Molecular Identification of *Giardia duodenalis* Parasite Isolates from Human by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism Technique (PCR-RFLP) in Baghdad Province

Abdullah Mustafa Qader,Tural Yelderim Bakir
Al-Nahrain University, Biotechnology Research Center,Foundation of Technical Education/College of Health and Medical Technology/Baghdad

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Abstract

The present study was designed to determine the genotypes of *Giardia duodenalis* isolates from human by using polymerase chain reaction – restriction fragment length polymorphism technique (PCR-RFLP) technique, by amplification of glutamate dehydrogenase (gdh) gene to provide information about the genetic diversity and their epidemiological and clinical characteristics in Baghdad province. Thirty isolates from children (1-12 years old) were processed for (PCR-RFLP). Data corresponding to demographic, social and environmental variables and presence or absence of symptoms were collected. The glutamate dehydrogenase (gdh) gene was amplified by using specific primers (GDHiF and GDHiR), the PCR amplification was observed in 17/30 (56.6%) of the fecal samples, among these 5/17 (29.4%) samples belonged to genotype A and 12/17 (70.5%) samples belonged to genotype B. Genetic subgenotypes identified from human fecal samples revealed that, 5/17 (29.4%) were subgenotype AII, 9/17 (52.9%) subgenotype BIII, and 3/17 (17.6%) subgenotype BIV. Genotype B was detected in children with severe symptomatic giardiasis and many of them had diarrhea while, subgenotype AII was detected in children with mild symptomatic giardiasis and without diarrhoea.

Key words: *Giardia duodenalis,* PCR-RFLP, Molecular Identification

Introduction

The flagellated protozoan *Giardia* is an intestinal parasite that can infect many species in the animal kingdom including mammalian, avian, reptilian, domesticated animals, and human [1,2]. The morphologically defined *Giardia* species are: *G.muris*, *G.microti, G.agilis*, *G.psicatti*, and *G.duodenalis* (syn. *G.lamblia*, *G.intestinalis*), only the later is recovered from human and a wide variety of other mammals. In human, it can cause gastrointestinal infections ranging from mild to severe as well as chronic disease [3]. Infection occurs by fecal-oral route transmission, either by direct contact or by ingestion of contaminated food or water [4]. Despite the morphological uniformity, considerable biotypic and genetic diversity exists within the *G.duodenalis* species [5]. The species includes several assemblages or genotypes (A–G) that can be discriminated on the basis of host selection and genomic mutations [6]. Although, several genes encoding proteins involved in meiosis are present in *Giardia*, a direct evidence for sexual recombination has not been shown yet[7]. Phylogenetic multi locus analysis using beta giardin (β giardine) gene, glutamate dehydrogenase (gdh) gene, and triose phosphate isomerase (tpi) gene based molecular methods have been used on representatives of each major genetic group to study the relations among genotypes from different hosts [8, 9].Molecular techniques such as polymerase chain reaction (PCR) provide alternative methods for specific detection of pathogens in stool, and in combination with techniques, such as restriction fragment length polymorphism (RFLP) have been used for genotyping *Giardia* [11]. The sensitivity of PCR detection is greater than the microscopic examination, making it of greater use for detection of low numbers of cysts in stool samples [12, 13]. The glutamate dehydrogenase (gdh) gene has been successfully used for genotyping *Giardia* isolated from human [14, 15, 16].

Materials and Methods

Samples collection: This study was conducted in Biotechnology Research Center/ Al-Nahrain University/ Baghdad-Iraq during the period from December 2008 to May 2009. A total of 30 positive *Giardia* cysts fecal samples belong to children (1-12 years old, 16 males and 14 females) that detected by conventional techniques (light microscopy) were collected in

plastic containers, then transported to the laboratory and stored at 4 Cº for 1 hours without preservatives [8].

The samples were collected from two hospitals in Baghdad (children central hospital in Al-Escan and Hemaet Al-Atfal pediatric teaching hospital). Selection of patients (with and without gastroenteritis) was accomplished with the assistance of the pediatrician consultant in the hospitals.

Purification of cysts: Cysts were obtained from fecal material by flotation method by using Zinc sulfate solution [17] and Sucrose solution [18] and washed with PBS and then all samples were stored at 4˚C until use.

