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Detection of genotypes for *Giardia lamblia* in Iraqi patients feces by using PCR-RFLP techniques based on *GDH* gene characterization

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A R T I C L E I N F O

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A B S T R A C T

This study was carried out during the period from March 2016 to January 2017 to detect of genotypes and subgenotypes of *Giardia lamblia* in human feces by PCR-Restriction fragment length polymorphism based on the detection of *glutamate dehydrogenase* gene and study the corralation between genotype and prescence of symptoms , a total of 101 stool samples were taken from patients(male and female), aged(2-72) years, who suffering from acute or persistent diarrhea and examined by microscope and molecular techniques , the *gdh* gene was amplified from 19 cases only by a conventional PCR assay by using a specific oligonucleotide sequence for coding region by *gdh* gene at size 432bp and typed by RFLP analysis, the results showed the total infection with *Giardia lamblia* was 84(83.1%) according to the results of microscopic examination, of the 19 positive samples, 2(1.98%) were infected with genotype AI , 3(2.97%) genotype AII , 5(4.95%) genotype BIII and 9(8.91%) infected with genotype BIV, with significance differences between the genotype and precence of symptoms.

Introduction

Giardia lamblia is an flagellated protozoan caused giardiasis disease is a common intestinal disease(1), Giardiasis is a common cause of waterborne diarrhea, with 20-30% prevalence in many developing countries and a much lower infection rate in developed countries (2). About 200 million person for the world were infected with giardiasis yearly, the infected individual is excess about 900 million cyst in feces during period of infection (in the acute of infection)(3).

Giardiasis disease is transmitted by oral-fecal route and contact with infected person, in most cases it is associated with contaminated drinking water but also occasionally by recreational activity in still water(4),

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Clinical manifestations of symptomatic giardiasis include fatty stools, flatulence, diarrhoea and abdominal cramps, although a majority of cases are asymptomatic(5). Present laboratory methods for investigation of this parasite from feces are usually based on microscopic examination but these methods are unable to distinguish between genetically distinct parasites(6).

For understand the epidemiology of giardiasis, especially the role of zoonotic transmission and the correlation between the genetic variation of the parasite and its pathogenicity in the host, a large number of isolates have been characterized genetically(7), *Giardia lamblia* is a species complex and composed of at eight genetically distinict but morphologically identical called genotyping reffered to as genotype A to H, only genotype A (subtypes I and II) and B (subtypes III and IV) have been associated with human infections, but are also found in a number of other mammalian hosts(8)

The aim of the present study was determine of genotypes of *Giardia lamblia* isolates from patients who infected with diarrhea based on *glutamate dehydrogenase(gdh)* gene characterization.

Materials and methods

Samples collection this study was carried out in National centre of Hematology\ Al-Mustansria University during the period from March 2016 to January 2017, a total of 101 fecal samples that collected from patients and restaurant workers who consult hospitals and private medical laboratories(male and female) who aged ranged from (2-72)years. Fresh fecal samples were collected by using a sterile containers and transported in to a cooled box.

Microscopic examination this test was conducated on each sample of feces to detect of parasite (cyst or trophozoite), based on method that remined by Brook etal.,2001, by preparation of a clean glass slide, adrop of normal sline was put on one half slide and adrop of Lugal׳s iodine was put on other half,then mixed with the stool specimens and examined by using the magnification power 400 (40 x 10) to detect the presence of trophozoites and/or cysts.

Parasite purification the parasite had been purified from the residue of the contents of the stool by flotation methods using solutions of high density as NaCl , Sheather׳s Sucrose or Zinc Sulfate , in the current study, we used Sheather׳s Sucrose Solution where was the Specific gravity of this solution(1.28) is higher than from specific gravity of cyst of *G.lamblia*. 5 gm of feces was mixed with 10 ml of Normal saline , then it is filtered by gauze and the liquid put in test tube and centrifuged with speed 2500 rpm for five minutes , then was clear supernatent, 5 ml from Sheather׳s Sucrose Solution was added to the sediment, cover slip was put on the tube and centrifuged by 2500 rpm for 5 minutes , then cover slide was pull and examined by microscope under power 40x , where we were observed cysts of parasite, then we were take 500µI from supernatent and it was placed in a tube size 1.5ml and then kept a temperature 4° C preface to molecular study(9).

