Journal of the Egyptian Society of Parasitology, Vol.48, No.2, August 2018
J. Egypt. Soc. Parasitol. (JESP), 48(2), 2018: 465 – 474

GIARDIA INTESTINALIS ASSEMBLAGES AMONG EGYPTIAN SYMPTOMATIC/ASYMPTOMATIC CASES IN CAIRO

By

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Abstract

Giardia intestinalis is frequent enteric protozoa, affecting humans worldwide. Human infections are mainly caused by two genetically different assemblages call A & B. This cross-sectional study characterized the *Giardia* genotypes isolated from the stool of symptomatic and asymptomatic Egyptians in Cairo and correlated these genotypes with the demographic and clinical data of the cases. Stool samples were collected from 389 individuals (245 complaining of gastrointestinal (GIT) symptoms and 144 apparently healthy asymptomatic individuals), and microscopically examined. Positive Samples for *G. intestinalis* were molecularly characterized by Copro-nPCR targeting beta-giardin gene, and then analyzed by RFLP for assemblage identification. *Giardia* was detected in 62 samples (15.9%); 53 samples in symptomatic cases (21.6%) and 9 samples in asymptomatic individuals (6.25%). DNA of positive samples was amplified by nPCR-RFLP assays. There was a significant predominance of assemblage B among symptomatic (82.7%) and asymptomatic (77.8%) groups, while the rest of samples had assemblage A. Among the clinical data, only flatulence was significantly associated with *Giardia* infection with assemblage B. Assemblage B is the predominant genotype found in Egypt in symptomatic and asymptomatic patients suggesting an anthropologic transmission cycle.

Key Words: *Giardia*; genotyping; Assemblage; nPCR-RFLP; beta-giardin.

Introduction

Giardia intestinalis (*G. intestinalis*) is a flagellated micro-organism that represents an essential global cause of diarrhea in humans (Feng and Xiao, 2011). WHO had included *Giardia*sis in the Neglected Diseases Initiative since 2004 due its influence on health (Savioli *et al,* 2006). The parasite can cause gastrointestinal infections extending from mild to severe or protracted disease. The clinical severity of *Giardia*sis depends on host factors (e.g. nutritional status, microbiome and immunity) as well as parasitic factors (e.g. infecting strain and infectious dose) (Tsourdi *et al,* 2014). The prevalence of giardiasis in African children can exceed 30% (Thompson and Smith, 2011).

Despite the widespread use of microscopy in epidemiological analyses, the real prevalence of giardiasis is underestimated (Verweij *et al,* 2004). PCR-based assays have higher sensitivity than microscopy detecting the parasite in stool samples (Ghosh *et al,* 2000). Molecular tools were used to offer better understandings of the taxonomy, host variety and transmission pathways of *Giardia* (Fayer *et al,* 2000).

G. intestinalis complex isolates are categorized into eight different genetic assemblages from A to H based on the characterization of their small subunit ribosomal RNA (ssu-rRNA), the glutamate dehydrogenase (gdh), the β-giardin (bg), the triose phosphate isomerise (tpi), the elongation factor 1-alpha (ef-1α), and the GLORF-C4 genes (Cacciò and Rayan, 2008; Monis *et al,* 2009). Assemblages A & B caused human infection worldwide, but the relative prevalence of assemblages varies geographically (Thompson and Monis, 2004). Although many in-

vestigations have been undertaken to relate the severity of the infection with a particular assembly (Sahagun *et al*, 2008), there remains no clearly demonstrated predictable relationship between the parasite assemblages and the severity of infection (Cacciò and Rayan, 2008). Very little information is known about the assemblages' diversity and multi-locus genotype in North Africa cases. In the existing study, the genotypes of *G. intestinalis* isolated from symptomatic and apparently healthy asymptomatic cases attending outpatient clinics in Kasr Al-Ainy University Hospitals were identified from the collected stool samples which were positive for *Giardia* by microscopy and then typed using PCR techniques (Nested PCR using *beta-giardin* gene & PCR-RFLP). The detected assemblages were correlated with the symptoms and demographic data of the cases.

