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GENETIC ANALYSIS OF GIARDIA LAMBLIA ISOLATED SPECIMENS FROM HUMANS IN SOME REGIONS OF BAGHDAD

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ASTRACT

The prevalence of Giardia lamblia was surved among 375 patient with gastroenteritis in outpatients of Medical City Lab, Al-Noamman Hospital and Al-Khadimya Teaching Hospital. In this study the relationship of some factors (gender, age and the season) with the prevalence of this intestinal protozoal parasite had been evaluated. the current study was carried out during the period from the beginning of April 2014 till the end of March 2015. The result revealed that the total rate of G.lamblia infection was 18.13% according to three diagnostic methods, there was no significant relation ($p \leq 0.01$) between infectivity rate of this parasite and gender, while there was significant relation($p \leq 0.01$) between infectivity rate and the age groups, the age group (1-9) years had the highest rate (54.41%), a significant differences($p \leq 0.01$) recorded between the infectivity rate and the seasons, as, the higher infection rate found in summer season (57.35%). Concerning G. lamblia molecular study, from the 68 positive samples, specific amplification of Trios Phosphate Isomerase (tpi)gene was noted in 38 (55.88%), the results showed that the PCR product of (tpi) gene used in detection G. lamblia genotype (assemblage A) and (assemblage B), that the positive samples isolates at 576bp and 208bp respectively, at percentage 10.52% and 68.42% respectively, and there are 21.05% mixed of assemblage (A+B).

KEYWORDS: Giardia lamblia, Baghdad city, some factors, PCR, tpi gene.

INTRODUCTION

Giardia lamblia is a widely distributed protozoon parasite that causes enteric disease in humans and other vertebrates.[1] It is recognized as the most common intestinal protozoa

infecting humans in Iraq.[2] It has major public health implication because the infective dose may be as low as 10 cysts.[3] Human can acquire G.lamblia infection through several transmission routes, such as direct contact with infected person or animals, and ingestion of contaminated water and/or food.[4] Approximately 200 million people have symptomatic giardiasis in Asia, Africa and Latin America and about 500,000 new cases are reported each year.[5] G. lamblia is recognized as the most common intestinal protozoan parasite infecting humans in Iraq.[2]

Pathological manifestations of giardiasis, depend on numerous factors, the most important of which are the virulence of G.lamblia strain, number of ingested cysts, age of the host and immune reactivity of the host at the moment of infection.[3] G.lamblia represents a complex species composed of at least eight genetic groups (assemblages A to H) that are distinguishable based on genetic polymorphisms in the triosephosphate isomerase (tpi), glutamate dehydrogenase (gdh), β-giardin (bg) and small-subunit unit rRNA (ssu-rRNA) genes.[6] Detection and identification of Giardia spp. including microscopic, immunologic and molecular methods, among them the molecular approach is considered to be an alternative method that is more efficient for this purpose, such as Polymerase Chain Reaction (PCR).[7] Till now, there is few data available on the molecular detection of G.lamblia from human in Iraq, therefore, the present study was conducted to detect, genotype of G.lamblia in fecal specimens of gastroenteritis patients.

MATERIALS AND METHODS

The samples were collected from: outpatients of Medical City Lab, Al-Noamman Hospital and Al-Khadimya Teaching Hospital during the period from beginning of April 2014 till the end of March 2015. Fecal samples were collected from 375 gastroenteritis patients, of both gender at various age, each sample was put in a clean screw cap container used for collecting stool samples, labeled with the number, date and site of collections.

Detection of G.lamblia in stool samples

The Smear samples were examined under light microscope using Lugol' iodine-stained preparation beside fresh normal saline smears[8,9], Zinc sulfate flotation technique and Chromatographic Immunoassay detection (Certest Giardia rapid strip test).

