Genotyping of *Giardia duodenalis* isolates from human subjects in Zabul, using PCR-RFLP

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**ABSTRACT**

**Objective:** To uncover the molecular prevalence of *Giardia duodenalis* by PCR-restriction fragment length polymorphism (RFLP) in Zabul city, Iran.

**Methods:** Twenty-four stool samples were collected from 215 patients with suspected giardiasis by microscopic examination. To increase the sensitivity of the PCR, the total genomic DNA from isolates was extracted by applying glass beads and the QIAamp Kit. A one-step PCR-RFLP method, targeting the glutamate dehydrogenase gene, was utilized to differentiate the assemblages A and B among isolates.

**Results:** The PCR fragment was determined from 30 isolates, RFLP assay of 24 isolates showed 24 (100) isolates as Genotype B group BIII.

**Conclusions:** The results with the glutamate dehydrogenase gene assay demonstrated that the predominant subtype of *Giardia duodenalis* in the area is BIII, which showed animals are the main reservoir of the isolates in this area.

1. Introduction

*Giardia duodenalis* (G. duodenalis) is an intestinal protozoan that commonly infects humans and a wide spectrum of various mammals[1]. It is also considered as the most important intestinal pathogenic protozoa in the North America, a wiki world, and developing countries as a common infection in childhood[2]. *Giardia* release about 280 million per year and transmit by person directly or indirectly through ingestion of cysts in contaminated food and drinking water[2].

In endemic areas, about 70% of infected people do not develop symptoms of giardiasis. However, most of affected children will develop the symptoms of giardiasis, but most adults have no symptoms and play a role as healthy carriers[3].

Infections might be asymptomatic or show symptoms of weight loss, chronic diarrhea and malabsorption[4]. Other manifestations of chronic giardiasis are loose, greasy and soft stools, complications in the visceral area, general feeling of discomfort or ailment and weakness and fatigue. Molecular methods such as the PCR-restriction fragment length polymorphism (RFLP) and sequence typing of several housekeeping loci have demonstrated the *G. duodenalis* as a complex species including eight major assemblages (A to H). The geographic distribution of these *G. duodenalis* genotypes is wide[5,6]. Genetic surveys have uncovered that assemblage A itself includes genetic group of I to VIII (A1 to A8), and assemblage B is from subtypes I to VI (B1 to B6). Among genotypes, BII, BIV, AI and AII have been mostly reported[7].

The AII and BIV assemblages are attributed to be specific for human population. Whereas, the C, D, E, F and G assemblages have been shown to be among a variety of animals.

Assemblages C and D consist only of canine isolates. Group E isolates are from productive animals (cattle, sheep, pigs). Moreover, Assemble F comprises feline *Giardia* isolates, and group G isolates are from rats[5,8,9]. Some *Giardia* genotypes exhibit narrower host specificity, especially those among dogs, cats, rats, muskrats, huffed animals and voles. The sequence analysis of the *GDH* locus by molecular techniques could differentiate subgroups such as AI, AII, BIII, and BIV[10]. Several molecular techniques, including multiplex PCR, PCR-RFLP, PCR, and sequence assessment of housekeeping loci have proved to be used as tools for discrimination of all assemblages and to provide powerful tools for understanding molecular epidemiology, infection sources and zoonotic potential of human giardiasis[11,12]. The aim of our study was to detect the *Giardia lamblia* genotypes of human isolates in Zabul, Iran. The study has been reported for the first time in the regard. The PCR-RFLP protocol employed is proper for the typing of...
the *Giardia* cysts in feces or other samples.

**2. Methods and materials**

**2.1. Collection of samples**

Samples were obtained from several laboratories. Stool specimens were evaluated under the light microscopy and Lugol’s iodine stain wet mount. Specimens, comprising cysts were concentrated and purified by the flotation method with sucrose having specific gravity 1 mol/L and next were stored at −20 °C until further study.

**2.2. Purification of the cyst**

Cysts were somewhat purified from the stool material by using the density gradient of sucrose protocol[13] and then washed with distilled sterile water. All the samples were placed at −20 °C with no preservatives until the DNA extraction and further tests.

**2.3. Extraction of the DNA**

*G. duodenalis* cysts were frozen and thawed for 10 times at −80 °C. Subsequently, DNA was obtained of 200 µL the purified specimens by the QIamp Kiaspin Kit (Qiagen, Germany) by following the manufacturer’s instructions. Extracted DNA in tubes was placed at −20 °C till further study.

**2.4. PCR of the GDH gene**

In the PCR reaction, a product of 458 base pair of the GDH gene was amplified with primer forward 5′-TCA ACG TCA ACC GCG GCT TCC GT 3′ and reverse 5'-GTT GTC CTT GCA CAT CTC C-3'.

