Predominance of *Giardia intestinalis* assemblage B in diarrhoeic children in Sharkia, Egypt

Ayman A. El-Badry[1], Faten A. Mohammed [2], Eman Abdul Gawad[3]

[1]Department of Microbiology-Medical Parasitology Section, College of Medicine, University of Dammam, Dammam, Saudi Arabia
[2]Parasitology Department, Faculty of Medicine, Zagazig University, Sharkia, Egypt
[3]Laboratory of Molecular Medical Parasitology (LMMP), Medical Parasitology Department, Kasr Al-Ainy Faculty of Medicine, Cairo University, Egypt

Original Article

ABSTRACT

Background: *Giardia intestinalis* (*G. intestinalis*) is one of the most common human enteric protozoa that infect Egyptian children, causing most of the diarrhoeic outbreaks worldwide. Identification of *G. intestinalis* assemblage is important because of its role in determining sources of infection, in understanding the ecological and clinical impact of giardiasis, and hence its management and control.
Objective: Study of this cross-sectional sample aimed to determine the genotypes of *G. intestinalis* isolated from the feces of diarrhoeic children attending outpatient clinic of Zagazig University hospital.
Subjects and Methods: A single fecal sample was collected from each child (n=126). All samples were subjected to microscopic examination by direct wet mount before and after formal- ethyl acetate concentration. Positive samples were amplified by nested PCR (nPCR) and sequenced for intra and inter assemblage identification targeting *tpi* gene.
Results: Microscopic examination revealed detection of *G. intestinalis* in 38 samples (30.2%). In 36 (28.6%) samples there was assemblage B predominance in 34 (94.4%) isolates, among which 24 (70.6%) were subgenotype BIV and 10 (29.4%) were sub-genotype BIII. Only 2 (5.6%) samples had assemblage A; all of them were AII subgenotype. All studied patient`s demographic and clinical data showed no significant association with *Giardia* infection or prevailing *Giardia* assemblages.
Conclusion: *G. intestinalis* is the prevailing intestinal pathogen in diarrhoeic Egyptian children, with *Giardia* assemblage B predominance. These findings necessitate physicians' attention and further genetic studies in Egypt and other endemic areas targeting different genetic loci, with the inclusion of larger population samples. This will lead to a better understanding of the ecological and clinical impact of giardiasis, its management and control.

Key Words: Assemblage, genotyping, *Giardia*, nPCR, *tpi* gene.

Received: 15 February 2017, **Accepted:** 12 March 2017.

Corresponding Author: Faten A. Mohammed, **Tel.:** +20-10-2031-1001, **E-mail:** fatenmr@yahoo.

ISSN: 1687-7942, Vol. 10, No. 1 & 2.

INTRODUCTION

Giardiasis, an infection with *G. intestinalis* (synonyms: *G. duodenalis, G. lamblia*), is one of the most common intestinal protozoan present in humans, both in Egypt and worldwide[1,2]. Clinical presentations of giardiasis vary, ranging from asymptomatic infection to chronic symptomatic infection. Infected patients present with diarrhea, steatorrhea, flatulence, and malabsorption and/or weight loss[3-5]. Giardiasis can affect all age groups; however, children are the most affected age group, for whom it can cause severe acute diarrhea that may in turn lead to malabsorption, malnutrition with growth impairment[6,4,5].

The prevalence of human giardiasis varies from 2% in developed countries to 70% in developing countries[7]. In Egypt, prevalence of giardiasis is up to 48%[8], a fact that makes Egypt a hyperendemic region according to the World Health Organization (WHO) criteria[9]. However, its true prevalence is unknown.

G. intestinalis is classified into eight assemblages from A to H[10-13]. Determination of *Giardia* assemblages is needed to understand routes of transmission, epidemiology, and range of the host, in addition to study of outbreaks[14]. In a report from Egypt, El Tantawy and Taman[15], reported the predominance of assemblage B over assemblage A, and few mixed A and B. The aim of the present study was to update the understanding of the epidemiology of *Giardia* assemblages prevailing in children attending outpatient clinics of Zagazig University Hospital.

