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Original Article

Incidence of *Giardia lamblia* Subspecies by PCR-RFLP in Stool Specimens of Hospitalized Children at Urmia Mutahhari Hospital, West Azerbaijan Province, Iran

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Abstract

Background: *Giardia lamblia* is one of the most prevalent intestinal flagellate protozoa that infects a wide range of vertebrate hosts causing severe intestinal disorder in children.This study was performed to determine subspecies of *G.lamblia* by the *PCR-RFLP* method, targeting the glutamate dehydrogenase*(gdh)*locus, in hospitalized children at Urmia Mutahhari Hospital, West Azerbaijan Province,Iran and determining the infection transformational storages in this area.

Methods: Overall,720 stool specimens were collected from the hospitalized children, 34 samples were positive and *Giardia* cysts were detected under the microscope. Cysts were partially purified by the sucrose density gradient method and then washed with sterile distilled water to remove effectively the PCR inhibitors. Genomic DNA of *G. lamblia* isolates was extracted by freeze-thaw cycles followed by phenol/ chloroform/isoamyl alcohol method. The single step *PCR-RFLP* assay was used to differentiate the assemblages between A and B, which were found in humans. In this method, 432 bp expected size was amplified, and then for detection of subspecies, specific restriction *RsaI* and *BspLI* enzymes were used.

Results: Totally 34 samples were positive in terms of *Giardia* cyst out of 720 examined samples microscopically, so the parasite spread rate is reported 4.72%. Analysis *PCR-RFLP* on these samples revealed that 28 samples (93.3%) have the genotype BIII and 2 samples (6.7%) belong to the subgroup BIV.

Conclusion: *PCR-RFLP* is a proper analytical method for determining the genotype among parasite types, using the glutamate dehydrogenizes zone's genes. Based on the results, an animal origin of infection cycle is suggested.

Introduction

Giardiasis, a common intestinal protozoan infection caused by *Giardia lamblia*(also known as *G. duodenalis or G.intestinalis*), has gained attention as a neglected disease in both developed and developing countries (1). Prevalence of *G. lamblia* is found in all age groups, but children are at the greatest risk for contracting clinical giardiasis. Clinical presentations of giardiasis differ from asymptomatic carriage to acute and chronic diarrhea (2, 3). *Giardia* isolates based on morphological criterion which six species, namely: *G. agilis,G.ardeae,G.lamblia, G.microti, G.muris, and G.psittaci*, vary significantly in their biology, host specificity, and genetics (4).

Among the 6 species, *G. lamblia* infects humans and numerous other mammals. Isolation of *G.lamblia*is classified under 7 assemblages (A-G), based on the characterization of the glutamate dehydrogenase, small-subunit rRNA, and triose phosphate isomerase (tpi) genes (5, 6). Assemblage of A isolates has been placed into subgroups I and II. Assemblage of B isolates has been separated into subgroups III and IV. "Genetic assemblages C, D, E, F, and G seem to be restricted to domestic animals, livestock, and wild animals" (4, 7). Although all human-derived *Giardia* isolates belong to assemblages A and B, these assemblages have also been found in isolation from the other domestic and wild animals such as dogs, cats, and cattle (8). Some researchers consider that presenting of *G. lamblia*is reflected on a risk of zoonosis from cattle (9), dogs (10-12), wild moose, reindeer (13), farm, and wild animals (14).The *gdh* gene is proven useful for the genotyping of *Giardia* isolated from mammals. PCR-RFLP has successfully been used by a number of researchers to differentiate between *Giardia* genotypes for humans and animals (4, 5, 8). This infection is diversely dispersed throughout all over Iran, such as West Azerbaijan Province. Incidence in this province is varied from 10.3% (15, 16) to 43.8% (17). However, most studies do not evaluate the risk factors for acquiring *G. lamblia* infection, which are essential for prevention and control strategies.

The primary goal of this study was to determine the genotypes of *G. lamblia* isolates (17) and identification of potential zoonotic reservoir in this area, with used sucrose density gradient, DNA extraction by phenol/ chloroform/isoamylalcohol, PCR RFLP method to acquire high sensitivity result in fecal samples.