Methodology

Study cases: This cross-sectional study was done on 389 Egyptian cases attending outpatient clinics in Kasr Al-Ainy University Hospitals to characterize the genotypes *of Giardia intestinalis* in the symptomatic/asymptomatic cases. The studied population was divided into 2 groups; GI: which included 245 patients suffering from GIT symptoms and GII with 144 persons not suffering from any GIT symptoms.

The study was permitted by the Ethical Board of the Medical Parasitology Department and the Ethical Board of Faculty of Medicine, Cairo University. Consents were taken from adult cases and custodians of the children included in the study before sample collection, and participation was optional. Complete medical history was obtained from each participant before doing any investigations.

Collection and processing of samples: One fecal sample from each participant was obtained in a dry, clean, leak-proof plastic container. A questionnaire containing demographic, clinical and environmental data was obtained with each sample.

Each sample was divided into three portions; a small portion for direct smear examination, another portion was preserved in tight containers using formalin-saline fixative for microscopic analysis, and the third portion of the sample was stored without any preservatives at -20^{o}c in Eppendorf tubes for molecular studies.

Coproscopic examination of samples: Wet smears stained with Lugol's iodine from all stool samples were immediately examined microscopically and Negative samples were subjected to concentration using the modified Ritchie's biphasic method (Garcia, 2007) before reexamined microscopically**.**

Copro-nPCR assays: 1- Genomic DNA extraction from stool samples: Following a light microscopy analysis, positive *Giardia* samples were submitted to DNA extraction, in the Laboratory of Molecular Medical Parasitology, Parasitology Department, Kasr Al-Ainy using a Favor Prep stool DNA isolation Mini Kit (Favorgen Biotech corporation Ping-Tung 908, Taiwan, Cat. No. FAS-TI001). Extraction of genomic DNA was performed according to the manufacturer's guide and modified according to Fontaine and Guillot (2006). The purified DNA was measured for the concentration and purity (Qubit® 2.0 Fluorometer) then used immediately or stored at -20^{0}C till used.

2- DNA amplification using Nested-PCR: Extracted genomic DNA was analyzed by nested-PCR using β-giardin (bg) gene. The first amplification reaction generated a 753bp fragment using the primer pair set G7; 5'-AAG CCC GAC GAC CTC ACC CGC AGT GC -3' and G759; 5'-CAT AAC GAC GCC ATC GCG GCT CTC AGG AA-3' (Caccio` *et al,* 2002).The second amplification produced a 511bp fragment using the primer pair set 2005F; 5'- GAA C GA ACG AGA TCG AGG TCC G -3' and 2005R; 5'-CTC GAC GAG CTT CGT GTT-3' (Lallea *et al,* 2005).

The primary amplification was carried out with 10 minutes of initial denaturation at 95ºC followed by 35 cycles of 95ºC for 30

seconds, 65ºC for 30 seconds and 72ºC for 1 minute and a final extension of 10 minutes at 72ºC. Samples were keeping at 4ºC until its analysis. The nested-PCR conditions were the same except that annealing temperature was performed at 53ºC.

All primary reactions contained 12.5µL 2x Master Mix (Thermo scientific, UK, Lot no.#K1081), 1µl (0.2µM) of each primer, 0.1µl Taq Polymerase (5units/µl) for reaction activation, 3µL of template DNA & 7.4 µl of double distilled water (ddH_2O). All n-PCR reactions contained 12.5µL 2x Master Mix, 1µl (200nM) of each primer, 0.1µl Taq Polymerase (5units/µl), 1µL of template DNA and 9.4µl of ddH_2O to obtain a final volume of 25µL.