MATERIALS AND METHODS

Study type and populations: This cross-sectional study sample included 126 children (5 to 12 years old),

 DOI: 10.21608/PUJ.2017.4735

suffering from diarrhea and/or other gastrointestinal symptoms, attending outpatient clinics of Zagazig University Hospital, Egypt.

Samples collection and processing: A single stool sample was collected from each diarrheic child attending the outpatient clinic. All collected samples were examined microscopically with iodine wet mount, after formal-ethyl acetate concentration at Parasitology Department Laboratory, Faculty of Medicine, Zagazig Unversity to detect *G. intestinalis* and other parasites. Part from each stool sample was freshly frozen at -20°C for copro immuno-molecular assays at the Laboratory of Molecular Medical Parasitology (LMMP), Kasr Al-Ainy Faculty of Medicine, Cairo University.

Copro-PCR assay: All samples microscopically positive for *G. intestinalis* trophozoites and/or cysts were subjected to genomic DNA extraction using Favor Prep stool DNA isolation Kit (Favorgen Biotech corporation ping-Tung 908, Taiwan), according to the manufacturer's instructions.The extracted DNA was submitted to a nPCR procedure for amplification of a 530-bp region targeting the triosephosphate isomerase (*tpi*) gene, using AL3543 (19 bp): 5′-AAA TIA TGC CTG CTC GTC G-3′ and AL3546 (19 bp): 5`-CAA ACC TTI TCC GCA AAC C-3 primers for first PCR, AL3544 (20 bp): 5′-CCC TTC ATC GGI GGT AAC TT-3′ and AL3545 (20 bp): 5`- GTG GCC ACC ACI CCC GTG CC-3 for nPCR. The reaction mixture and conditions were performed according to Sulaiman *et al.*[16] in a volume of 25 µl containing 3 µl of DNA extract for first PCR reaction or 1 µl of the second PCR reaction of the nPCR, 12.5 µl master mix PCR kit (thermo scientific, UK, Lot no.#K1081), 1 µl of each forward and reverse primer, 0.1 µl of Taq polymerase (Qiagen, Germany) and 7.4-9.4 µl ddH2O. PCR reactions were performed in Biometra thermal cycler (Tpersonal). The PCR amplification reactions for *Giardia* were performed in conditions consisting of 5 min at 95°C followed by 35 cycles of 45 sec at 94°C, 45 sec at 50°C, 60 sec at 72°C and, finally, by 7 min at 72°C. Positive control, negative control and blank containing sterile distilled water in place of template DNA were included for each reaction set. Amplified DNA fragments were analyzed by electrophoresis in a 1.5% (w/v) agarose gel stained with ethidium bromide (0.5 µg/ ml) and visualized under a UV light system[17].

Sequencing: nPCR products were sequenced to determine *Giardia* assemblage. The amplified products were purified using Qiagen PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and visualized on 1.5% agarose gel electrophoresis. Purified nPCR products were sequenced with BigDye® Terminator v 3.1. Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Big Dye X purification kit (Applied Biosystems, Foster City, CA,USA) were used to clean post sequencing reaction products according to the manufacturer's instructions. DNA template sequencing was performed in both directions on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA,USA).

Statistical analysis: Data entry and analysis were performed using the statistical package for social sciences (SPSS) version 20. Mean ± standard deviation were used for analysis of descriptive data, while frequencies were used to analyze qualitative data. *Giardia* infection was the dependent variable and chi-square test was used to assess its significance of association with the independent variables (demographic data and clinical symptoms).

Ethical considerations: Fecal samples were collected from children after informing their parents about the purpose of the study and informed consent was obtained from parents of all children included in the study.

