

Genotype of giardia duodenalis isolates from humans using glutamate dehydrogenase (gdh) gens by PCR-RFLP

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Abstract: Aim and Background: Giardia lamblia (synonymous with Giardia duodenalis, Giardia intestinalis) is a protozoa intestinal flagellant which is the most common endemic and epidemic cause of diarrhea in all over the world. the main aim of this study is determining the genetic diversity of Giardia lamblia isolates using the PCR-RFLP method on the gdh (glutamate dehydrogenase gene) gene sequence in Kerman (southeastern of Iran), according to the prevalence of this unicellular in the region, we succeed to determine the genotype of numbers of Giardia lamblia isolates in infected patients. Materials and Methods: From 353 feces samples from patients with Giardiasis, approximately 50 samples was floated using sucrose by density gradient method and DNA was extracted successfully from the 30 of 50 samples using GLASS BEADS and QIAamp Stool Mini Kit, then the gdh gene was regenerated, after that 432bp fragment was appeared in PCR reaction, in following Giardia lamblia genotypes were separated by two restriction Enzyme. The results from the 30 samples tested indicated 60% (18) with "All" type assemblage, 16/6% (5) with "A1" type assemblage and 23/4% (7) with "BIII" type assemblage and necessary, it is worthy to say that the BIV type assemblage was not detected in this category. Conclusion: The PCR-RFLP is an advanced tool to determine the genotype which is used to identify the Giardia lamblia isolates which are identical morphologically and with several hosts. This molecular method assists us in determining the Giardia lamblia genotypes from direct feces samples of humans and domesticated animals.

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1. Introduction

Aim and Background: Giardia lamblia (synonymous with Giardia duodenalis, Giardia intestinalis) is a protozoa intestinal flagellant which is the most common endemic and epidemic cause of diarrhea in all over the world. (Thompson 2004). Its symptoms appear from asymptomatic infection to severe diarrhea (Amar, Dear et al. 2002). The protozoa is charged for the 2/5 million diarrhea and malnutrition in world children (Minvielle, Molina et al. 2008). These intestinal parasites have several hosts and infect a wide range of domestic and wild mammals and birds. However, there is a little information on epidemiology and the way of the pathogen transmission, since zoonosis transmission of Giardia is a risk factor for human infections, the base of the disease prevalence and zoonosis transmission of the parasite is important (Hunter and Thompson 2005). Based on morphological differences, six new species of the parasite have been named (Monis, Andrews et al. 2003) (Bertrand, Albertini et al. 2005). Currently Giardia isolates from related to humans and a wide range of domestic and wild primate that are morphologically inseparable have been classified into seven genotype by molecular

method so accordingly genotype of A,B have the widest hosts, these two genotype are different genetically and biologically so that sometimes researchers believe that they may be considered as separated species. Giardia lamblia isolation molecular criteria are divided into A to G genotypes including human genotype (A, B), genotype in dogs (C, D) genotype in Cat (F) genotype in domesticated cattle (E) and rat genotype (G), it is noteworthy that the (A, B) group consists a large number of hosts, including humans, dogs, cats, domesticated animals, and wild mammals in addition to human (Monis, Caccio et al. 2009). Group "A" isolates were categorized in several sub-groups of A₁ – A₈. Group B are divided into several sub-groups B1-B6 that the human isolates are mostly classified in the genotype A1, All, BIII (Lalle M 2005). Of course, according to the diversity of Giardia infection symptoms that consists the scope of without symptom or chronic diarrhea among patients, the great range of detected genotypes among diseases leads to define a main group and subordinate group of Girdia lamblia (Cedillo-Rivera and M. 2003). Although, Giardia lamblia isolates from different hosts have undetectable morphology, these isolates can be

separated by molecular methods which are based on polymerase chain reaction(PCR) using Giardia gene sequence analysis such as glutamate dehydrogenase gene (gdh) and TriosephosphateIsomerase Gene (tpi) (Caccio SM, De Giacomo M et al. 2002). The main purpose of this study is to determine the genetic diversity of Giardia lamblia isolates using PCR-RFLP method on glutamate dehydrogenase gene (gdh) gene sequences in Kerman (southeastern of Iran) in where the incidence of Giardia in different age has been 21.4% (Ziaali N 1996), using the method we could determine the genotypes of Giardia lamblia in parasite infected patients.