Certest Giardia rapid strip test

This test is used to determine G.lamblia in stool samples, kit was supplied by Certest Biotec /Spain, consist of CerTest Giardia strip test and stool collection tube with diluents, the strip consists of a nitrocellulose membrane pre-coated with mouse monoclonal antibodies on the test line (T), in the results strip, against G.lamblia and with rabbit polyclonal antibodies, on the control line (C) a against a specific protein. The faecal sample must be diluted in the dilution buffer that is supplied with the test and put some drops of mixture (fecal sample + diluent) in the well of rapid strip. The positive sample, antigen of the diluted react with red colored conjugate complex (anti-Giardia mono clonal antibodies-red polystyrene) and the red line be visible within fifteen minutes.

Molecular approach

This part of research has been done in ASCO Learning Center -Molecular Biology Lab /Al-harthia /Baghdad. DNA was extracted from each Giardia-positive sample using the QIAamp® Stool mini kit (Qiagen, Germany) following the manufacturer's instructions. The purity of DNA were estimated using a Nanodrop Spectrometer. Molecular diagnosis of G.lamblia was performed using 576-bp region and 208 bp region from the tpi A and tpi B genes respectively according to.[10,11] In each reaction, negative (mix+water) except DNA sample were added. The PCR primers were provided by Alpha DNA, Canada as the sequence, tpi A primer, forword (5'- CGA GAC AAG TGT TGA GAT G-3') and reverse (5'- GGT CAA GAG CTT ACA ACA CG-3').The tpi B primer, forword (5'- GTT GCT CCC TCC TTT GTG C-3') and reverse (5'-CTC TGC TCA TTG GTC TCG C-3'). Amplification reactions (25µl) contained (5µl) of DNA template, 2X Go Taq Green Master Mix at (12.5µl), G.lamblia forward primer (1µl), G.lamblia reverse primer (1µl) and nuclease free water (5.5µl). Annealing temperature were 55C^{o} for both primers. The sizes of the DNA amplicons were determined by a 1.0% agarose gel electrophoresis, ethidium bromide staining, and ultraviolet transillumination compared to a 1-kb DNA ladder run concurrently in the same gel slab.

Statistical analysis

Experimental data were analyzed using the Chi-square test: P value ≤ 0.05 and ≤ 0.01, was considered statistically significant.

RESULTS AND DISCUSSIONS

Giardia lamblia is one of the most widespread intestinal parasites in humans. It is also considered as one of the common non-viral causes of diarrhea in developed countries.[3,12] The results showed that the number of positive samples of G.lamblia were 68 /375(18.13%) table (1). This percentage was lower than the results of Turki et al. (2015) who observed total infectivity rate 25% in Al-Muthanna province.[11] On other conlerary study of epidemiology for intestinal parasites in Abu Ghraib and Al-Amiriyah regions showed the infectivity rates of G.lamblia were11.95%, 5.49% respectively[13], Salman et al.(2016) recorded the prevalence of G.lamblia in Kirkuk province at percent 10.31 %.[2] The differences in infectivity rate of G.lamblia between the provinces in our country and sometime in the same province from one area to another may be due to many reasons such as: immune status, nutritional status, and age of the host, number of samples[14], environmental factors, crowding effects, lower standards of hygiene, sanitation, also drinking of unfiltered water, person to person contact, person to animal contact which consider important source of infection.[15] As well, transmission of this parasite occurs via fecal-oral route, either directly or indirectly by eating or drinking fecally contaminated food and water.[16]

The Results also showed that no significant relation between gender and infectivity rate, the infectivity rate of giardiasis in male was 36/194 (18.55%) and female was 32/181 (17.67%) table (1), these results agree with observation of Al-Warid (2012) who showed that the incidence of G.lamblia was disassociated with gender where the infection rate among males 52.32% and 47.68% in females.[17] Non significance difference between gender and infection with G.lamblia can be discuss by both male and female have the same opportunity to work, learning and education especially in urban region, so the infection by G.lamblia and other parasites may not varied so much between genders. In Iran, study carried out by Vahedi et al.(2012) observed no significant relation between the infection of parasite and gender, where as the results in males 2.2% and in females 1.9%.[18]

The current study disagree with the study of Al-Jubouri (2010) who showed significant relation between the gender and infectivity rate and reported a higher infection rate among males 18.18%, in females 10.61%.[19], while study in north of Iraq by Al-Taie and Ali in 2009 showed that the infectivity rate in females significantly was higher than in males 25%, 20% respectively.[20]

