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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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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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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.

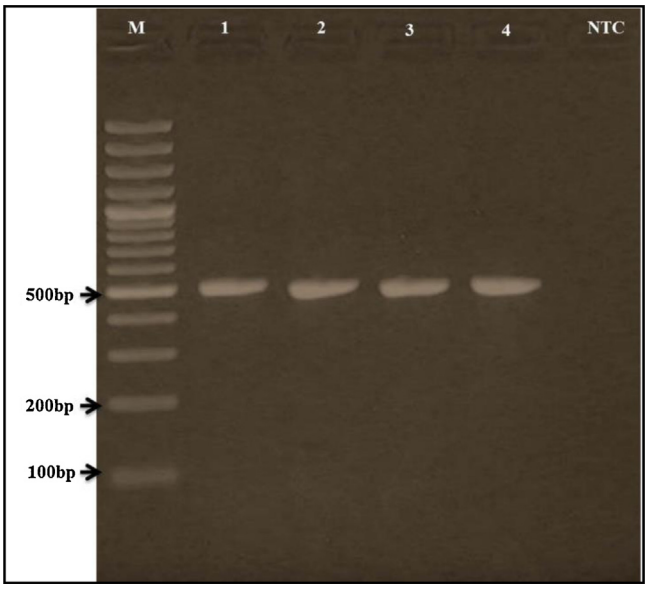

Fig. 1. Agarose gel electrophoresis of the nested *β-giardin* gene PCR products: Lanes 1–4 a single PCR fragment of 514 bp. M, molecular marker (100 bp). NTC: negative control for contamination detection.

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%