Two negative control samples were routinely used for every extraction series and in every PCR run, which contained only the reagents and no template. DNA of the positive control sample was also involved in the reactions. For detection of possible inhibitors, PCR-inhibition control sample containing the sample DNA and the DNA of the positive sample was run along each sample.

Visualization of the obtained amplified sequences was done using 1.5% agarose gel electrophoresis following staining by ethidium bromide (Chevallet *et al*, 2006).

Genetic characterization of *G. intestinalis:* The positive n-PCR samples were then analyzed by PCR-RFLP, to genetically characterize the *G. intestinalis* assemblages. PCR-RFLP was done by digestion of the secondary products of n-PCR with 10 units/µl of the endonuclease enzyme HaeIII (511pb fragment) (New England Biolabs Inc., USA). Digestion of PCR products of positive samples for *Giardia* after amplification was done in accordance to the manufacturer's guidelines. The following were added: PCR reaction mixture (10µl), Nuclease-free water (17µl), Green buffer (2µl.) & HaeIII (1µl) were added to reach a total a volume of 30µL for 4h at 37°C.

The restriction fragments were further examined by 3% Metaphor agarose gel electrophoresis stained by ethidium bromide, the fragments were visualized by Ultra-Violet Trans-illumination to determine *G. intestinalis* assemblages. A squematic representation of the expected bands of *Giardia* assemblages was given (Fig. 1).

bp	*Giardia* Assemblages					
	A	B	C	D	E	F
201	■					
200				■		
194			■	■		
186					■	■
150	■	■	■		■	■
117		■		■		
110	■	■	■		■	■
102						
84		■				
50	■		■			■
26		■			■	
24		■			■	
15			■		■	F. 1

Fig. 1: Diagram of expected *Giardia* assemblage profiles after Hae III digestion of nPCR products

Statistical analysis: Data were analyzed using SPSS version 16. Percentages were used to analyze the categorical and quantitative variables. Pearson´s chi-squared and

Fisher's Exact Tests were used for categorical data. Statistical significance of results was set as $p<0.05$.

Results

Clinical and demographic data of the studied population: Most of the participants are under 18 years old with an equilibrated percentage between males and females (Tab. 1).

Table 1: The demographic and clinical data of the studied population

Studied population			Symptomatic (n= 245)	Asymptomatic (n= 144)	Total (n=389)
Demographic data	Age	<18	225 (91.9%)	132 (91.7%)	357 (91.8%)
		19-49	17 (6.9%)	9 (6.2%)	26 (6.7%)
		>50	3 (1.2%)	3 (2.1%)	6 (1.5%)
	Gender	Males	120 (49%)	73 (50.7%)	193 (49.6%)
		Females	125 (51%)	71 (49.3%)	196 (50.4%)
Clinical Data		Diarrhea	151 (61.6%)	-----	
		Abdominal pain	150 (61.2%)	------	
		Flatulence	138 (53.9%)	------	
		Fever	33 (13.5%)	------	

Parasitological coproscopic analysis: Microscopic examination was performed for all samples. *Giardia* was the most commonly detected pathogen in the symptomatic group being isolated in 22.5% of the diarrheal cases, 13.3% of cases with abdominal pain, 10.1% of flatulence cases and 9.1% of cases with fever. Also, *Giardia* was also the most prevalent pathogen detected in the asymptomatic cases being present in 13.2% of cases. The prevalence of *Giardia* was statistically significant higher in symptomatic than in asymptomatic group ($P < 0.001$) and the main complaint of positive cases were diarrhea and abdominal pain representing 79.1% and 46.5%, of cases respectively ($P <0.001$).

Regarding the age distribution among the positive *Giardia* cases there was significant predominance of *Giardia* infection among cases below the age of 18 years, the study had shown 95.4% of *Giardia* cases below the age 18 years ($P < 0.001$) and only 4.6% were between 19 & 49 years among the symptomatic group. Besides, all asymptomatic *Giardia* cases were below the age of 18 years ($P < 0.001$) but without significant difference concerning the age distribution between the groups ($P = 0.822$). Similarly, difference in gender between the positive symptomatic and asymptomatic cases there was not significant (P = 0.396).

