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ORIGINAL ARTICLE

Genotypic analysis of *Giardia duodenalis* in children at Egypt

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Abstract *Giardia duodenalis* is among the most common and frequent intestinal protozoan infecting Egyptian children. The present study aims to identify the genotypic features of *G. duodenalis* among children fecal samples complaining of diarrhea at Beni-Suef Governorate, Egypt, and to study the association between *Giardia* assemblages and clinical presentation of the disease among this category. One hundred thirty diarrheic stool samples were subjected to direct stool examination, and positive samples for *Giardia* were subjected to copro-DNA extraction and amplification targeting the triose phosphate isomerase (*tpi*) gene using nested polymerase chain reaction (nPCR) technique. Then amplified DNA products were purified and sequenced. Out of 36 microscopically positive samples for *Giardia*, 28 samples were successfully purified and sequenced; subassemblages AII, BIII, and BIV were detected (10.7, 14,3, and 17,8 %, respectively), and it was difficult to subgroup 16 samples that belong to assemblage B (57 %). Children below the age of 6 are significantly 16 times at risk of infection with assemblage B than assemblage A (p value = .001). Flatulence and presence of fat particles on microscopic examination were significantly associated with infection (p value = 0.001, 0.027, respectively). However, assemblage B was associated with variations of symptomology than A. The present study focuses on giardiasis among Beni-Suef community. Infection is due to both assemblages: assemblage B is more prevalent (89.3 %) than A (10.7 %) and assemblage AI was not recorded. More studies are needed to identify source of infection.

Keywords Giardiasis · *Giardia duodenalis* · tpi · Assemblage · Genotyping · Egypt

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Introduction

Giardia lamblia is a flagellated eukaryotic unicellular microorganism (Amar et al. 2002). Giardiasis is a common disease in children in Africa and Middle East, and its prevalence may reach to 30 % in some area. (Thompson and Smith 2011). The infection can be found in all ages; however, children are one of the most risky groups manifested with symptomatic signs of the disease especially before school age (Bonhomme et al. 2011). Growth impairment and malnutrition could complicate the disease (Nematian et al. 2008). Clinical presentation of giardiasis differs in some patients; it may pass asymptomatic or present with diarrhea, steatorrhea, flatulence, and malabsorption and malnutrition in children. This is due to multifactor, virulence, and genetic diversity of the parasite or host factors (Puebla et al. 2014).

Isolates of *G. duodenalis* are classified into 8 assemblages (Read et al. 2002; Thompson and Monis 2004; Caccio and Ryan 2008; Feng and Xıao 2011).). Assemblages, A and B, are related to human infections (Thompson and Monis 2004), while the remaining assemblages (C to H) are likely to be host specific (Lim et al. 2008; Anuar et al. 2012). However, a previous study by Foronda et al. (2008) at Egypt reported isolation of assemblage E from human isolates for the first time. Worldwide, the prevalence of *Giardia* assemblages varies geographically from area to another. However,

assemblages have been associated by some authors with clinical presentation of giardiasis especially diarrhea (Haque 2007; Helmy et al. 2009; Etemadi et al. 2011).

Genetic sequencing of giardiasis is helpful for identification of a true assemblage frequency and detection of source of infection in an endemic area through the studying of each assemblage distribution in different hosts (Amar et al. 2002). Tpi gene for giardiasis genotyping was used by many studies (Sulaiman et al. 2003; Foronda et al. 2008; Helmy et al. 2009; Fahmy et al. 2015) for both unilocus and multilocus detection due to the high genetic heterogeneity by *Giardia* spp. at this locus. It is useful not only in detection but also in taxonomic studies of *Giardia* spp. (Monis et al. 1999).

In Egypt, a great concern should focus on this parasite which is endemic especially in children, as some studies reported high prevalence of the disease reached up to 42.3 % by Helmy et al. (2009) among diarrheatic isolate at Giza and Sadek et al. 2013 reported 30 % prevalence at Menoufiya Governorate. El-Tantawy and Taman. (2014) reported a childhood prevalence of 33.12 % at Dakahalia Governorate. That is why it is important to study the genotypic features of *G. duodenalis* among children's fecal samples complaining of diarrhea at Beni-Suef Governorate in the north of Upper Egypt and to elucidate the association of clinical picture and assemblage type.