Materials and Methods

Sample collection

Overall, 720 stool specimens were collected from the hospitalized children, between June 2011 and February 2012. All samples were tested by light microscopy. *Giardia* cysts were isolated and partially purified by sucrose flotation (18, 19). The semi filtered and concentrated cysts were stored in sterile distilled water without adding any preservatives, up to two weeks at -20 ^{0}C.

DNA extraction

1) According to repeated freezing and thawing method, this process was performed by 6 times freezing and thawing in liquid nitrogen for 60 seconds and in 65°C water bath for 60 seconds, respectively (20).

2) Then, DNA extraction was performed based on glass beads and phenol-chloroform extraction assay (21). DNA presented in the supernatant was precipitated with 0.1 volumes 3M sodium acetate (pH 5.2), and 2-propanol.The precipitant had been washed with 70% ethanol and then the purified DNA was resuspended in 30 µl of distilled water.

PCR amplification

Amplification of the *gdh* genes was accomplished as the single PCR. In the PCR reaction, the 432 bp fragment was amplified by using the forward primer (*GDHiF*) 5-CAG TAC AAC TCY GCT CTC GG-3 and the reverse primer (*GDHiR*), 5-GTT RTC CTT GCA

CAT CTC C-3 (4). Amplification reaction was modified as follows- the PCR mix consisted of 1X buffer containing1.5 mM MgCl2 (Cinaclon, Iran), each deoxynucleotide triphosphate at the concentration of 100µM (Cinaclon, Iran), each primer at a concentration of 0.5 µM, 10 ng of DNA and 2.5 U of HotStarTaq DNA polymerase (Cinaclon, Iran). Cycling parameters were 10 min at 94°C (initial heat activation step), followed by 50 cycles of 35 s at 94°C, 35 s at61°C, and 50 s at 72°C, with a final extension of 7 min at 72°C (4). Both positive and negative controls were included in each PCR to validate results. Cysts were utilized as the templates for the positive controls, and distilled water was utilized as the template for negative controls throughout.

RFLP analysis

RFLP analysis was performed by digesting 8 µl of PCR products with 1.5 U of *RsaI* (vivantis) or 0.8 U of *BspLI* (vivantis) in 2 µl of 10X enzyme buffer in a final volume of 20 µl for 3 h at 37°C (18) . The *RsaI* digestion allowed the distinction between the assemblage of B group III and group IV after amplification. The *BspLI* digestion was employed for the distinction between assemblage A group I, assemblage A group II after amplification with the *GDHiF* and *GDHiR*primers (4).

PCR product and restriction fragment detection. PCR products and restriction fragments were separated by horizontal electrophoresis in 1.5 and 2%agarose gels, respectively, with ethidium bromide (0.6 µg/ml) staining. A 100-bpDNA ladder (Fermentas, Lithuania) was included as the size marker. PCR products and restriction fragments were recorded by UV transillumination (4).

Results

DNA extraction

After DNA extraction, we ran samples on agarose gels (1.5%) to confirm DNA extraction, but in most cases, there was either a paucity or absence of DNA. In these circumstances, the causative agent could have low parasite numbers of isolates. However, using PCR amplification, extracted DNA of positive samples is confirmed.

PCR amplification

In 34 samples, *gdh* gene was intensified by using freeze-thaw technique and phenol/chloroform/isoamylalcohol method, 30 samples (88.2%) with the use of primers *GDHiF, GDHi*R, a432bp expected size was amplified (Fig. 1).