Copro-nPCR-RFLP assays analysis: Out of 389 cases, 62 were positive for *Giardia* cysts by microscopy and were subjected to nested-PCR. Nested-PCR assay resulted in 52 positive and 10 negative cases. Regarding the distribution of *Giardia* assemblages among the studied groups, there was a significant predominance of assemblage B in all groups ($P < 0.001$ (Figs. 2, 3).

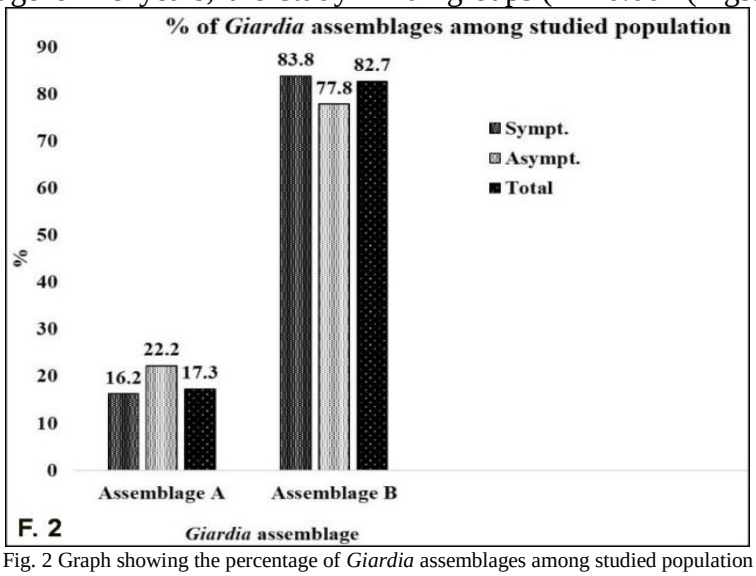

Fig. 2 Graph showing the percentage of *Giardia* assemblages among studied population