Table 1: The percentage of total infection and incidence of infection according to the gender

Gender	No. of Infected	No. of non Infected	Total cases	Percentage%
Male	36	158	194	18.55
Female	32	149	181	17.67
Total	68	307	375	18.13
Chi square =0.0485		p-value is 0.825642		

Table (2) illustrates the prevalence of giardiasis according to the seasons, few infections observed in spring (March, April, May) 6.86% and then reached its peak in the summer (June, July, August) and autumn (September, October, November) 37.12%, 11.11%, respectively and very low 3.92% in winter (December, January, February) the result showed highly significant in the distribution of infection during the seasons of the year ($p \leq 0.01$). Current result was with the alliance with the results referred to by Saeed et al. (2005) who recorded higher infectivity rate in July was 8.1%, while the lower infectivity rate recorded in January (2.7%)[21], the reason for this may be due to changes in temperature during the months of the year as the low temperatures recorded during winter reduces the growth of the cysts and its development[21], another study indicated that cold weather kills the infective cysts[22,23], while the presence of high temperatures during the summer increase the chances of infection by drinks, ice-cream, fruits and vegetables that may be contaminated by the cysts of parasite.[21] The result in current study disagree with Abbas et al. (2011) who found that infection in Kingdom of Saudi Arabia increased in winter especially in February 5.71% and no cases were recorded at July and August.[24]

Table 2: The percentage of infection according the seasons

Seasons	No. of non infection	Total cases	Percentage %
Summer	83	132	**37.12
Autumn	80	90	11.11
Winter	49	51	3.92
Spring	95	102	6.86
Total	307	375	18.13
Chi-square = 50.7146. p-value is < 0.00001. **P≤ 0.01			

In table (3), showed the maximum infection rate in age group (1-9) year, 47.36%, and the babies under one year 17.77%, while the other age groups (9-19),(19-29),(29-39),(39-49) and (50 ≤) years have less percentage of infectivity rate, 7.79%,7.81%,1.96%,7.40% and 6.25% respectively, there was highly significant

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Table 3: The percentage of infection according the age

Age group	No. of Infection	No. of non infection	Total cases	Percentage %
< 1	8	37	45	17.77
1-9	45	50	95	**47.36
9-19	6	71	77	7.79
19-29	5	59	64	7.81
29-39	1	50	51	1.96
39-49	2	25	27	7.40
>50	1	15	16	6.25
Chi square =77.439 p value is < 0.0001 **P≤ 0.01				

The positive samples for G.lamblia (68/375), which detected by three diagnosis methods (microscope exam, rapid strip test and flotation method), figure 1 showed the distribution of positive samples according the three diagnostic methods.