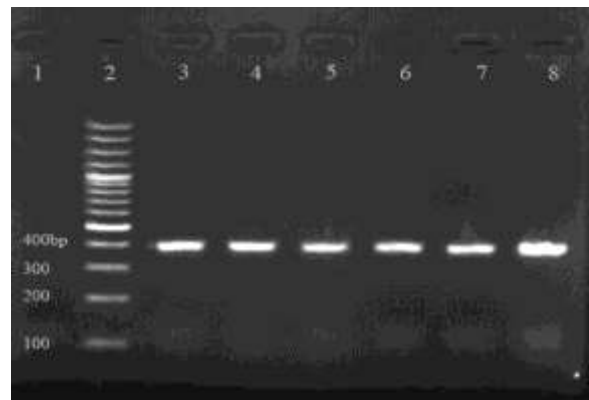

Fig.1: Electrophoretic separation of PCR product from DNA amplified at the *gdh* locus of *G. lamblia*,on an ethidium bromide stained 1.5% agarose gel. Lane 1, negative control; lane 2, 100 bp, Plus molecular weight marker(Fermentas, Lithuania); Lanes 3-8, PCR products (432 bp fragment)

RFLP method

RFLP assay on 30 samples, with using *RsaI, BspLI* enzymes. The genotyping results are summarized in Table 1.

Out of 30 samples isolates, 28 samples (93.3%) were found as *G.lamblia* (genotype BIII), 2(6.7%) belonged to assemblage BIV (Fig. 2).

Risk Factors

Table 2 shows analysis of the risk factors for giardiasis in this population; it pointed at children ranging in age from 3 to 5 years old which had a superior risk of acquiring giardiasis.

Table 1: Genotypes of *G. lamblia* determined by PCR-RFLP of *gdh* locus

Isolate code	Genotype	*Isolate code*	Genotype
1	BIII	16	BIII
2	BIII	17	BIII
3	BIII	18	BIII
4	BIII	19	BIII
5	BIII	20	BIII
6	BIII	21	BIII
7	BIII	22	BIII
8	BIII	23	BIII
9	BIII	24	BIII
10	BIV	25	BIII
11	BIII	26	BIII
12	BIII	27	BIV
13	BIII	28	BIII
14	BIII	29	BIII
15	BIII	30	BIII

Fig. 2: *RsaI and BspLI* digestion of PCR products on an ethidium bromide –stained 2% high resolution agarosegel. Line2,*G.lamblia* assemblage BIV, *(RsaI* digestion): line 3, *G. lamblia* assemblage BIII (*RsaI* digestion),line 4-6, G.*lamblia*assemblage B (*BspLI* digestion) and line 1, 100bp plus molecular weight marker (Fermentas, Lithuania)

Table 2: Characteristics of hospitalized children and prevalence of *Giardia lamblia* infection

Study group	no.examined	No.infected(%)	*P.* value
Male	374	19(5.08)	0.580
Female	349	15 (4.29)	0.580
<1	32	0 (0)	0.001
1-3	38	1 (2.6)	0.001
> 3-5	215	17(7.9)	0.001
>5-8	185	11 (5.9)	0.001
>8-11	121	2(1.6)	0.001
>11-14	129	3 (2.3)	0.001
total	720	34 (4.72)	

The results show that there is no significant relationship between infection and sex. (*P*.value=0.580).

Discussion

Giardiasis is a common intestinal protozoan infection caused by *Giardia lamblia*. Infection with *G.lamblia* is widespread in both humans and animals and multiple transmission routes exist, with water and food playing an increasingly recognized role worldwide (20).To understand the epidemiology of the infection and to implement control measures, it is im-

portant to determine genotype of *G.lamblia*. For this reason, to use advanced tools such as *PCR-RFLP* (21).In this study, molecular analysis on these samples revealed that 28 samples (93.3%) have the genotype BIII and 2 samples (6.7%) belong to the subgroup BIV. Based on the results, an animal origin of infection cycle is suggested.

As it has been mentioned, *G. lamblia* is the most crucial parasitic disease that is spread in various parts of Iran, especially in Urmia, which is an endemic region. Wide epidemiological studies have been conducted in this area by Dr. Hazrati et al., during 2008-2009 (15,16) which proved that the occurrence of Giardiosis among elementary school students in the district had been 10.3%.But in the current study the outcome is 4.72%, which indicates that the level of people's consciousness, knowledge of the public health, personal hygiene, use of safe water, health facilities, etc. have the positive effects on the decrease of the rate of giardiasis infection.