Extraction of DNA DNA was extracted from feces by using AccuPrep® stool genomic DNA Extraction Kit was supplied by BIONEER company, Korea and done according to company instructions (www.Bioneer.com), then the extracted genomic DNA was checked by using spectrophotometer (Shimadzu,Japan), which measured concentration and purity of the DNA by reading the absorbance in (260/280 nm).

Polymerase chain reaction PCR technique was carried out for detection of genotypes and subgenotypes for *G.lamblia* based on *glutamate dehydrogenase* (*gdh*) gene from human stool samples, PCR master mix was prepared by using Accupower PCR –premix master mix was equipped from BIONEER company, Korea , and this master mix done according to company instructions with some slight modification as following table (2-1).

Then, all the PCR tubes transferred into vortex for several minutes, Then placed in PCR Thermocycler.

PCR Thermocycler Conditions:

PCR thermocycler conditions were done according to the methods of Joshi *etal.*,2011 with some modification as table.

Table (2.1): conventional PCR mastermix preparation.

PCR master mix	volume
DNA template samples	5µl(26-1250)ng
Forward primer	1µl(10 pmole)
Revers Primer	1µl (10 pmole)
Sterile distilled water	8µl
PCR-Premix mastermix tube contain	5µl
Final volume	20µl

Table (2.2): primers of *gdh* gene of *Giardia lamblia* were equipped from BIONEER company(Nahavandi et al,2011).

primers	Nucleotide Sequence	Total nmole
Forword	5'-CAGTACAACTCYGCTCTCGG-3'	10.0 nmole
Reverse	5'- GTTRTCCTTGCACATCTCC-3'	10.0 nmole

Table(2.2): PCR Thermo cycler Conditions.

NO:	Steps	Temperature	Times	Cycles
1	Initial denaturation	94oC	10 min	1
2	Denaturation	94oC	35sec	40
3	Anneling	52oC	35sec	
4	Extension	72oC	60sec	
5	Final extension	72oC	7min	1

Gel electrophoresis for PCR_Products of *gdh* gene PCR products of *gdh* gene was carried out gel electrophoresis to make sure of prescence *gdh* gene(432bp) for insertion restriction enzymes *(NlaIV,RsaI*), 2% Agarose gel was prepared for electrophoresis of PCR products and

observation of bands on UV-transliminator for evalution and documentation of the results.

Restriction fragment polymorphism length (RFLP) Restriction enzymes (*RsaI*, BIONEER,Korea and *NlaIV*,BIOLAB,England) were used for restricted of PCR Products for *gdh* gene , reaction maxiture was prepared based on the company instructions where 10µl of PCR product were placed into Eppendorf tube (1.5ml) and 2µl from Accu buffer enzyme were added to it then, 0.5µl of restriction enzyme were added to previous mixture, finally 7.5µl of Diluent buffer were added to all of these components which final volume of reaction mixture was became 20µl.

Reaction mixture was incubated at 37°C for three hours, when Incubation period was completed , gel electrophoresis(2% Agarose gel) was carried out for observation of restricted bands by restriction enzymes .