The mixed PCR reaction (25 µL) containing 5 µL of extracted DNA, 25 µL mastermix and 19 µL distilled water and 1 µL of each primer was used. The thermal profile of the reaction included 5 min at 94 °C, then 35 cycles of 94 °C for 1 min, 1 min at 61 °C and a temperature of 72 °C in 5 min. The final stage of the replication was 72 °C for 10 min. Distilled water with the samples at any PCR reaction was used as a negative control.

**2.5. RFLP analysis**

RFLP analysis was done with digestion of 10 µL of PCR products using 0.5 µL of BspI and 2.5 µL of restriction buffer (Tango buffer, fermentase) in a total volume of 20 µL for 3 h at 37 °C. For those isolates that exhibited the assemblage B genotyping profile, RsaI restriction enzyme was employed to distinct between the BII and BIV as following: 10 µL product of PCR was added in a mixture of 1 µL (10 IU) of RsaI, 2.5 µL of restriction buffer (Tango buffer fermentase) and 13 µL of distilled water. The reaction mixture then was placed in 37 °C for 3 h. The digested fragments were run and differentiated on 2.5% agarose gel that was stained with the DNA green staining.

**2.6. Ethical approval**

The study protocol was performed according to the Helsinki declaration and approved by Zabul University of Medical Sciences. Informed written consent was obtained from Research part of Zabul University of Medical Sciences, Zabul, Iran.

**3. Results**

**3.1. Patients’ history**

Among 30 patients, 19 (63.33%) were female and 11 (36.67%) were male. The age range was 1–69 years with the mean age of (34.33 ± 5.67) years. Twenty-two patients were literate and eight were illiterate. Twelve (40.00%) patients were in age range under 10 years. Table 1 exhibits patients’ history and positive stool samples of *Giardia lamblia*. One female pediatric patient (8 years) was dead due to the infection.

| Patients’ characteristics | Stool positive (%) | Death rate (%) |
|--------------------------|--------------------|---------------|
| Education | | |
| Literate (n = 22) | 100.00 | 0.00 |
| Illiterate (n = 8) | 75.00 | 12.50 |
| Sex | | |
| Male (n = 11) | 90.90 | 0.00 |
| Female (n = 19) | 94.73 | 9.09 |
| Age (years) | | |
| < 10 (n = 12) | 100.00 | 8.33 |
| 10–25 (n = 6) | 100.00 | 0.00 |
| 25–50 (n = 5) | 80.00 | 0.00 |
| 50–70 (n = 7) | 85.71 | 0.00 |

**3.2. PCR assay and RFLP results**

On the 30 samples, the GDH gene was amplified using glass beads and kit method. Twenty-four samples were amplified, but because of employing the glass beads and QIAgen kit in all remaining 30 isolates, the 458 bp expected size was amplified (Figure 1). Resulting from the present research, 24 (100%) of the 24 isolates were determined as assemblage B group BII.

![Figure 1. The GDH gene with 458 bp size.](image)

M: Marker; Columns 1–3, 6 and 7: Positive samples; Columns 4 and 5: Control negative and positive samples, respectively.

**4. Discussion**

*G. duodenalis*, as a small intestinal protozoan parasite, is spread all over the world.

The main aim of the current study was to detect the genotypes of *G. duodenalis* isolates in Zabul city. This is the first study of genotyping of *Giardia* to identify and differentiate human strains of *Giardia* molecular features. Molecular assays such as PCR are employed to classify *G. duodenalis* to assemblages and sub-assemblages. Most of studies have applied tests related to one or more of four genetic sequences including small subunit ribosomal RNA (SSU-rRNA) and the triosephosphate isomerase, β-giardin and GDH genes[14–16]. Many of studies assess only a single gene, mostly SSU-rRNA. While the amplification of a distinct gene or of course a different set of PCR primers may occasionally assign the identical
isolate as a non-related assemblage. PCR-RFLP is an analytical and robust method sensitive for determining the genotype of the parasites[17]. The use of stool samples for the presence of lipids, hemoglobin, bile and polysaccharide inhibit PCR with regard to the problems of cultured parasites, which firstly requires a lot of time there and secondly multiple infection medium. Using a method known as sucrose concentration method, the material and bacterium inhibitors that have lowered the stool can be used both directly from stool specimens without culturing the parasite, which makes it possible[18].

All of isolates were classified in the assemblage BIII in this study. Studies of the last few years from some parts of Iran on the genotype determination of human isolates of Giardia have been more or less similar to other parts of the world[15,19].

In a study by Ramírez et al., the assemblage B was predominant in G. duodenalis in Colombia. Moreover, the PCR for the SSU-rDNA was more sensitive than for TPI and Gdh. The microscopy exhibited flaws for the diagnosis of Giardia[18].

In contrast to these results, the studies in Kerman, Isfahan University and Tehran obtained the opposite results of Zabol isolate as a non-related assemblage. The authors acknowledge Abdolmajid Ghasemian for helping the writing and English editing of the manuscript.

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