Genomic DNA extraction: Genomic DNA of *G. duodenalis* isolates were extracted according to the manufacturer's protocol of Wizard® Genomic DNA Purification Kit that provided by Promega / U.S.A

PCR amplification of the gdh gene: The amplification of the gdh gene was performed on ice under aseptic conditions in a laminar air flow using 0.5 ml tight cap eppendorf tubes, and a single PCR protocol. In the reaction a 432bp fragment was amplified by using the forward primer (GDHiF) 5'-CAGTACAACTCTGCTCTCGG-'3 and the reverse primer (GDHiR) 5'-GTTGTCCTTGCACATCTCC-'3 [15]. In order to avoid non specific amplification of none targeted gdh gene sequence, the specific primers were subjected to multiple sequence alignment on CLUSTAL W program [19].

The primers then was subjected to second round of alignment on Genbank program BLAST [20] to further ensure that the primers were complementary with the target sequence.

The amplification conditions were modified as follows-the PCR mix consist of 2µl of each primers, 5µl of templete DNA , and 20 µl of Go *Taq*® Green Master Mix that provided by Promega/U.S.A. PCR was performed on a Mastercycler gradient (eppendorf–Germany) thermal cycler with the following amplification conditions: 1 cycle of 94˚C for 10 min. (initial heat activation step), 50 cycles of 35 sec. at 94˚C, 35 sec. at 61˚C and 50 sec. at 72˚C, with a final extension of 7 min. at 72˚C. PCR products were visualized on ethidium bromide (10 mg/ml) stained 1% agarose gel.

RFLP analysis: The restriction fragment length polymorphism (RFLP) analysis was performed by digesting 10 µl of PCR product (432bp) with 2 U of RsaI or 2 U of NlaIV in 1X Enzyme buffer (Promega,U.S.A) in a final volume of 25 µl for 3 hours at 37˚C.

The genotypes specific restriction enzymes profiles patterns were obtained by RsaI and NlaIV digestion of PCR product. The predicted restriction fragments sizes are described previously.

The NlaIV digestion was used for the distinction between all of the major genotypes and subgenotypes AI and AII after amplification. RsaI digestion distinguished between subgenotypes BIII, BIV [15].

Restriction fragments were separated by horizontal electrophoresis in 2% agarose gel, with ethidium bromide staining. A 1000 bp DNA ladder was included as a size marker. Restriction fragments were recorded by U.V. transilluminator at 310 nm wavelength [21].

Results

PCR Amplification of gdh gene:

The gdh gene was successfully amplified from 17/30(56.6%) samples. A 432 bp fragment of gdh locus was amplified in the PCR using primers GDHiF and GDHiR (Figure 1). The negative samples remaining observed could not be amplified.

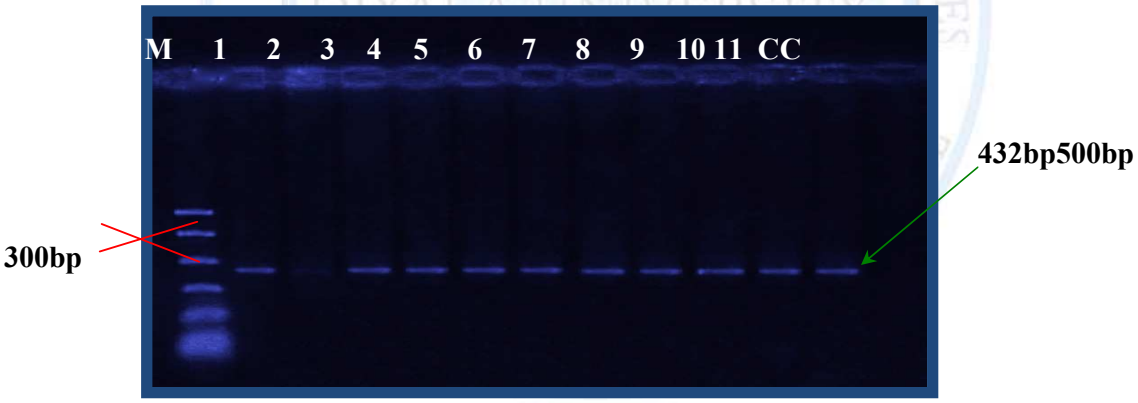

Figure (1): Agarose electrophoresis of PCR amplification for gdh gene using GDHiF and GDHiR primers. Lanes 1, 3, 4-11 represent PCR product. Lane CC represents contamination control and Lane M represents DNA Ladder. 432 bp fragments were resolved on a 2% agarose gel (40 minutes, 10V\cm, Tris borate buffer) and visualized by ethidum bromide staining.

RFLP analysis:

Restriction fragment length polymorphism analysis of the 17 samples of *G.duodenalis* revealed that 5/17 (29.4%) samples belong to genotype A, and 12/17 (70.5%) samples belong to genotype B.