PCR-RFLP is a sensitive and powerful analytical tool which is capable of providing the level genotyping discrimination among assemblages by targeting some loci such as *gdh* and *tpi*, making it possible to identify the presence of mixed genotypes (5,8,23).For direct use of stool and existing PCR inhibitors, there were purified and concentrated by flotation on sucrose with specific gravity of 0.85 M.

Moreover, direct amplification of cysts DNA from feces assisted PCR inhibitors (e.g. lipids, hemoglobin, bile, salts, polysaccaharides from mucus, bacteria and food degradation product) which could affect the result of amplification (21), but with the use of sucrose density gradient centrifugation and washing with sterile distilled water in order to be effectively removed(21). The cyst walls are resistant to DNA extraction, therefore DNA extraction was ineffective. This became possible with repeated freezing and thawing, glass beads and phenol/chloroform/isoamylalcohol method (4).

All *G.lamblia* assemblages attained from humans were identified as assemblage B group, due to assemblage B more frequency than assemblage A in this region, corresponding to the findings of an Indian study that examined 10 clinical individual samples and found 100% assemblage B (23) and it was different from an Iranian study which found *G.lamblia* detected in humans (87%) of assemblage A(21). This study provides, for the first time,information on the distribution of the genotype of *G.lamblia* from humans with sporadic giardiasis in West Azerbaijan Province of Iran.

The difference between the occurrence of assemblages A and B may be attributed to the geographic locations of the patient studies.

Given information has evolved livestock in Urmia and surrounding and considered the obtained molecular results which indicate the dominant subspecies parasites in this area BIII is often more prevalent in humans, livestock; according to the study conducted on calves by Dalir Naghadeh et al. 2006-2007 during the infection rate reported by approximately twelve percent (24). The most likely hypothesis is that calves are the source of infection in this region. To prove this theory, a molecular epidemiological study is recommended for livestock, particularly cattle and calves infected with *Giardia, which* are important to understand the epidemiology and infection control procedures; cycle of transmission of diseases to humans; and to identify reservoirs.

The current study demonstrated that assemblage B, subgenotype BIII was widespread in Urmia, Iran.

Serve notice of the prevalence of assemblage B and none of sub assemblage AII, an animal source of infection is suggested.This information will be advantageous to the effective prevention and control program of giardiasis in this population.

Therefore, to discover the role of domestic animals and livestock as a potential source of infection for humans in the community, a research on molecular was advised, and secondly, to guarantee that there is no parasite

genetic diversity in this region, it has been suggested that other molecular researches should beconducted on people whom are older than 14 years.

The consequence of PCR-RFLP in this region, which is mostly BIII evidenced that there is no relationship among genders, ages, and subtypes (Table 2).

According to Chi Square Test, there is a significant connection between age groups and parasitic infections that indicates that in the age group 3 to 5 years, the prevalence of the parasite occurs ($P = 0.001$).

Given the absence of positive samples in less than 1-year-old offspring, it seems that this age group has no direct contact with parasites. Besides, an unusual lipase enzyme in human milk, which is effective in preventing giardiosis in this age range, had been considered. It is essential to mention that the sample of individuals with gastrointestinal symptoms had been conducted. Thus, the study of parasites and subtype disease has been inevitably cancelled by itself.

Conclusion

According to the achieved outcomes, it can be concluded that:

- The Phenol chloroform assay using frozen- thaw and the use of glass beads are the best and cheapest method of extracting DNA from *G. lamblia* cysts.
- Isolating of *G. lamblia* is similar in terms of morphology, and genomic variation.
- There are the subspecies B *G. lamblia* in West Azerbaijan that biotype dominant is BIII.
- *G.lamblia*, subspecies BIII, normally is in livestock, and according to high infections of*Giardia* in this region, a zoonotic origin of the infection route is suggested.
- The logical association between assemblageswith age and admissions were not observed.

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