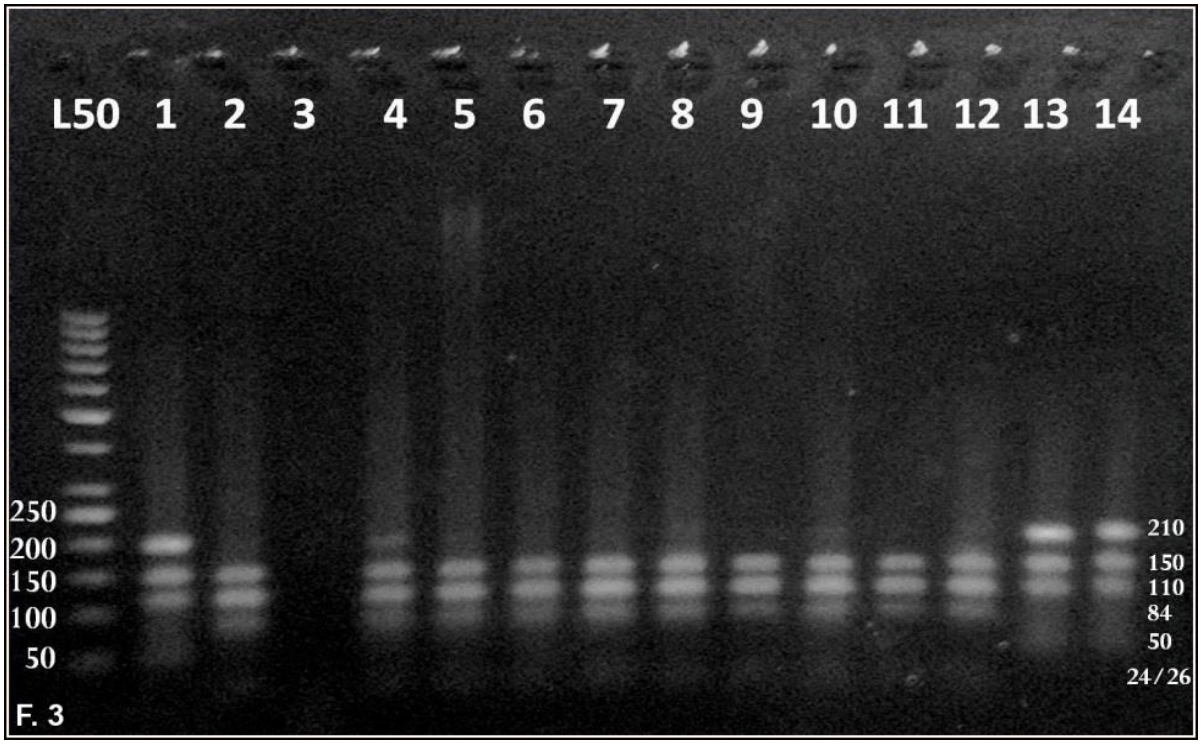

Fig. 3: nPCR-RFLP product following digestion of *Giardia* samples targeting *B*-giardin gene with endonuclease visualized on agarose gel: Lane (L50) MW marker of 50 bp, L1: Positive control assemblage A showing bands at 50 (faint), 110, 150 & 210 bp, L2: Positive control assemblage B showing bands at 24/26 (faint), 84, 117, & 150 bp, L3: Negative control samples, L5-12: assemblage B digestion product, L4, 13 & 14: assemblage A digestion product.

Giardia assemblages' distribution among cases relative to clinical & demographic data (Tab. 2):

Table 2: Distribution of *Giardia* assemblages among cases in relation to their clinical and demographic data

Studied population			Assemblage A (n= 9)	Assemblage B (n= 43)	Total	*P*- value
Groups	Symptomatic		7	36	43	0.668
	Asymptomatic		2	7	9	
Demographic data	Age	<18	9	41	50	0.316
		19-49	0	2	2	
		>50	0	0	0	
	Gender	Males	5	23	28	0.910
		Females	4	20	24	
Clinical data	Diarrhea	Present	4	30	34	0.146
		Absent	5	13	18	
	Abdominal pain	Present	4	16	20	0.685
		Absent	5	27	32	
	Flatulence	Present	0	14	14	0.045*
		Absent	9	29	38	
	Fever	Present	0	3	3	0.414
		Absent	9	40	49	

Data presented as n & (*) *P* value < 0.05 was significant

Giardia assemblages had no significant relation regarding whether the studied individuals had symptoms or not (*P* =0.668) with the predominance of Assemblage B in both groups.

Assemblage B was significantly predominant among all age groups than Assemblage A with no significant difference between age groups (*P*-value =0.316). Also, Assemblage B was significantly predominant in both males and females without significant difference in the cases' sexes (*P* = 0.910). As to elation between both assemblages and the presented symptoms within the symptomatic group, flatulence was the only the symptom that had significant correlation with *Giardia* Assemblage B (*P* =0.045) which was predominant in cases complaining of flatulence.

Discussion

Giardia intestinalis is a cosmopolitan unicellular flagellate parasite infecting a variety of animals including man. The genotypes of *Giardia intestinalis* include 8 Assemblages from A to H (Lasek-Nesselquist *et al,* 2010). Assemblages A & B are known to cause *Giardia*sis in humans and also were detected in various animals (Caccio *et al,* 2008).

The current study aimed to define the predominant genotypes of *G. intestinalis* among cases attending Kasr Al-Ainy Hospitals, Cairo and to correlate *G. intestinalis* assemblages with the clinical and demographic data of the cases. *Giardia*sis causes diversity of clinical forms extending from asymptomatic carriers, to acute or chronic disease (Eckman, 2003).