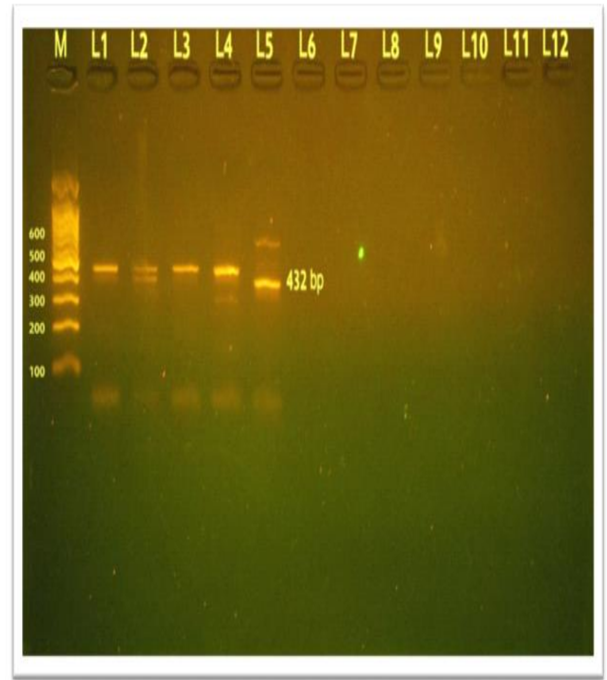
Both of these restriction enzymes were Specialized for restricted some sequences that was specific with one genotype without the others, which restriction enzyme (*NlaIV*) was used for discrimination subgenotypes belonged on genotype(A) and (B), while restriction enzyme (*RsaI)* was used for discrimination subgenotypes belonged on genotype (B) only, characterized to 2 subgenotypes AI,AII,BIII and BIV.

Staticitical analysis was done according to,(10), All data analyzed using Chi-Sequare test by using the SPSS statistical program.

Results

PCR Amplification of *gdh* gene:

The *gdh* gene was successfully amplified from 19/101(18.8%) samples . A 432bp fragment of *gdh* gene was amplified in conventional PCR technique as the figure(3.1).



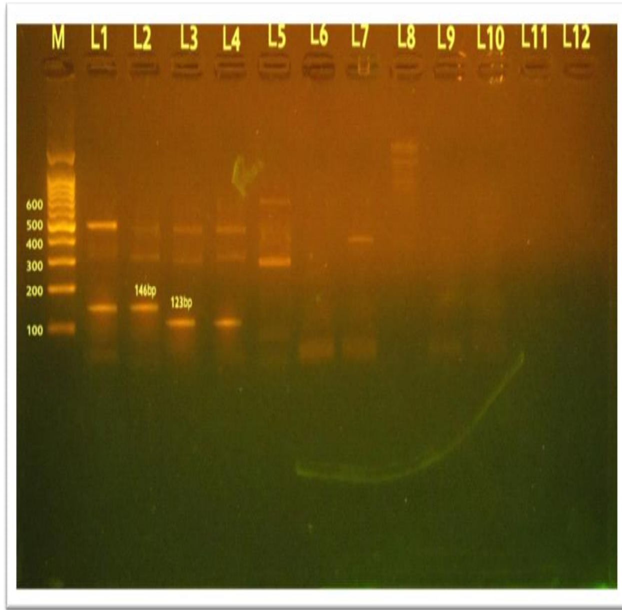
Figure(3.1): Amplification of *gdh* gene for DNA of *G.lamblia* from the infected human. Molecular weight marker (100 bp ladder), Lanes (1,2,3,4,5) positive samples for *gdh* gene at 432bp, Running conditions: Agarose gel (1.5%), 5v/cm for 1 hour, stained with ethidium bromide.

RFLP analysis

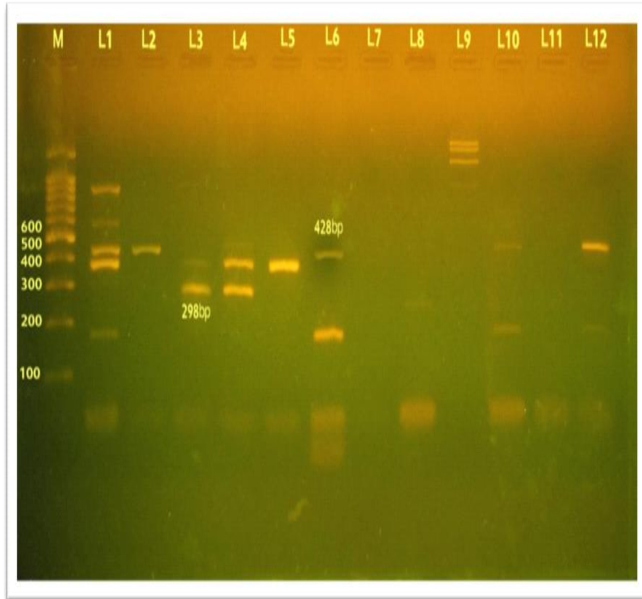
Restriction fragment length polymorphism analysis of the 19 samples of *Giardia lamblia* showed that 2/19(10.5%) infected with subgenotype AI, 3/19(15.7%) with subgenotype AII, 5/19(26.3%) with subgenotype BIII and 9/19(47.3%) infected with subgenotype BIV, as the following table:

