Acta Tropica 154 (2016) 1–5

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

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

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ARTICLE INFO

Article history:
Received 2 June 2015
Received in revised form 6 October 2015
Accepted 12 October 2015
Available online 30 October 2015

Keywords:
Giardia duodenalis
Genotype
Assemblage
β-gardin
PCR-RFLP

ABSTRACT

Giardia duodenalis is a common gastrointestinal parasite that infects humans and many other mammals. It is most prevalent in many developing and industrialized countries. *G. duodenalis* is considered to be a complex species. While no morphological distinction among different assemblages exist, it can be genetically differentiated into eight major assemblages: A to H. The aim of this study was to determine the genetic heterogeneity of *G. duodenalis* in human isolates (a study conducted for the first time in Syria). 40 fecal samples were collected from three different hospitals during the hot summer season of 2014. Extraction of genomic DNA from all *Giardia* positive samples (based on a microscopic examination) was performed using QIAamp DNA Stool Mini Kit. *β-giardin* gene was used to differentiate between different *Giardia* assemblages. The 514 bp fragment was amplified using the Polymerase Chain Reaction method, followed by digestion in *HaeIII* restriction enzyme. Our result showed that genotype A was more frequent than genotype B, 27/40 (67.5%); 4/40 (10%) respectively. A mixed genotype of A + B was only detected in 9 isolates (22.5%). This is the first molecular study performed on *G. duodenalis* isolates in Syria in order to discriminate among the different genotypes. Further expanded studies using more genes are needed to detect and identify the *Giardia* parasite at the level of assemblage and sub-assemblage.

1. Introduction

Giardia is the most common of intestinal parasites worldwide. It is estimated that in developing countries, where poor levels of hygiene, sanitation, and overcrowding enhance *Giardia* transmission, about 200 million individuals develop symptomatic giardiasis and 500,000 new cases are reported each year (WHO 1996; Adam 2001). *Giardia* genus comprises of six species: *G. duodenalis* (syn: *G. intestinalis* or *G. lamblia*), *G. muris*, *G. microli*, *G. agilis*, *G. psittaci*, and *G. ardeae* (Adam 2001).

Giardia duodenalis has a variety of mammalian hosts including humans (Gardner and Hill, 2001). It is transmitted to individuals via fecal-oral route by direct contact or by ingestion of resistant cysts from contaminated food or water (Karanis et al., 2007). The clinical manifestations of giardiasis vary between asymptomatic infection to severe diarrheal illness with or without mal-absorption, weight loss, and abdominal cramps (Gardner and Hill, 2001).

Conventional diagnostic methods are used widely in many laboratories for the detection of *Giardia* cysts or trophozoites in stool samples using a light microscope (Adam, 1991). However, these methods are of low sensitivity, time consuming, and require microscopic experience. In addition, the identification of *G. duodenalis* genotypes is not possible using these simple methods, due to its morphological homogeneity (Amar et al., 2002).

Recently, a variety of molecular techniques, such as PCR-based diagnostic system, PCR-RFLP, cloning and sequencing analysis of a specific set of *Giardia* genes [glutamate dehydrogenase (*gdh*), triosephosphate isomerase (*tpi*), elongation factor 1 alpha (*efla*), beta giardin (*bg*) and 18S RNA genes] proved to be sensitive, powerful, and specific analytical tools for detection of *Giardia* parasites in stool samples as well as for genotyping this complex parasite (Caccio et al., 2002; Wielinga and Thompson, 2007; Sprong et al., 2009; Soliman et al., 2011; Torres-Romero et al., 2014). By means

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http://dx.doi.org/10.1016/j.actatropica.2015.10.008
0001-706X/

of molecular techniques, *G. duodenalis* can be genetically differentiated into eight major assemblages: AH (Monis et al., 2003; Caccio and Ryan, 2010). Assemblage A and B have been identified to infect humans and a broad range of other animals (dogs, cats, and livestock) (Karanis and Ey 1998; Bertrand et al., 2005), whereas the remaining six assemblages (C–H) appear to be host restricted to domestic animals, livestock, wild animals, and marine vertebrates (Monis and Thompson 2003; Monis et al., 2009; Lasek-Nesselquist et al., 2010; Feng and Xiao 2011).

In Syria, there is a lack of information on the prevalence of giardiasis and the route it transmits to humans. Some earlier studies, which depended on conventional methods only, have reported the presence of *G. duodenalis,* especially amongst children (Almerie et al., 2008).

The aim of this study was to inspect the genetic heterogeneity of *G. duodenalis* isolates detected in human stool samples using the Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) analyzing methods on *β-giardin* gene, and to distinguish between the different genotypes or assemblages.