RESULTS

G. intestinalis was detected in 38 samples (30.2%). Among them 36 (28.6%) samples were successfully amplified by nPCR and genotyped by sequencing using *tpi* gene (Fig. 1). Their age ranged from 5 to 12 years old with mean of 7.34 ± 2.33 without statistical significance (*P*=0.96). Both *Giardia* assemblage B and A (94.4% [n=34], 5.6% [n=2], respectively) were detected. All assemblage A samples were AII while subgenotyping of assemblage B was BIII and BIV (29.4% [n=10], 70.6% [n=24], respectively) (Table 1). All studied variables showed no significant association with *Giardia* infection or detected *Giardia* assemblage (Table 2).

Table 1: Diagnostic yield of nPCR and sequencing among microscopically positive samples.

	nPCR					
	Positive (n=36)				**Negative**	Total
	Assemblage AII	Assemblage B				
		BIII	BIV	Total		
Positive microscopy	2	10	24	34	2	38

Table 2: Demographic and clinical data of the studied children positive by nPCR (n=36).

Variables		n (%)	*P* value
Age group (years old)			
>2-6		16 (44.5)	0.96
>6-12		20 (55.5)	
Sex			
Male		24 (66.7)	0.33
Female		12 (33.3)	
Clinical examination	Abdominal cramps	13 (36.1)	0.27
	Flatulence	5 (13.9)	0.56
	Diarrhea	5 (13.9)	0.56
	Vomiting	7 (19.4)	0.47
Stool consistency	Loose	11 (30.6)	
	Soft	15 (41.7)	0.23
	Greasy	10 (27.8)	

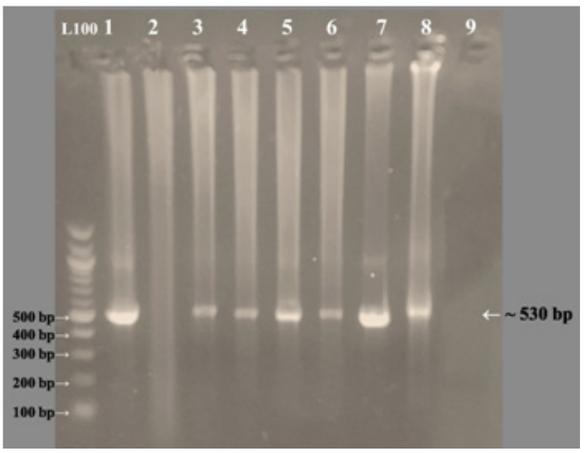

Fig. 1: Agarose gel stained with ethidium bromide for *Giardia* nPCR product of study samples targeting *tpi* gene: lane L100 Molecular weight marker (100 bp), lane 1 is positive control at 530 bp, lane 2 is negative control, lanes 3-8 are positive specimens, lane 9 is a negative specimen.

DISCUSSION

Diarrheal disease is one of the worldwide leading causes of children morbidity and mortality with 2 billion cases yearly[18]. Giardiasis causes most of diarrhea cases worldwide[19]. *G. intestinalis* was detected in 30.2% of diarrhoeic children from Zagazig city in our study. Phylogenetically, within genus *Giardia* there are 6 species, amongst which only *G. intestinalis* has been documented as infecting humans[11,13]. Based on molecular analyses, 8 assemblages A-H of *G. intestinalis* species were recognized; assemblages A and B infect mammals including humans, while, assemblages C-H are zoonotic and more host specific[10,12]. Lebbad *et al.*[20] reported that there is limited zoonotic transmission among human *Giardia* genotypes. Different loci on multiple genes have been targeted for *G. intestinalis* molecular sub-typing, including small subunit ribosomal RNA, β-giardin, glutamate dehydrogenase, elongation factor 1-alpha (*gdh*), *tpi*, GLORF-C4 and the inter-genomic rRNA spacer region[21].