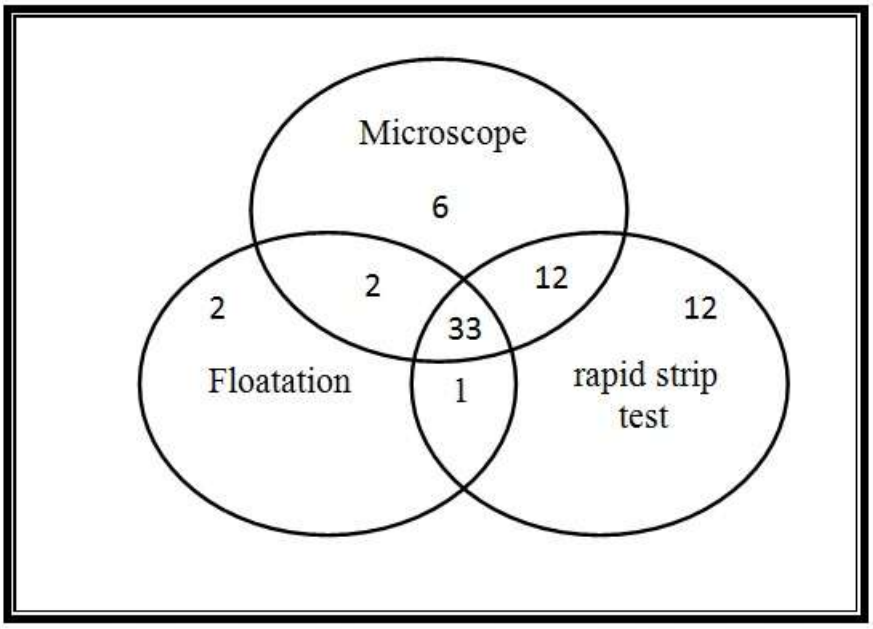

Figure 1: positive samples distribution over the three detection methods

The tpi gene was successfully amplified from 38 of the analyzed 68 positive fecal samples of human (55.88%). A576bp and 208bp fragment of tpi gene was amplified in the PCR using primers tpiA and tpiB representing in figures (2) and (3) respectively. Among G.lamblia isolates 4/38(10.52%) were genotype A, 26/38 (68.42%) were genotype B and 8/38(21.05%) mixed of genotype (A+B).

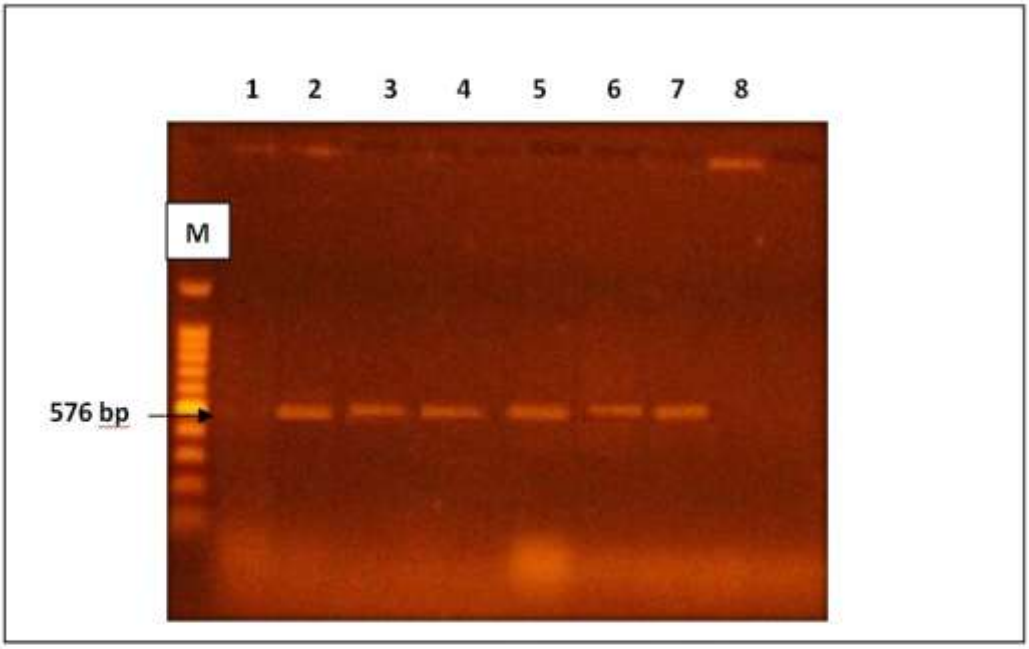

Figure 2: Agarose electrophoresis of PCR amplification for *tpi* gene using TPIA primers. Lanes 1&8 represent negative control , 2-7 represent PCR product. Lane M represents DNA Ladder. 576 bp fragments were resolved on a1% (7 v/cm for 1-2 hr) and visualized by ethidum bromide staining