In the present study, diarrhea, abdominal pain and flatulence were the predominant symptoms. This matches previously reported findings who reported the occurrence of abdominal pain in 50-80% of patients infected with *Giardia* (Heresi *et al,* 2000; D'Anchino *et al,* 2002; Hill and Nash, 2006).

In the studied groups, asymptomatic carriers were 6.25% of the asymptomatic individuals. In immuno-competent persons giardiasis was self-limiting and might pass without clinical symptoms (Eckman, 2003).

There epidemiological evidence suggested that infection with *G. intestinalis* has the capability to modulate the host immune system, which may be influenced by concomitant infections with enteric pathogens. In previous studies, *G. intestinalis* infection was associated with infections as *Cryptosporidium spp.* (Wang *et al,* 2013), *Blastocystis spp.* (Elghareeb *et al,* 2015) and enteric bacteria (Ankarklev *et al,* 2012; Júlio *et al,* 2012; Eldash *et al,* 2013; Krumkamp *et al,* 2015).

In the present study, the mixed parasitic infection in concomitant with *Giardia* infection in seven samples within the symptomatic group only, including *Entamoeba histolytica/dispar* complex, *Blastocystis* spp. and *Entamoeba coli.* More research should be directed to clarify the physiological and immunological mechanisms and the influence of concomitant infection on the virulence and final outcome of *Giardia* infection.

Various molecular techniques including the Nested-PCR followed by PCR-RFLP were used to study the epidemiology and animal role in human infection and to discriminate *Giardia* assemblages (Feng and Xiao, 2011). PCR-RFLP is a simple, reliable and rapid technique used for genotyping characterization and it yields the same results of the sequencing (Lubeck and Hoorfar, 2003).

In this study, β-giardin genes were used, through sequence analysis of this locus, isolates can be grouped into assemblages from A to G. The benefit of targeting β-giardin in the molecular detection of *Giardia* spp. is their unique existence in this parasite (Faubert, 2000; Caccio *et al,* 2002; Read *et al,* 2004). However, some authors preferred *tpi* gene because of its high genetic heterogeneity (Thompson and Monis, 2004).

In the present study, out of 62 *Giardia* positive stool samples by microscopy, 52 cases (84%) were positive by PCR targeting β-giardin locus. The negative results (16% of the samples) could be related to the existence of inhibitors in the samples (Abbaszadegan *et al,* 2007), sample storage conditions, DNA extraction method or the gene targeted, the choice of primers and the cycling settings that might affect the success of DNA amplification (Gasser, 2006). The most important reason for false negatives was the variances between *Giardia* strains including; insertion-deletion events, single-nucleotide polymorphisms and rearrangements of the chromosomes. These variances, combined with overall nucleotide identities ranging from 70-80%, have led some to observe that these assemblages could be considered to be different species (Franzen *et al,* 2009; Jerlstrom-Hultqvist *et al,* 2010).

In the current study, the isolated genotypes of *Giardia* from symptomatic and apparently healthy asymptomatic Egyptian Cases showed a significant predominance of assemblage