2. Materials and methods

2.1. Study samples

Stool specimens were collected from 40 patients presented to the internal medicine clinics at three different hospitals in the city of Damascus in the period between June and September 2014. Patients were suffering from diarrhea with or without other symptoms (such as: abdominal pain, flatulence, cramps, mal-absorption. . .etc.,). All patients completed a simple questionnaire about clinical and epidemiological information (including: age, gender, education level, socioeconomic background, personal hygiene, specifically regarding hand washing and food consumption, types of water supply, sewage disposal system, and if there is close contact with household pets).

Patients and their families were informed about the study and they signed a written consent. This study has been approved by the ethical committee of Damascus University – Syria.

2.2. Microscopic examination

Stool smears were stained by Lugol's iodine and examined under a light microscope. All positive samples (cysts and/or trophozoites) were preserved in 70% ethanol (1:3) for further use in molecular characterization methods.

2.3. Genotypic characterization of G. duodenalis

2.3.1. DNA extraction

Between 0.2 and 0.3 g of each ethanol preserved fecal specimen was transferred to a centrifuge tube, washed three times with distilled water, and centrifuged for 10 min at 3800 rpm, to insure the removal of all ethanol. Total DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with minor modification; when using ASL lysis buffer by increasing temperature to 95 °C for 5 min to increase total DNA yield. Elution of DNA was done in 200 μl of elution buffer (Qiagen, Valencia, CA), and incubated for 10 min at room temperature before centrifuging for 1 min at 15,000 rpm. DNA extracts were stored at −20 °C until use.

2.3.2. PCR-RFLP analysis

A nested PCR method was performed to amplify a fragment of the *β-giardin* gene. The first reaction amplified a fragment of 753 bp using the following primer pairs: G7 as a forward primer (5' AAGCCCGACGACCTCACCCGCAGTGC3') and G759 as a reverse primer (5' GAGGCCGCCCTGGATCTTCGAGACGAC3') (Caccio et al., 2002). The PCR reaction (25 μl final volume) contained 12.5 μl Go Taq Green Master Mix 2X (Promega), 1 μl of each primer, 3 μl nuclease-free water, and 7.5 μl extracted template DNA. PCR cycling conditions were as follows: initial denaturation at 95 °C for 15 min, then 40 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s. The final extension was at 72 °C for 7 min.

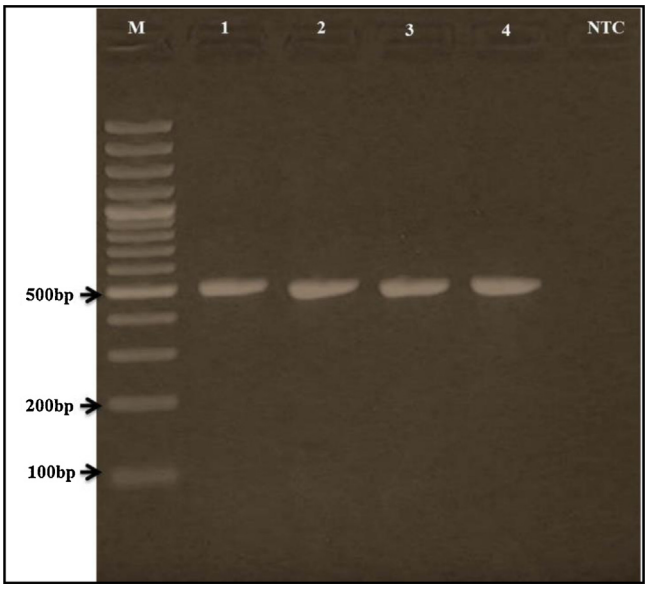

Fig. 1. Agarose gel electrophoresis of the nested *β-giardin* gene PCR products: Lanes 1–4 a single PCR fragment of 514 bp. M, molecular marker (100 bp). NTC: negative control for contamination detection.

For the second PCR reaction, we used GiarF (5) as a pair of primers to amplify a 514 bp PCR fragment (Lalle et al., 2005). The PCR reaction (25 μl final volume) contained 12.5 μl Go Taq Green Master Mix 2X (Promega), 1 μl of each primer, 5.5 μl nuclease-free water, and 5 μl of the first reaction product. The PCR reaction was carried under the following conditions: after an initial denaturation at 95 °C for 15 min, 35 cycles (95 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s) were run. The final extension was at 72 °C for 7 min.

Each PCR experiment contained a negative control (5 μl of nuclease-free water) for contamination detection. PCR reactions were done using Eppendorf Master Cycler. The amplified products were electrophoresed in 1.5% agarose gel containing ethidium bromide, visualized and photographed using a UV transilluminator.

PCR-RFLP method was applied to differentiate *Giardia* assemblages (Monis et al., 1996). Each 514 bp amplified PCR product was digested using *HaeIII* restriction enzyme (Promega) for 6 h at 37 °C. Finally, digested products were separated by 3% agarose gel electrophoresis, using 100 bp and 50 bp DNA ladder (Sigma) as size standard, visualized and photographed using a UV transilluminator.