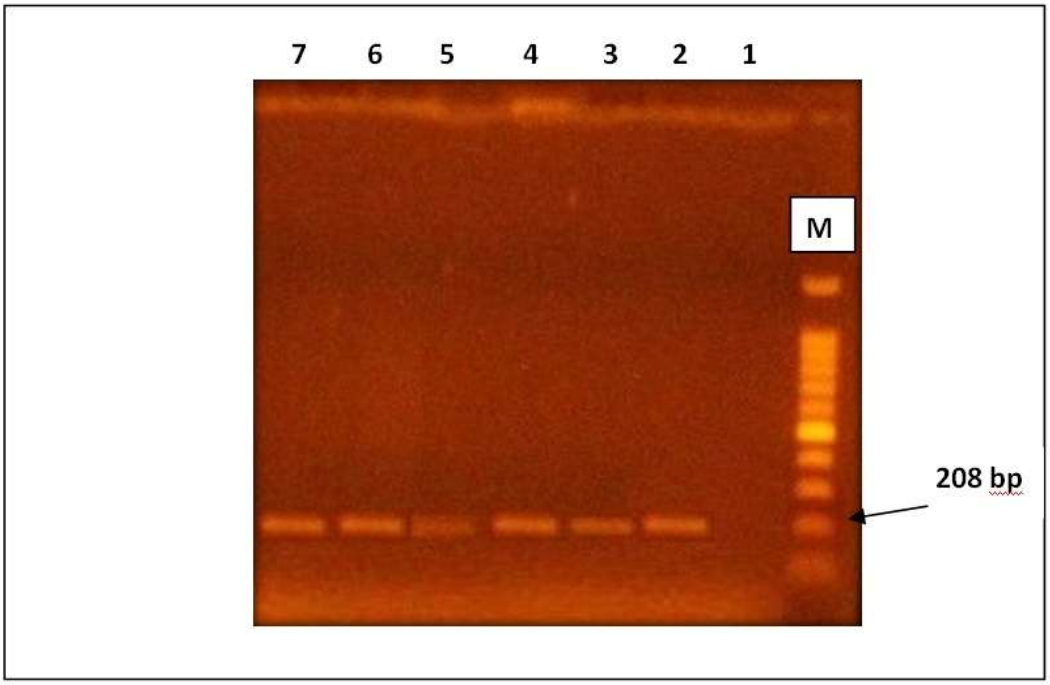

Figure 3: Agarose electrophoresis of PCR amplification for *tpi* gene using TPIB primers. Lanes 1 represent negative control, 2-7 represent PCR product. Lane M represents DNA Ladder. 208 bp fragments were resolved on 1% (7 v/cm for 1-2 hr) and visualized by ethidum bromide staining.

These results agree with previous study which used the same primers conducted in Al-Nijaf Al-Ashraf province of Iraq which found that 41% of samples had succeeded the tpi gene amplification and the genotype B was prevalent in patients at percentage 61% while the genotype A showed in 39.1%.[10] Molina et al.(2007) succeeded in amplification of same gene by same primers in 28 of the 34 analyzed samples (82.35%) and the B genotype was obtained in all cases.[27] While disagreement with other study conducted in Iraq in Al-Muthanna province used the same primers which recorded the percentage of assemblage A was 30.14% while the assemblage B was 26.03% .[11] The predominance of assemblage B in study cases, meaning the genotype B have virulence factors more than genotype A.[10]

The failure of amplified specific gene in samples, reported in several studies[7,10] so there is a consensus among the authors that the occurrence of false-negative cases is associated with factors:

- The small amount of G.lamblia cysts in the samples may affect the concentration of the extracted DNA.[7]
- Low quality DNA of the samples either due to their degrading in time or because of chemical modifications caused by several substances.[28]

- The presence of inhibitors in the fecal sample and the concentration of these inhibitors which may act in 3 levels: interference in the cellular lysis, degradation or uptake of nucleic acids, or inactivation of thermo stable polymerases.[27]
- Mismatches in primer sequences may be too long to allow successful PCR analysis although that gene primers are designed to bind regions in genes.[7]
- Infected with G.lamblia belong to other assemblage.[29]

The tpi gene was specially chosen for this study because high genetic heterogeneity displayed by Giardia spp. at this locus[30,31], The tpi-based genotyping tool is also useful in epidemiologic investigations of giardiasis in humans[30], it is evolution-marking gene used to identify different genotypes and sub-genotypes.[27] The tpi gene encode to triose-phosphate isomerase enzyme[32]

CONCLUSIONS

Gender is not related to the spread of giardiasis, Incidence of G.lamblia is increasing in hot seasons and age group (1-9 years) is more susceptible to giardiasis, the highest rate of infection with the assemblage (B) compared to assemblage (A) according to PCR assay.

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