Material and methods

Study design and populations

This is a cross-sectional study including 130 children attending Beni-Suef University Children Outpatient's Clinics complaining of diarrhea.

Collection and processing of samples

Single stool sample at least was collected from all patients and divided into two parts, one for colposcopic examination by direct wet mount before and after formalin-ethyl acetate concentration using saline and Lugol's iodine to detect *G. duodenalis* and other parasites using ×10 and ×40 objectives, and the other part was freshly frozen at −4 °C for molecular processing.

Copro-PCR assay

Genomic DNA extraction was done using Favor Prep stool DNA isolation Kit (Favorgen Biotech corporation ping-Tung 908, Taiwan, Cat. No. FASTI001), and extraction was performed according to the manufacturer's instruction with modifications in the form of thermal treatment of samples by using liquid nitrogen for 5 min then water bath (95 °C for 5 min) (repeated for 10 cycles). Nested PCR (n PCR) was done using two sets of primers targeting tpi gene: AL3543: 5′-AAATIATGCCTGCTCGTCG-′ 3 and the reverse primer AL3546: 5′- CAAACCTTITCCGCAAACC-′ 3 for the primary reaction to amplify ~605 bp DNA and a fragment of ~530 bp for the secondary reaction using AL3544: 5′-CCCTTCATCGGIGGTAACTT-′ 3 and the reverse primer AL3545: 5′- GTGGCCACCACICCCGTGCC-′ 3 (Sulaiman et al. 2003). The melting temperature (Tm) calculations of the chosen primers were performed online using an online oligonucleotide properties calculator (OligoCalc) at its website, http://basic.northwestern.edu/biotools/. Then, gradient annealing temperatures (from 48 to 63 °C) were tested. After optimization of reaction conditions, 1 μl of each primer (200 nM), 5 μl of template DNA, 12.5 μl of Dream Taq Green PCR Master Mix (Product No. K1081, Thermo Scientific, USA), and 0.1 μl Taq Polymerase (5 U/μL) were prepared for primary reaction mixture. Molecular grade water was added, and finally a volume of 25 μl was cycled. For the secondary reaction, 2 μl of the primary PCR product was used and the amplified products were visualized with 1.5 % agarose gel electrophoresis after ethidium bromide staining.

DNA sequencing

Amplified products were purified using Qiagen PCR purification kit (QIAGEN, Hilden, Germany); the amplified purified products were visualized on 1.5 % agarose gel electrophoresis. Sequencing reaction performed with BigDye® Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) uses the same amplification primers according to manufacturer's instructions. Post-sequencing reaction cleaning was carried out with Big Dye X purification kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The DNA template sequencing was performed on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis

The statistical package SPSS version 17 (Chicago, IL, USA) was used for statistical analysis. For descriptive data, chi-square test (χ^2) was used to determine variable association as regards prevalence of assemblage A and B, and frequency was used to estimate character of the studied population. The univariate logistic regression analysis was used by considering age, sex, clinical symptoms, stool pattern, and microscopic examination of the independent variables, while the dependent variable was the prevalence of giardiasis assemblage. All univariate models were used to identify associations between assemblage's type and the independent variables.

Results

PCR yielding

Out of 130 patients complaining of diarrhea, 36 samples were positive microscopically for *G. duodenalis*, and 28 samples of them were successfully purified and sequenced for the yielded PCR product. Assemblage A was detected in only 3 samples (10.7 %), and all of them were subgroup AII, while assemblage B was detected in 25 samples (89.3 %). Five (20 %) were subgroup BIV, 4 (16 %) were subgroup BIII, while it was difficult to identify the remaining 16 (64 %).

Association of different variables with assemblage type

Assemblage B was more presented (64 %) in children below 6 years while assemblage A was prevalent (66.7 %) in children above age of 6 (Fig. 1) with statistical significant difference (p value = .001). Male distribution was predominant in both assemblage A and assemblage B (66.6 and 58 %, respectively), but with no statistical insignificant difference (p value = .998) (Table 1).