2. Material and Methods

1.2. Cysts Collection and Condensation:

The study has been conducted in Kerman, southeastern of Iran, during the February to December, during the period, 353 sample of feces related to patients with Giardiasis were collected from three health center and conveyed to Afzalipour medical science college laboratory, after Formalin Ether tests, the sample were examine microscopically and the severity of the disease was determined based on the number of the parasites in each microscopic field x 40, the samples with upper 15 cysts in averagely 10 microscopic field was prepared for concentration tests, then the samples were condensed via concentration gradient with sucrose 0.89 M (specific gravity = 1.13) and kept in -20 freezer until the next test (Roberts-Thomson, Stevens et al. 1976). In this study, the Giardia lamblia standard strain (ATCC ® Number: 30888™) was used as a positive control.

2.2. Extracting DNA:

From 353 positive samples of Giardia lamblia, the genome DNA was extracted for 30 samples so that at first the cyst wall was chopped mechanically using glass beads e(0.45 -0.52 mm diameter "sigma company, Germany)(Babaei, Oormazdi et al . 2008), then 1% SDS (1.20 final volume) and flowingly by proteinase K (10mg/ml) was added by Freeze-thawing technique (10 cycles for 3 min), after that they incubated 55 ° for 4 hours, after incubation, the samples DNA was extracted via QIAamp (QIAGEN, Company Germany) DNA Stool Mini Kit and Manufacturer manual, then kept in - 20 freezer until PCR test time. PCR amplification of the GDH gene was conducted using the PCR protocol, in PCR reaction, the 432bp fragment was amplified by Forward primer of 5'-CAG TACAACCTCYGCTCTC GG-3 'and Reverses primers of 5'-GTT RTCCTTGCA CAT CTC C-3 (Read, Monis et al. 2004). The mix of PCR reaction contained 10µL of DNA template, 2.5 µL of 10X buffer (Roche), 0.5µL of dNTPs (Roche), 1U of Tag polymerase (Roche) and 50 pmol of each primer in a final volume of 25 µL. DNA were reproduced using Corbbet Germany under

the following conditions: 1 cycle in 94 ° for 8 min (denaturation), 35 cycles in 94 ° for 1 minute, 60 ° for 90 seconds, 72 ° for 2 min and a final cycle of 72 ° for 7 minutes . The distilled water was used as negative control, the PCR products were electrophoresis on 1% agars gel stained with ethidium bromide.

PCR-RFLP at the gdh locus PCR-RFLP was performed on the position of GDH gene using restriction enzyme of BspLI (NlaIV, Fermentase) and RsaI (Fermentase) respectively to separate assemblage of (the genotype of) A, B and BIII, BIV . 15 µL of PCR product with 0.5 U of each enzyme along with 1.5 of 1X buffer enzyme, 1µL distilled water in final volume of 18µL was kept in 37° for 2 hours and then the fragments resulted from enzyme restriction were isolated on 3% agar high resolution gels stained by ethidium bromide after electrophoresis, a ladder 50 bp was used as a size marker.

3. Results

From 353 fecal samples related to the patients with Giardiasis, 50 samples was condensed using sucrose concentration gradient, then from the 50 samples, DNA extracted for 30 samples and GDH genes were successfully amplified for these samples, and bp432 strain of GDH gene was evident on agars 1/5 % gel according size marker of bp100 (Figure 1).