β-giardin gene was considered a target for the molecular detection of *Giardia* spp. as it is unique to *Giardia*[22]. Nevertheless, *tpi* gene is preferred by many studies due to its high genetic heterogenicity[14,23]. The ssrRNA gene is the least used because it lacks specificity[24,25]. Direct sequencing of an amplified DNA sequence remains the "gold standard" for accurate typing and sub-typing of *Giardia*[26].

Targeting the *gdh* gene by PCR-RFLP revealed that assemblage AII was much higher than assemblage BIII in cases from Menofeya and Sharkia Governorates[27]. Also from Egypt, El Tantawy and Taman[15], targeting the *tpi* gene, reported 62.14% as assemblage B, 31.07% as A, and mixed A and B in 2.91% with 3.88% of undetermined species. Hence with variable reports on prevalence, assemblage B seems to be the most prevailing assemblage for *Giardia* infections in Egypt as documented in many Egyptian studies in Cairo[2,28], Kafr Elshiekh[29], and Ismailia[13,30].

Our study revealed a much higher assemblage B predominance (94.4%), with no association between *G. intestinalis* and the patient`s demographic and clinical data. There continues to be controversial reports

concerning correlation between *Giardia* assemblage (A or B) and clinical symptoms. Although there are reports on the lack of correlation between *Giardia* assemblage (A or B) and clinical symptoms[31,32], a few studies report significant correlation[33-35]. In the report by El Tantawy and Taman[15], the detection rate of assemblage B was higher in samples from children with persistent diarrhea, whereas assemblage A detection rate was higher in samples from acute diarrhea. Thus, we corroborate the report of Rafiei *et al*.[36] on the need for more studies to understand these relations.

Giardia was the prevailing enteric pathogen (38/126 [30.2%]) among our study group, indicating that physicians and public health professionals should consider *Giardia* in diarrhoeic children. Giardiasis seems to be endemic in Zagazig as none of the study population had travel history.

We amplified *Giardia* copro-DNA by nPCR for 36 of the 38 microscopically positive samples. Negativity of positive microscopic samples by PCR may be attributed to the different *Giardia* strains, which may include single-nucleotide polymorphisms, insertion-deletion, and rearrangements of chromosomes. The negative results could also be explained by the presence of inhibitors in stool that may affect the DNA amplification[37].

In conclusion, *G. intestinalis* is the prevailing intestinal pathogen found in diarrhoeic Egyptian children, with a predominance of *Giardia* assemblage B. These findings necessitate physicians' attention when diagnosing diarrhoeic patients. Further genetic studies are needed, in Egypt and other endemic areas, targeting different genetic loci, with the inclusion of larger population samples. This will lead to a better understanding of the ecological and clinical impact of *Giardia* infection and its management and control.

Author contribution: All authors contributed equally in the work.

REFERENCES

1. Adam RD. Biology of *Giardia lamblia*. Clin Microbiol Rev, 2001; 14: 447-475.

2. Ismail MA, El-Akkad DM, Rizk EM, El-Askary HM, El-Badry AA. Molecular seasonality of *Giardia lamblia* in a cohort of Egyptian children: A circannual pattern. Parasitol Res, 2016; 115(11): 4221-4227.

3. Nematian J, Gholamrezanezhad A, Nematian E. Giardiasis and other intestinal parasitic infections in relation to anthropometric indicators of malnutrition: a large, population based survey of schoolchildren in Tehran. Ann Trop Med Parasitol, 2008; 102(3): 209-214.

4. Puebla LJ, Núñez FA, Fernández YA, Fraga J, Rivero LR, Millán IA, Valdes LA, *et al*. Correlation of *Giardia duodenalis* assemblages with clinical and epidemiological data in Cuban children. Infect Genet Evol, 2014; 23: 7-12.

5. Tsourdi E, Heidrich FM, Winzer M, Röllig C, Kirsch C, Meinel J, *et al.* An exotic cause of exudative enteropathy. Am J Case Rep, 2014; 15: 226-229.