As regards the association of the clinical manifestation of giardiasis and assemblage type, only flatulence recorded a significant difference between assemblages (p value = .001). However, patients with assemblage B showed variations of clinical symptoms, intermittent diarrhea (68 %), abdominal pain (36 %), persistent diarrhea (32 %), flatulence (28 %), and fever (8 %). While patients with assemblage A were complaining of intermittent diarrhea, one patient presented with abdominal pain and no other complains were recorded. Among variants of stool analysis, presence of fat particles on microscopic examination was detected in 36 % of assemblage B with statistical significant difference (p value = .027). Loose pattern of stool was predominant in assemblage A (66.6 %) while soft pattern (44 %) was predominant in assemblage B.

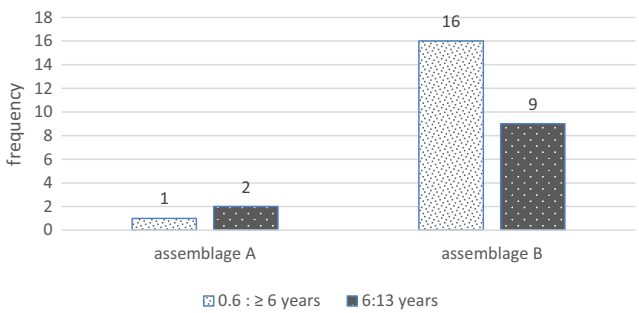

Fig. 1 *Giardia* assemblages A and B and age of the 28 successfully genotyped cases

Discussion

The results of this study detected that both assemblage of *G. duodenalis* A and B are present among children at Beni-Suef City with a microscopic prevalence of 27.9 %. Successful purification and sequencing of 28 samples were done using tpi gen. Assemblage B was predominant (89.3 %) among studied population while assemblage A was detected in only 10.7 %. In Egypt, many authors attributed infection of giardiasis with assemblage B, which is more prevalent than assemblage A. Foronda et al. (2008) reported 80 % prevalence of assemblage B, and Soliman et al. (2011) reported 86.6 % assemblage B prevalence. Helmy et al. (2014), El-Tantawy and Taman (2014), and Fahmy et al. (2015) reported similar observations. Worldwide distribution of *Giardia* assemblages in human isolates differs greatly. Assemblage B was predominant by some studies at UK (Amar et al. 2002), India (Traub et al. 2004), Rwanda (Ignatius et al. 2012), and Cuba (Puebla et al. 2014).

On the other hand, Helmy et al. (2009) reported 75.5 % of patients with diarrhea in Egypt were due to assemblage A and 19.5 % were assemblage B using tpi gene. Also, Eligio-Garcia et al. (2008) at Mexico, Gelanew et al. (2007) at Ethiopia, Al-Mohammed (2011) at Saudi Arabia, and Alyousefi et al. (2013) at Yemen reported predominance of assemblage A.

Human giardiasis is caused mostly by the assemblages (A and B). Subassemblage AI is transmitted in human isolates and number of animals while subassemblage AII is related to anthroponotic methods, but few studies reported its occurrence in animals (Cacciò et al. 2008; Feng and Xıao 2011). On the contrary, assemblage B subgroups are numerous and responsible possibly for human and animal infections (Ryan and Cacciò 2013).

In the present study, all cases of assemblage A were subtype AII, and subtype I was not recorded, suggesting that source of infection in patients with assemblage A is human to human than zoonotic transmission (Read et al. 2002). In Egypt, Fahmy et al. 2015 showed predominance of subgroup AII among 21 samples of assemblage A. Also, our results are consistent with those in the UK by Amar et al. (2002) and France by Bertrand et al (2005) in France. As regards distribution of assemblage B, it was detected in 25 samples (89.3 %), 5 of them (20 %) were subgroup BIV, 3 (12 %) were subgroup BIII while it was difficult to identify the remaining 17 (68 %). Some studies reported failure to identify subgrouping assemblage B, as Fahmy et al. (2015) showed 70.1 % assemblage B prevalence with difficulty for subgrouping all of them. On the contrary, Sadek et al. (2013) subgrouped all assemblage B cases as BIII.

Variation of giardiasis assemblage's distribution according to the geographical location is a sign of interest which still need explanations; however, it may point to the sources of infection in a certain community which differ widely.