B in all studied groups representing 83.7% of the symptomatic group and 77.8% of the cases in asymptomatic group without reported mixed Assemblages in our samples. In agreement with the present study, Assemblage B was 80% prevalent among Egyptians living in two west Nile Delta Governorates (Foronda *et al,* 2008). Furthermore, PCR-RFLP targeting tpi, bg and gdh loci of 15 positive samples for *Giardia* reported assemblage B in 13 samples and assemblage A and E in one sample each (Soliman *et al,* 2011) Moreover, in Kafr-Elshiekh Governorate assemblage B was higher than the assemblage A (Amer, 2013). In Dakahlia Governorate, it was reported higher incidence of assemblage B (62.14%) than assemblage A (31.07%) in diarrheic children and they also found mixed assemblages (A & B) in 2.91% of the cases (El-Tantawy and Taman, 2014). Controversially, a case-control study using PCR-RFLP targeting *tpi* gene among symptomatic and asymptomatic cases and reported that 47.19%, 17.98% and 11.23% of the cases were assemblage AI, AII and B respectively, 21.35% of the cases had mixed genotypes and false negative results up to 2.25%, they also stated that no significant difference in the assemblage distribution among studied individuals (Abdel-Moniem and Sultan, 2008). Also, another hospital-based study was done in Egypt targeting *tpi* gene stated that assemblage A (75.6%) was higher than assemblage B 19.4% (Helmy *et al,* 2009). Besides, a study targeting *gdh* by PCR-RFLP in Menofia and Sharkia Governorates revealed that assemblage AII (83.33%, 70.59%) was higher than assemblage BIII (16.67%, 29.41%) in cases from Menofia and Sharkia Governorates respectively (Sadek *et al,* 2013).

The distribution of *Giardia* assemblages among human cases were varied in the different parts of the world. The frequencies of assemblage B were higher in UK (Amar *et al,* 2002), Australia (Read *et al,* 2002), India (Tarub *et al,* 2004), Thailand (Tungtrongchitr *et al,* 2010), Saudi Arabia (Al-Moham med, 2011) and Spain (de Lucio *et al,* 2015), while in Mexico, Brazil, Colombia (Eligio-Garcia *et al,* 2008; Lebbad *et al,* 2008) and Turkey (Sonmez *et al,* 2015), assemblage A was predominant. This genetic variability in distribution of assemblages' distribution among these countries can be due zoonotic reservoir's role in transmission of the disease beside the geographical distribution.

There was no significant association between *Giardia* assemblages and the demographic data of the studied groups. This agreed with Ignatius *et al.* (2012) who reported that age did not matter with assemblage A or B. Also, Anthony *et al.* (2007); Gelanew *et al.* (2007) and Anuar *et al.* (2014) reported a non-significant difference in genders among population infected with either assemblage. Controversy, Mahdy *et al.* (2009) reported that females had twice higher risk of having assemblage B of *Giardia* compared to males.

The relation between the clinical presentation and *Giardia* assemblages had debatable results. In our study, there was a statistically significant association between the assemblage B and flatulence in the studied population. These results agreed with Lebbad *et al.* (2011) who found that flatulence was significantly more common in cases with *G. intestinalis* assemblage B.

Also, in the present study, diarrhea and abdominal pain was associated with assemblage B more than assemblage A. It went with similar reports from Egypt (Abdel-Moniem and Sultan, 2008), Malaysia (Mahdy *et al,* 2009), Australia (Yang *et al,* 2010), Argentina (Molina *et al,* 2011), Yemen (Alyousefi *et al,* 2013), Cuba (Puebla *et al,* 2014) and Turkey (Sonmez *et al,* 2015).

Controversy, some other reports done in Bangladesh, Spain, Australia and Egypt had stated a statistically significant existence of the symptom within the individuals infected with assemblage A (Read *et al,* 2002; Haque *et al,* 2005: Sahagun *et al,* 2008; Sadek *et al,* 2013; Fouad *et al,* 2014).

Conclusion

Assemblage B is the predominant genotype found in Egypt in symptomatic and asymptomatic patients. The prevalence of assemblage B indicates an anthropologic transmission cycle. However, the role of domestic animals and livestock as potential sources of infection for humans in the community also needs to be investigated. There is still a missing point of a clear relation between assemblages and the clinical presentation with contradictory results**.** This necessitates further work to be clarified through more studies that can guide better understanding of the link between genotypes and different clinical presentations of the infected individuals.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee.

Contribution of each author: All the authors contributed to every activity in this manuscript.

Conflict of Interest: The authors declared that they have neither competing interests nor received fund.

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