mixed (1.1 % mixed AI + AII)	AI (33.2 %), AII (22.6 %), B	Hussein et al. 2016
Iran	*gdh*	RFLP	85.3	9.3	5.4 % mixed	AII, BIII, BIV	Sarkari et al. 2012
Iran	*gdh*	RFLP	14	27	59 % mixed AII + BIII	AII, BIII	Rafiei et al. 2013
Iran	*gdh*	RFLP	0	100		BIII (93.3 %), BIV (6.7 %)	Hazrati et al. 2014
Iran	*tpi*	RFLP	59.7	37.3	3 % mixed	–	Pestechian et al. 2014
Iran	*gdh*	Sequencing	80	20		AII, BIII, BIV	Rayani et al. 2014
Palestine	18S, *gdh*	Sequencing	75	25		AII, B	Hussein et al. 2009
Saudi Arabia	IGS	HRMCA	57.5	37.5	5 % mixed	AI (16.7 %), AII (12.5 %), B	Al-Mohammed, 2011

Country	Locus/loci used	Type of analysis	Assemblage A (%)	Assemblage B (%)	Mixed A and B/other	Assemblage sub-types	References
Syria	*bg*	RFLP	67.5	10	22.5 % mixed		Skhal et al. 2016
Turkey	bg	Sequencing	52.9	47.1	–	–	Çiçek and Şakru, 2015
Turkey	bg	Sequencing	50	31.8	18.2 % mixed		Tamer et al. 2015
United Arab Emirates	tpi	Assemblage specific PCR	45.7	41.3	13 % mixed		ElBakri et al. 2014
Yemen	18S rRNA and bg	Sequencing	66	34		BIII and AII	Alyousefi et al. 2013

tpi triose phosphate isomerase gene, *bg* β-giardin gene, *gdh* glutamate dehydrogenase gene, *IGS* rDNA intergenic spacer region, *RFLP* restriction fragment length polymorphism analysis, *HRMCA* high resolution melting curve analysis

[a]1 sample (1/15)

Supplementary Table 1. Alignment of selected *gdh* sequences deposited in GenBank with reference GenBank sequences.

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13 KX228239    CTCCGCTTCCACCCCTCTGTCAACCTCTCGATCCTCAAGTTCCTCGGCTTTGAGCAGATC
26 KX228240    CTCCGCTTCCACCCCTCTGTCAACCTCTCGATCCTTAAGTTCCTCGGCTTTGAGCAGATC
96 KX228242    CTCCGCTTCCACCCCTCTGTCAACCTCTCGATCCTTAAGTTCCTCGGCTTTGAGCAGATC
30 KX228241    CTCCGCTTCCACCCCTCTGTCAACCTCTCGATCCTTAAGTTCCTCGGCTTTGAGCAGATC
EF507654 BIV   CTCCGCTTCCACCCCTCTGTCAACCTCTCGATCCTTAAGTTCCTCGGCTTTGAGCAGATC
06 KX228243    CTCCGCTTCCACCCCTCTGTCAACCTCTCGATCCTTAAGTTCCTCGGCTTTGAGCAGATC
33 KX228244    CTCCGCTTCCACCCCTCTGTCAACCTCTCGATCCTTAAGTTCCTCGGCTTTGAGCAGATC
93 KX228245    CTCCGCTTCCACCCCTCTGTCAACCTCTCGATCCTTAAGTTCCTCGGCTTTGAGCAGATC
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01 KX228236    CTCCGCTTCCACCCCTCTGTCAATCTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATC
39 KX228237    CTCCGCTTCCACCCCTCTGTCAATCTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATC
46 KX228238    CTCCGCTTCCACCCCTCTGTCAATCTTTCGATTCTCAAGTTCCTCGGTTTCGAGCAGATC

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26 KX228240    CCTAAGGGCAAGTCGGACAACGAGGTCATGCGCTTCTGCCAGTCCTTTATGACTGAGCTC
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30 KX228241    CCTAAGGGCAAGTCCGACAACGAGGTCATGCGCTTCTGCCAGTCCTTTATGACTGAGCTC
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