These *G.duodenalis* main genotypes were divided into: 5/17 (29.4%) subgenotype AII, 9/17 (52.9%) subgenotype BIII, and 3/17 (17.6%) subgenotype BIV (Figures 2, 3) (Table 1).

Table (1): **The RFLP profiles of *G.duodenalis subgenotypes* after digestion with *Nla IV* and *Rsa I.***

Subgenotypes	Restriction enzymes	Expected fragments sizes(bp) [15]	Diagnostic fragments sizes (bp)
AII	Nla IV	20, 40, 50, 70, 80, 90, 120	70, 80, 90, 120
BIII	Nla IV	50, 120, 290	120, 290
BIII	Rsa I	30, 130, 300	130, 300
BIV	Rsa I	30, 430	430

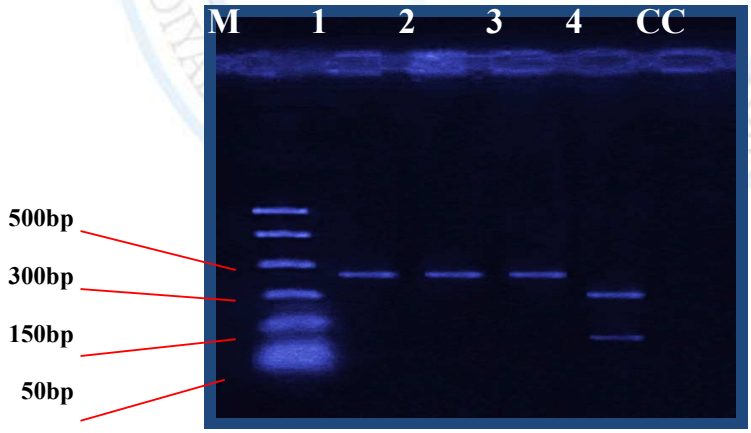

Figure (2): Restriction analysis of amplified gdh gene by PCR using Rsa I. Lanes 1, 2 and 3, represent *G.duodenalis* genotype B subgenotype IV (430 bp); lane 4, *G.duodenalis* genotype B subgenotype III (300 bp and 130 bp); lane CC represent contamination control; and lane M represent DNA Ladder. Fragments were resolved on a 2% agarose gel (40 minutes, 10V\cm, Tris borate buffer) and visualized by ethidum bromide staining.

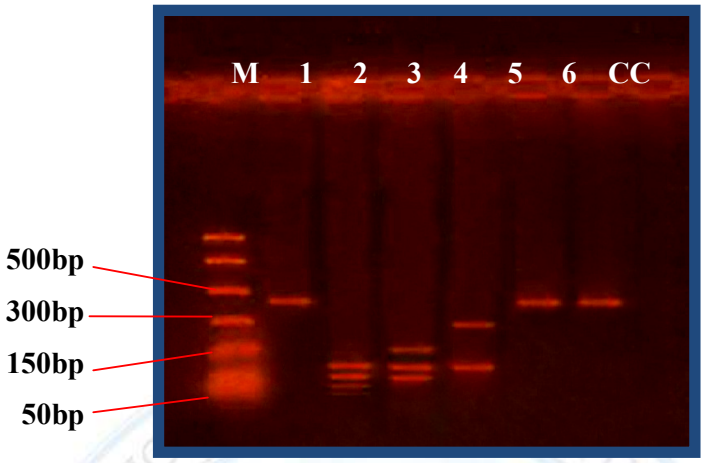

Figure (3): Restriction analysis of amplified gdh gene by PCR using Nla IV. Lanes 1, 5 and 6, represent PCR product (432 bp fragment); lane 2, *G.duodenalis* genotype A subgenotype II (120,90,80,and 70 bp); lane 4, *G.duodenalis* genotype B (290 and 120bp);; lane CC represent contamination control; and lane M represent DNA Ladder. Fragments were resolved on a 2% agarose gel (40 minutes, 10V\cm, Tris borate buffer) and visualized by ethidum bromide staining.

Epidemiological results:

1. Environmental conditions: - All individuals presenting subgenotype AII 5/5 (100%) lived in urban areas and had poor sanitary conditions, while the individuals with genotype B lived in rural areas and had poor sanitary conditions were 7/12 (58.33%), medium sanitary conditions were 4/12 (33.33%), or good sanitary conditions were 1/12 (8.33%).