Table(3.1): the diagnostic subgenotypes in the present study.

sub genotypes	Enzyme	Expected fragments sizes (bp)	Diagnostic fragments sizes (bp)
AI	NlaIV	16,39,47, 87,146	146
AII	NlaIV	16,39,47, 69,77,87,123	123
BIII	RsaI	30,131,298	298
BIV	RsaI	30,428	428



Figure(3.2): *NlaIV* enzyme digestion of single PCR product for *gdh* gene of *G. lamblia* on 1.5% Agarose gel stained with ethidium bromide.Lanes 1 and 2 positive sample for subgenotype AI at 146bp and Lanes 3 and 4 positive sample for subgenotype AII at 123bp(100bp ladder marker).



Figure(3.2): *RsaI* enzyme digestion of single PCR product for *gdh* gene of *G. lamblia* on 1.5% Agarose gel stained with ethidium bromide. Lanes 3 and 4 positive sample for subgenotype BIII at 298bp and Lanes(1,2,4,5,6,7) positive sample for subgenotype BIV at 428bp(100bp ladder marker).

Correlation between the symptomatic of giardiasis and genotypes with statistically significance differences between the prescence of symptoms of giardiasis and genotype for Giardia lamblia in the current study, as the following table:

Table (3.3): correlation between symptomatic of giardiasis and genotypes.

Clinical signs	Genotype A	Genotype B	Total
Symptomatic	3(60%)	12(85.7%)	15(78.9%)
Asymptomatic	2(40%)	2(14.3%)	4(21.05%)
Total	5(100%)	14(100%)	19(100%)

Discussion

Diarrhoeal diseases includes giardiasis are more common among people in developing countries, and they responsible of a considerable amount of morbidity and death rates particularly among children, based on the reports received by WHO and UN, the diarrhoel diseases still the most common cause of death among children under five ages in Worldwide(11), *G. lamblia* celebrated as the most common intestinal pathogen with an predestined number of 2.8 million infecting person per year(12).

In the current study, the amplification percentages of the *gdh* gene from stool samples 19(18.8%) agree and disagree with studies of several authors, Some fecal samples that identified positive with microscopic examination, they were a false negative results with PCR assay, these failures may be due to low DNA levels, the precence of a soild wall that inhibit the release of DNA from the cysts or the presence of PCR inhibitors in some of the fecal samples such as lipids, bile salts, polysaccharides from mucus, bacteria, and food degradation product or dissolution of parasite during storage(13).

The heterogeneity in the results of molecular analysis has discover that *G.lamblia* is a complex species, comprised of a range of diverse genotypes. For this reason, advanced tools were used for molecular epidemiology determination of this varied parasite in the world is critical. PCR-RFLP is a sensitive and powerful analytical tool that can qualified for providing the level genotyping discrimination between and within genotypes by targeting some genes such as *gdh,* making it possible to identify the presence of mixed genotypes, and it is important to observed that all genes able to successful classified at level genotypes of *G.lamblia* isolates, the *glutamate*

dehydrogenase gene allows distinguishing between the subgenotypes of the genotypes A and B(14).

The *gdh* gene is one of the most common and advantageous genetic marker for genotyping of *G.lamblia* , molecular analysis for this gene had been shown to assemble genotypes A and B isolates into four groups: AI, AII,BIII and BIV by using PCR-RFLP techniques(15).