6. Bonhomme J, Le Goff L, Lemée V, Gargala G, Ballet JJ, Favennec L. Limitations of *tpi* and *bg* genes sub-genotyping for characterization of human *Giardia duodenalis* isolates. Parasitol Int, 2011; 60(3): 327-330.

7. Eligio-García L, Cortes-Campos A, Cota-Guajardo S, Gaxiola S, Jiménez-Cardoso E. Frequency of *Giardia intestinalis* assemblages isolated from dogs and humans in a community from Culiacan, Sinaloa, Mexico using beta-giardin restriction gene. Vet Parasitol, 2008; 156(3-4): 205-209.

8. Fahmy HM, El-Serougi AO, El Deeb HK, Hussein HM, Abou-Seri HM, Klotz C, *et al. Giardia duodenalis* assemblages in Egyptian children with diarrhea. Eur J Clin Microbiol Infect Dis, 2015; 34(8): 1573-1581.

9. WHO 1987: Prevention and control of intestinal parasitic infections. WHO Technical Report Series, 379, 1-47.

10. Cooper MA, Adam RD, Worobey M, Sterling CR. Population genetics provides evidence for recombination in *Giardia*. Curr Biol, 2007; 17: 1984-1988.

11. Lasek-Nesselquist E, Welch DM, Sogin ML. The identification of a new *Giardia duodenalis* assemblage in marine vertebrates and a preliminary analysis of *G. dodenalis* population biology in marine systems. Int J Parasitol, 2010; 40: 1063-1074.

12. Lebbad M, Mattsson JG, Christensson B, Ljungstrom B, Backhans A, Andersson JO, *et al.* From mouse to moose: multilocus genotyping of *Giardia* isolates from various animal species. Vet Parasitol, 2010; 168: 231-239.

13. Solaiman RH, Fuentes I, Rubio JM. Identification of a novel assemblage B subgenotype and a zoonotic assemblage in human isolates of *Giardia intestinalis* in Egypt. Parasitol Int, 2011; 60: 507-511.

14. Thompson RCA, Monis PT. Variation in *Giardia*: Implications for taxonomy and epidemiology. Adv Parasitol, 2004; 58: 69-137.

15. El Tantawy NL, Taman AI. The epidemiology of *Giardia intestinalis* assemblages A and B among

Egyptian children with diarrhea: A PCR–RFLP-based approach. PUJ, 2014; 7: 104-109

16. Sulaiman IM, Fayer R, Bern C, Gilman RH, Trout JM, Schantz PM, *et al.* Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerg Infect Dis, 2003; 9(11): 1444-1452.

17. Chvallet M, Luche S, Rabilloud T. Silver staining of proteins in polyacrylamide gels. Nat Protocol, 2006; 1(4): 1852-1858.

18. WHO 2009: Diarrhoea disease. Fact sheet No. 330. Available at: http://www.who.int/mediacentre/factsheets/fs330/en/index.html

19. Mahdy MAK, Surin J, Wan KL, Mohd-Adnan A, Al-Mekhlafi MS, Lim YA. *Giardia intestinalis* genotypes: Risk factors and correlation with clinical symptoms. Acta Trop, 2009; 112(1): 67-70.

20. Lebbad M, Petersson I, Karlsson L, Botero-Kleiven S, Andersson JO, Svenungsson B, *et al.* Multilocus genotyping of human *Giardia* isolates suggests limited zoonotic transmission and association between assemblage B and flatulence in children. PLoS Negl Trop Dis, 2011; 5: e1262.

21. Caccio SM, Ryan U. Molecular epidemiology of giardiasis. Mol Biochem Parasitol, 2008; 160: 75-80.

22. Caccio, SM, De Giacomo M, Pozio E. Sequence analysis of the β-giardin gene and development of a polymerase chain reaction-restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. Int J Parasitol, 2002; 32: 1023-1030.