Figure1. 432bp band from Gdh gene on 1/5% agarose gel with size marker 100bp
1-5 Lane PCR products from feces samples related to patients with Giardia lamblia

Then using the restriction enzymes, the resulted fragments was well separated and cleared that the broken parts was appeared on high resolution 3% agar gel, in this study, it was indicated using BspLI (NlaIV, Fermentase) enzyme, three bands for A1 genotypes 4 bands for All genotypes, and 3 bands for B genotype, in accordance with the size marker bp50 that considering the RsaI enzyme are used to detect BIII

and BIV assemblage, by applying the enzyme on B genotype subtypes, it was found that BIII type with 4 band were created by different size markers. According to table 1, there were bands with different molecular weight on several assemblages as well as diagnostic profile was obtained by RFLP method for all isolates on GDH gene positions. Different size markers indicated that there are diversity of several AI,All,BIII genotypes in the region which have more diversity then other parts of Iran. (Table 1 - Figure 2)

Table 1. Predicted fragment sizes (bp) and diagnostic genotyping profile (bp) of *G. duodenalis* genetic assemblages when digested with *NlaIV* and *RsaI*

Diagnostic genotyping profile	Predicted fragment sizes	Enzyme	Assemblage
90, 120, 150	16, 18, 39, 87, 123, 149	<i>NlaIV</i>	A1
40,70, 80, 90, 120	18, 16, 39, 72, 77, 87, 123	<i>NlaIV</i>	All
40,120, 290	39,18, 123 291	<i>NlaIV</i>	B
40,130, 300	2,39, 133, 297	<i>RsaI</i>	BIII
430	2, 430	<i>RsaI</i>	BIV

According to the obtained results, from the 30 tested samples, 60% (18) were with AII type assemblage, 16/6% (5) with A1 type assemblage and 23/4% (7) with BIII type assemblage, it is noteworthy that that BIV type assemblage was not detected in the collection (Table 2).

Table2. Genotypic characterization of human *Giardia* isolates at the a GHD locus

Samples Code	Assemblages	Total
1,7,21,28,30	A1(16.6%)	5
2,3,4,6,8,9,10,11,14,15,16,17,22,23,24,25,27,29	All(60%)	18
5,12,13,18,19,20,26	BIII(23.4%)	7
no detected	BIV(0%)	0
30(100%)		

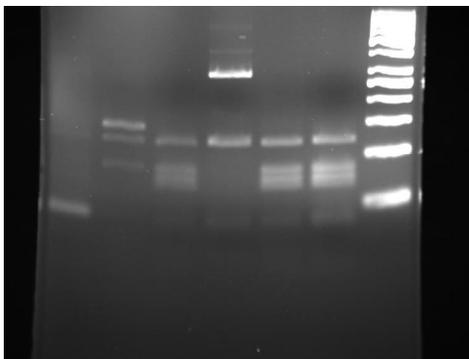


Figure No 2. The effect of *BspLI* enzyme on PCR products of *Giardia lamblia* on high resolution 3% agar gel 1, 2, 4 AII Lane genotyping, 3 Lane B-genotype and 5 Lane A1 genotype, M Lane size marker 50 bp

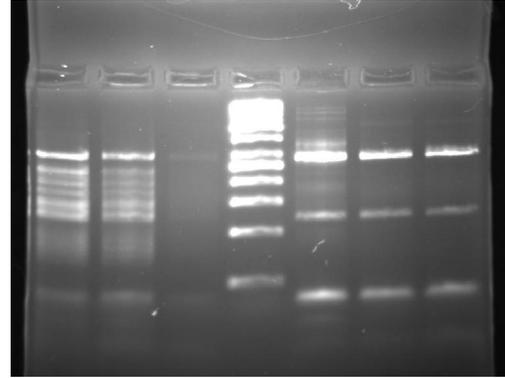


Figure 3: The effect of *RsaI* enzyme on PCR products of *Giardia lamblia* on high resolution 3% agar gel 1-3 Lane BIII genotype and Lane M size marker 50bp