Overcrowding was observed in all cases 5/5(100%) of subgenotype AII; while only 5/12 (41.66%) had this condition among individuals with genotype B. House flooding was never present among individuals with subgenotype AII while individuals with genotype B had frequent flooding in 5/12 (41.66%) of the cases, sometimes in 3/12 (25%) and never in 4/12 (33.33%).

2. Clinical signs:- All individuals with subgenotype AII 5/5 (100%) reported only weakness and loss of appetite and those with genotype B reported 5/12 (41.66%) abdominal pain, 4/12 (33.33%) weakness, and 3/12 (25%) vomiting. Individuals infected only with *G.duodenalis*

(100% genotype B) reported 3/5 (60%) diarrhea, 2/5 (40%) abdominal pain, 2/5 (40%) sleeping disorders, 2/5 (40%) weakness, 1/5 (20%) loss of appetite, and 1/5 (20%) vomiting.

Disscussion

The results of the present study have showed that the proportion of samples in which the glutamate dehydrogenase (gdh) gene could be amplified was higher than that reported by [31] who used the same primers, which were highly specific for gdh gene of *G.duodenalis.*

In this work, the amplification percentages of the gdh gene from fecal samples 56.6% agree and disagree with reports of several authors. [31] Reported amplification percentages of 52.9 % of human fecal samples when they worked on the same gene with fresh feces. [32] Analyzed 29 human fecal samples preserved in either potassium dichromate or formalin and obtained amplification of *Giardia* tpi gene in 16 (55%). On the other hand, [14] reported that the amplification percentage for the tpi gene was 94% in sporadic giardiasis and 88% in a giardial outbreak in a neonatology service when they worked with fresh feces. Chenna with his colleagues [18] analyzed 60 fresh fecal samples belong to humans, they obtained amplification of *Giardia* tpi gene in 43/60 (71.66%) of the samples, while [33] and [34] reported an amplification percentages of 98% and 100% respectively when they worked on the gdh gene.

The failures in the amplification of some fecal samples would derive from the low quantity of samples DNA, either due to their degrading in time or may be presence of some of PCR inhibitors such as (lipids, hemoglobin, bile salts, polysaccharides from mucus, bacteria, and food degradation product) which can affect the results of amplification [26,27].

The heterogeneity in the results of molecular analysis has revealed that *G.duodenalis* is a complex species, comprised of a range of diverse genotypes [1, 28]. For this reason, advance tools were used for molecular epidemiology determination of this diverse and interesting parasite in the world is critical. PCR-RFLP is a sensitive and powerful analytical tool that can capable of providing the level genotyping discrimination between and within genotypes by targeting some loci such as gdh, making it possible to identify the presence of mixed genotypes [14,15,25,29], and it is important to note that all loci enable successful grouping at level genotype of *G.duodenalis* isolates, and could characterize the subgenotype AII, whereas only a few loci allow subgenotypes differentiation within B genotype. The glutamate

dehydrogenase gene allows distinguishing between the subgenotypes of the genotypes A and B [30].

According to the present results, subgenotype AII was found only in ≥ 3 years old children while genotype B was found in different ages of children. In regard to sex, the distribution frequency of genotype B was estimated in both genders, and it was higher in males for subgenotype AII.

Overcrowding was present in all children infected with subgenotype AII that enabled to hypothesize the possibility of interhuman transmission. On the other hand, most children with genotype B showed no overcrowding. Although house flooding was frequent, suggesting the possibility of infection from a contaminated source such as water or food.

All individuals with subgenotype AII reported appetite loss and weakness; none of them exhibited diarrhea, abdominal pain, sleeping disorders, or vomiting symptoms that were found in children with genotype B. The results correlate subgenotype AII with mild symptomatic giardiasis and genotype B with sever symptomatic giardiasis, also individuals infected with genotype B are likely to seek medical advice while those with subgenotype AII are not; resulting in a slow deterioration of their own health status, and this is due to alternations in the nutrient absorption caused by *G.duodenalis.* Homan and Mank [35] found that children infected with genotype A had a 26 fold greater risk of having diarrhoea while Read [14] determined that genotype B was responsible of diarrheal outbreak in daycare centers in England. In the Netherlands, Ceu souza, and Poiares [36] found that genotype B infections associated with chronic diarrhea, while genotype A infections were related to intermittent diarrhoea. Eligio-Carcia [37] found there was no correlation between digestive manifestations and genotypes. The direct amplification of cysts DNA obtained from feces helps to solve important questions such as the presence of mixed genotypes, correlation between genotypes and hosts (pathogenicity), and selection for irrelevant genotypes during cultivation [22,23, 24,25].

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