23. Huey CS, Mahdy MAK, Al-Mekhlafi HM, Nasr NA, Lim YAL, Mahmud R. Multilocus genotyping of *Giardia duodenalis* in Malaysia. Infect Genet Evol, 2013; 14: 269-276.

24. Weiss JB, Vankeulen H, Nash TE. Classification of subgroups of *Giardia lamblia* based upon ribosomal RNA gene sequence using the polymerase chain reaction. Mol Biochem Parasitol, 1992; 54: 73-86.

25. Ghieth, MA, Kotb MA, Abu-Sarea EY, El-Badry AA. Molecular detection of giardiasis among children at Cairo University Pediatrics Hospitals. J Parasit Dis, 2016; 40(4):1470-1474.

26. Geurden T, Levecke B, Caccio SM, Visser A, De Groote G, Casaert S. *et al.* Multilocus genotyping of *Cryptosporidium* and *Giardia* in non-outbreak related cases of diarrhoea in human patients in Belgium. Parasitology, 2009; 136: 1161-1168.

27. Sadek GS, El-Settawy MA, Soha A, Nasr SA. Genotypic characterization of *Giardia duodenalis* in children in Menoufiya and Sharkiya governorates, Egypt. Life Sci J, 2013; 10(1): 4006-4015.

28. Ghieth MA, El-Badry AA, Abu-Sarea EY, Abdel Gawad SS, Elsharkawy MM. Genotypic analysis of *Giardia duodenalis* in children at Egypt. Comp Clin Pathol, 2016; 25(6): 1241-1246.

29. Amer SE. Genotypic and phylogenetic characterization of *Giardia intestinalis* from human and dairy cattle in Kafr El Sheikh Governorate, Egypt. J Egypt Soc Parasitol, 2013; 43(1): 133-46.

30. Helmy YA, Krucken J, Nockler K, Samson-Himmelstjerna GV, Zessin KH. Comparison between two commercially available serological tests and polymerase chain reaction in the diagnosis of *Cryptosporidium* in animals and diarrhoeic children. Parasitol Res, 2014;113(1): 211-216.

31. Kohli A, Bushin OY, Pinkerton RC, Houpt E, Newman RD, Sears CL, *et al*. *G. duodenalis* assemblage, clinical presentation and markers of intestinal inflammation in Brazilian children. Trans R Soc Trop Med Hyg, 2008; 102: 718-725.

32. Ajjampur SSR, Sankaran P, Kannan A, Sathyakumar K, Sarkar R, Gladstone B, *et al. G. duodenalis* assemblages associated with diarrhea in children in south India identified by PCR-RFLP. Am J Trop Med, 2009; 80: 16-19.

33. Read C, Walters J, Robertson ID, Thompson RCA. Correlation between genotype *Giardia duodenalis* and diarrhoea. Int J Parasitol, 2002; 32: 229-231.

34. Cordon PG, Saldon OCP, Vasquezas VF, Soto JR, Bordes LS, Moreno MS, *et al*. Prevalence of enteroparasites and genotyping of *G. duodenalis* in Peruvian children. Parasitol Res, 2008; 103: 459-465.

35. Etemadi S, Zia-Ali N, Babaei Z, Fasihi-Harandi M, Zia-Ali A, Salari Z, *et al.* The correlation between clinical signs and genotypes of *G. duodenalis* isolated from patients with gardiasis in Kerman City. J Kerman Univ Med Sci, 2011: 18: 330-338.

36. Rafiei A, Roointan SE, Samarbafzadeh RA, Shayesteh AA, Shamsizadeh A, Borujeni PM. Investigation of possible correlation between *Giardia duodenalis* genotypes and clinical symptoms in Southwest of Iran. Iranian J Parasitol, 2013; 8(3): 389-395.

37. Abbaszadegan MR, Velayati A, Tavasoli A, Ryu H. Rapid DNA extraction protocol from stool suitable for molecular genetic diagnosis of colon cancer. IBJ, 2007; 11: 203-208.