Table 1
Summary of the study samples from Damascus city, by sex and age.

Study group	No. examined	No. infected	%
Sex			
Female	19	19	47.5%
Male	21	21	52.5%
Age groups<1-			
10	25	25	62.5%
11-15	11	11	27.5%
16 ≤	4	4	10%
otal	40	40	100%

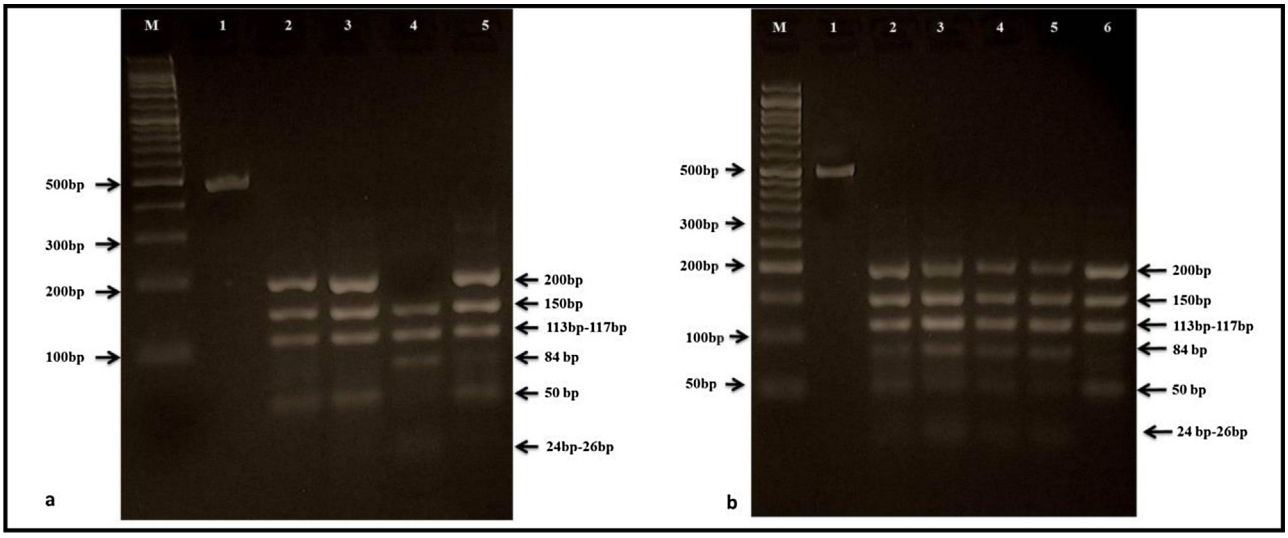

Fig. 2. PCR-RFLP assay (3% agarose gel electrophoresis) of the *β-giardin* gene products after restriction of the polymorphic region with *HaeIII* restriction enzyme. (a) Lane 1: undigested *β-giardin* gene products, Lanes 2,3,5: assemblage A, Lane 4: assemblage B. M: molecular marker (100 bp). (b) Lane 1: undigested *β-giardin* gene products, Lanes 2-5: mixed assemblages A+B, M: molecular marker (50 bp).

Table 2
The PCR-RFLP profile of *G. duodinalis* genotypes after digesting with *HaeIII* enzyme.

Assemblages	No. cases (%)f	Fragments size
A	27 (67.5%)	200, 150, 117-113, 50 bp
B	4 (10%)	150,117–113, 84, 26–24 bp
A+B mixed	9 (22.5%)	200, 150, 117–113, 84, 50, 26–24 bp

3. Results

Microscopic analysis of the 40 human stool samples confirmed *Giardia* infection. Cysts and/or trophozoites were observed in all fecal isolates after staining by Lugol's iodine.

Of the 40 giardiasis cases, 19/40 were females and 21/40 were males. Patients were comprised of 25 children (4 months to 10 years of age), 11 adolescents (11 to 15 years old) and 4 adults (16 years and up) (Table 1).

In addition, our data reported the presence of mild pasty diarrhea as a common clinical symptom, accompanied with growth disturbance, weight loss, and mal-absorption. Only 4 positive giardiasis cases presented foamy diarrhea.

PCR amplification of a fragment from *β-giardin* gene yielded the expected size which was approximately 514 bp from all isolates (Fig. 1). Our results showed different restriction patterns from fecal isolates belonging to assemblages A, B, and A+B mixed (Fig. 2a–b).

Among the fecal isolates, 27/40 (67.5%) cases were identified as assemblage A, which was defined by the presence of DNA bands at 200, 150, 113 and 50 bp. Whereas assemblage B, which generated DNA bands at 150, 117–113, 84, 26–24 bp, was found in very few samples 4/40 (10%). Finally, the genotype A+B was detected in 9/40 (22.5%) of fecal samples (Table 2).