The intragenotyping variations of *gdh* in assemblages A and B is helpful for subgenotyping where *Giardia lamblia* have a clonal population structure, detection of subgenotypes within A and B genotypes by using one set of primer for amplification *gdh* locus and inserted two types of restriction enzymes and acquired on different fragments of molecular lengths(16). Based on the results of the present study, the subgenotype BIV is more common among the study groups in the current study with percentage 9(47.3%) compared with other subgenotypes, These results were agreement with the study of Mbae *etal*.,2016 in Kenya while this study was disagreement with the study of Qader *etal*.,2011 in Baghdad province , the study of Hazrati-Tabbeh *etal*.,2014 in Azerbaijan and the study of Roointan *etal*.,2013 in southwest of Iran. these results may be due to transmission of giardiasis from human to human in their areas and zoonotic transmission plays a role in these distribution and the levels of hygiene conditions, the results of this study was showed with siginificance differences between the symptoms of giardiasis and genotypes where giardiasis disease has highly variability symptoms in some patients without any obvious symptoms but can be able excess cysts in stool, It's not visible where some patients have clinical signs and others are asymptomatic, but it may be due to reflect the virulence factors that it is found into genotype itself and may be due to the host factors such as immunity conditions and variability in parasites strains play a role in these differences(Amar *etal*.,2003)..

Many studies have found a relationship between symptomatic infection with genotype B and asymptomatic infection with genotype A as the study of Rafiei *etal.,* 2013 where they found no correlation between the infection with genotype A or B and symptoms and the study of Sahagun *etal*.,2008 in Spain where they found correlation between the genotypes and symptoms of giardiasis, where the highest rate of infection with assemblage A was among the patients who have symptomatic of disease, and the highest rate of infection with assemblage B was among the patients who have asymptomatic of giardiasis with precence significant differences.

These results may be due to the virulence factors that belonging into each genotype, the correlation between the prescence of symptomatic of giardiasis and genotype B and the asymptomatic of giardiasis and the assemblage A show to be no clear in children compared with adults where we found all children who admitted in this study have the same degree of symptoms, these differences in children may be due to patient status as the degree of host adaptation, parasite infective dose, age, prescence of co-infectious, nutritional and immunological status may affect the clinical signs of giardiasis more than *Giardia* assemblages(17 and 18).

in this study, we concluded Subtyping analysis showed that genotype B belongs to subtype BIII and BIV, and subtybing analysis for genotype A belongs to subtype AI and AII with predominance the subgenotype BIV. we recommended in future studied Genetic profiling is conducated by using multicopies of genes such as *SSU-rRna* and *β-giardin* to prevent failure cases through polymerase chain reaction.

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التحري عن الطرز الوراثية لطفيلي *Giardia lamblia* في براز المرضى العراقيين بوساطة تقنية -PCR RFLP واستنادا الى خصائص جين GDH

سبأ محسن الفهداوي سراب فوزي العاني يسرى غياث ياسين

الخلاصة

اجريت هذه الدراسة خلال المدة الواقعة بين اذار 2016الى كانون الثاني 2017كان الهدف منها التحري عن الطرز الوراثية لطفيلي *Giardia lamblia* في البراز البشري بوساطة تقنية تقييد اطوال القطع متعددة الاشكال PCR-RFLPوذلك من خلال الكشف عن جين *gdh* ، تم خلال هذه المدة جمع 101عينة براز من مرضى يشكون من اسهال حاد واعراض الإصابة بداء الجيارديات و من عدد من عمال المطاعم وصالونات الحلاقة والفنادق بأعتبارهم مصدر للعدوى بالطفيليات، تم تضخيم جين *gdh* من 19عينة بوساطة تقنية PCRالتقليدية وتم تنميطها جينيا بوساطة تقنية -PCR RFLPوذلك من خلال ادخال نوعين من الانزيمات القاطعة، من مجموع 19عينة موجبة لفحص PCRوجد 2(1.98%)مصابين بالطراز الوراثي AI، 3(2.97%)مصابين بالطراز الوراثي AII، 5(4.95%)مصابين بالطراز الوراثي BIIIو 9(8.91%)مصابين بالطراز الوراثي BIV، لاتوجد فروقات حقيقية بين نسبة الإصابة والعمر، الجنس،المهنة، التباين الفصلي،والمنطقة الجغرافية.