Table 1 Association of variables with *Giardia* assemblages

Variable		Not examined (n = 130)	%	Assemblage A (n = 3)		Assemblage B (n = 25)		P value	95 % C.I.	
				NO	%	NO	%		Lower	Upper
Gender										
Male		77	59.2	2	66.6	13	52	.998	.321	1.728
Female		53	40.7	1	33.3	12	48			
Intermittent diarrhea		114	78.6	3	100	17	68	.105	.060	10.004
Persistent diarrhea		16	12.3	0	0	8	32	.058	.121	20.686
Fever		12	9.2	0	0	2	8	.999	.797	1.038
Flatulence		13	10	0	0	7	28	.001*	.742	1.050
Abdominal pain		28	21.5	1	33.3	9	36	.927	.070	11.221
Macroscopic examination	Soft	69	53	1	33.3	11	44	.999	.692	1.062
	Loose	21	16.1	2	66.6	9	36		.907	1.823
	Watery	5		0	0	5	20		.767	1.044
Microscopic examination	Giardia cyst	33	25.3	3	100	22	88	1.000	.705	1.067
	Giardia trophozoite	3	2.3	0	0	3	12		.426	1.079
	Undigested food	17	13.7	1	33.3	8	32	.322	.032	1.647
	Fat particles	11	8.4	0	0	9	36	.027*	.454	1.085
	RBCs	4	3	0	0	3	12	.983	.509	1.036
	Pus cells	7	5.3	0	0	2	8	.955	.654	1.065

*P value: significant p value >.05

Our results showed that children below age of 6 are 16 time (Fig. 1) at risk of infection with assemblage B than assemblage A, as those usually attend daily care centers and nursery schools and they are exposed to infection by assemblage B, which may be related to human to human route or animal sources of infection (Van Keulen et al. 2002; Castro-Hermida et al. 2006). In Egypt, few records are available for relation between age and assemblages type. El-Tantawy and Taman (2014) reported that the highest rate of infection is significantly in the 3–7 years old without mentioning either they were assemblage A or B. In the UK, Breathnach et al. (2010) and Minetti et al. (2015) reported assemblage B is prevalent in children than A. However, El Basha et al. (2016) recorded assemblage B was significantly higher in children with age range 6–16 while assemblage A was prevalent in children with younger age (2–8 years).

In our study, using the univariate logistic regression analysis showed that only flatulence was statistically significant between assemblages A and B (p value = .001). However, children infected with assemblage B showed variations of clinical symptoms than assemblage A. This comes in agreement with studies at Argentina (Molina et al. 2011), Cuba (Puebla et al. 2014), Emirates (El Bakri et al. 2014), and Egypt (Fahmy et al. 2015). However, other studies at Bangladesh (Haque et al. 2005), Spain (Sahagún et al. 2008), and Egypt (Fouad et al. 2014) have shown that patients infected with assemblage A were more clinically presented. Controversial, studies at Yemen (Alyousefi et al. 2013) and Malaysia (Choy et al. 2014) concluded that no association between assemblage types and clinical complains. Definitely, discrepancies reported by studies from different areas of the world may be caused by virulence of each assemblage and subassemblages and/or host factors (Thompson et al. 2000).

As regards our results, association of assemblage B with symptomatic patients may be a cause for high prevalence rate of assemblage B than A among positive samples of giardiasis, as symptomatic children mostly seek for clinical examination than asymptomatic one (Feng and Xıao 2011). In addition, 64 % of children infected by assemblage B were below 6 years and this age is highly observed by their mother to ask medical advice than older children.

In this study, the presence of fat particles on microscopic examination of samples was statistically significant (0.027 respectively). Many authors reported that diarrhea caused by giardiasis is accompanied by loose, greasy, foul-smelling stool (Thompson and Monis 2004; Thompson and Smith 2011). The greasy appearance of stool is due to malabsorption caused by the parasite which is multifactor in nature (Haque 2007).

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Compliance with ethical standards The research protocol was approved by the Ethics Committee of Scientific Research Developing Unit, Beni-Suef University and comes in accordance with the 1964 Helsinki declaration.

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent It was obtained from all parents of children included in the study, and they were informed about purpose of the study and sample collection (stool samples) was obtained after their agreement.

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