4. Discussion

Giardiasis is a common cause of diarrheal disease in almost all vertebrates, including humans. It is widely spread in developing countries (Thompson, 2000; Tak et al., 2014).

Our data showed that children (62.5%) and adolescents (27.5%) were more frequently infected than adults (10%). This result is in agreement with similar studies conducted in different countries (Mohammed Mahdy et al., 2009; El Fatni et al., 2014). Previous reports suggested that the reasons for high prevalence of giardiasis among the age group of 1 - 15 years old may be because they are easily exposed to contaminated water or for their lack of immunity (Karanis et al., 2007).

Microscopic detection of *Giardia* (cysts and/or trophozoites) in fecal samples is a traditional diagnostic method for giardiasis (Adam, 1991). However, this method is time-consuming, requires experienced microscopists, and is unable to distinguish between genetically distinct *G. duodenalis* isolates (Amar et al., 2002).

PCR-RFLP is a molecular sensitive tool and it is capable of distinguishing human isolates of *G. duodenalis* at the genotype level (Monis et al., 1996; Caccio et al., 2002; Lalle et al., 2005).

In this study, we used PCR-RFLP to distinguish between *Giardia* assemblages using *β-giardin* gene. This gene was used as a target for molecular identification of *Giardia*. The advantage of using giardin genes is that they are considered to be unique to this parasite (Faubert, 2000). The giardin proteins (29–38 KDa) are defined as a family of structural proteins. They are found at the edges of dorsal ribbons, which are an integral part of the ventral disk of the trophozoite (Adam, 2001).

Our results showed that the nested *β-giardin* amplification product yielded the expected fragment from all isolates; which is 100% consistent with our microscopic detection. Furthermore, genotyping analysis reported the presence of *G. duodenalis* assemblages A, B, and a mixed genotype A+B at different rates.

Among 40 fecal isolates, assemblage A was the most frequent genotype detected (67.5%), while assemblage B was less detected (10%). These results are in agreement with previous studies conducted in Egypt (75.5% A, 19.5% B, n = 41, Helmy et al., 2009), Saudi Arabia (57.5% A, 37.5% B, n = 40, Feng and Xiao 2011), Ethiopia (52% A, 22% B, n = 59, Gelanew et al., 2007), Italy (80% A, 20% B, n = 30, Caccio et al., 2002), Brazil (78.4% A, 21.6% B, n = 37, Souza et al., 2007), and Thailand (71.4% A, 2.3% B, n = 35, Traub et al., 2009). However, several studies conducted in India (Sulaiman et al., 2003), United kingdom (Amar et al., 2002), United states (Guy et al., 2004) and Nipal (Singh et al., 2009) indicated that assemblage B was more prevalent than assemblage A.

Many studies worldwide, established that only *G. duodenalis* assemblage A and B are responsible for almost all human giar-

diasis infections (Bertrand et al., 2005; Fallah et al., 2008). It has been known that assemblage A is often responsible for zoonotic transmission with a wide range of animals acting as reservoir hosts (Babaei et al., 2008; Mohammed Mahdy et al., 2009). Although, assemblage B is most likely transmitted from humans, it has been detected in some animals and may represent a zoonotic potential as well (Trout et al., 2005; Solieman et al., 2011).

In addition, 22.5% of *G. duodenalis* mixed genotype A+B was detected in our fecal sample study. This is compatible with other studies which mentioned the presence of a mixture of A and B assemblages in human stool samples (Amar et al., 2002; Guy et al., 2004; Lalle et al., 2005). The presence of more than one assemblage can be explained by the possible uptake of genetically different *Giardia* cysts, or by subsequent infection of an already infected host with a different *Giardia* species (Sprong et al., 2009; Solieman et al., 2011).

The presence of mild pasty diarrhea in our samples was found to be a common clinical symptom accompanied with one or more other symptoms as growth disturbance, weight loss, and mal-absorption. This data showed no evidence of differential pathogenicity between assemblages A and B, which was in agreement with findings by García et al. (2002) and Ceu-Souza and Poiares da Silva (2004). Although some prior studies have suggested that assemblage A infections are more likely to cause diarrhea than assemblage B (Read et al., 2002; Haque et al., 2005; Cordón et al., 2008), this was not seen in our study.

In conclusion, this preliminary data on giardiasis using the molecular method is the first reported study in Syria. Indeed, for better understanding of the molecular epidemiology of giardiasis in our country, expanded studies on human and animal samples using more genes are required to detect and identify the *Giardia* parasite at the level of assemblage and sub-assemblage.

Acknowledgement

This research was conducted with financial assistance from Damascus University. We would like to thank our colleagues at Damascus hospitals and patients for providing the stool samples used in this study.

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