4. Discussions

Giardiasis is a pathogenic factor that threat the children in under developing countries and the highest level of it prevalence in the region is related to the under 10 years children which consists between 15-20%, the seemingly the infection is usual in human and involves all age groups. Clinical symptoms of giardiasis is different from without symptom to chronic diarrhea with changeable malabsorption as well the factor risk are not detected well, this diversity in virulence was attributed to diversity in giardia isolates , host factors such as immune response and a combination of both (Thompson 2000). Taxonomy, life cycles, zoonosis potential of *Giardia lamblia* parasites are weakly detected in mammals, birds have been controversial for many years, but developing molecular tools for detecting giardia isolates from feces samples play a significant role in solving the problems (Thompson and Monis 2004; Hunter and Thompson 2005). There are different molecular methods for indentifying and diagnosis giardia *Lamblia* genotypes; some of the techniques prove that they are valuable tools for displaying all kind of genotypes distinct (Caccio SM, De Giacomo M et al. 2002). GLORF -C4, GDH TPI b-*Giardiasis* EF-1a, 16SrDNA are the gene sequences that are examined, the sequences have been successful in diagnosis the giardia genotypes and species using molecular PCR . Several molecular studies human isolates have conducted in world regions that shows 26% of the samples are with A assemblage, 69 percent with B assemblage, and 6% with mixed of A, B assemblage, as well as TPI and 16SrDNA genes were highly used sequences(Cacci 2005). Considering the point that PCR method sensitivity is 5 folds more than microscopic feces test (Armson A 2009). In the study, the PCR-RFLP method and GDH gene sequencing were used to identifying and determining the giardia *lamblia* parasite genotypes. After examining

the obtained results, it was observed that the common genotype of the parasite (A, B) has been found in human samples from southeast of Iran (Kerman). The distribution of AI, AII, BIII was respectively 16/6%, 60%, and 23/4%. The obtained results from studying on GDH gene sequence in Iran show that detected AII genotype from human isolates was from the most frequent ones and in this regard is consist with the current study. But not reporting AI genotypes in the study indicated the prevalence of the genotype in Kerman, but the prevalence of BIII genotype is more than Babaie's study in Tehran (60% compared to 7/87%) and is less than Fallah's study in East Azerbaijan (23.4% to 44%) (Babaei, Oormazdi et al. 2008; Fallah, Nahavandi et al. 2008).

The results from Read et al. 2004 from distinct human isolation on GDH gene sequences indicates that BIV is the most frequent genotype (Read, Monis et al. 2,004) while Glanew et al, 2007 study on human isolates in Ethiopia showed that 52% of samples are with A type genotype and 22% of them are with B type genotype that consist with recent study is (Gelanew, Lelle et al. 2007). On the other hand, based on the study performed in Norway in 2006, B is recorded as the most frequent genotype (Giessen, Vries et al. 2006). Bertarnd et al in 2005 among the 26 subjects with Giardiasis indicated that 61% of subjects were with B genotype and the others with A genotypes which are different from the results obtained in the present study (Bertrand, Albertini et al. 2005). Souza et al. in 2007 reported AII genotype in Brazil from 37 human isolates that supported our results (Souza, Gennari et al. 2007). Considering the fact that human infections are mostly occurred by B and AII type, the study confirmed the fact (Souza, Gennari et al. 2007). The above studies show that Giardia Lambilia genotypes are detected on the position of glutamate dehydrogenase gene using molecular PCR-RFLP. This molecular method helps us in determining giardia lambelia genotypes from direct human and domesticated animal feces sample and lead to obtaining valuable information on these parasite genotypes in a population without need to in vitro or out vitro amplification (Thompson, Hopkins et al. 2000). Researches in their studies and by advanced molecular examination found the fact that Giardia Lambilia has heterogeneity and is a species complex (Xiao and Fayer 2008) that give it different facial position based on the different geographical zone and host. It's presence was reported in wide scope of mammalian including phytophagous and human. In the study, it was tried to search and determine the parasite genotypes base on the geographical and ecological conditions and it is recommended to investigate the possibility of being zoonosis its genotypes.

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