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Molecular diversity and Phylogenetic tree of *Giardia lamblia* among some Iraqi gastrointestinal patients.

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Abstract:

The present study was conducted during the period from 10 th of March 2017 until 31 th of July 2017. The study intended to evaluate the genetic potential association between *Giardia lamblia* assemblages and *Helicobacter pylori* co-infection, in addition to studying gene characterization of *Giardia lamblia* among gastrointestinal disturbances patients.

Two hundred and fifty feces samples were collected from patients experiencing gastrointestinal disturbances with various age groups who attending to different governmental hospitals in Baghdad city with the main clinical feature (abdominal pain, nausea and bloating).The stool specimens were collected in sterile dry plastic cups with tight lids particularly made for this purpose. Each cup was given a unique number representing the patient.

The outcomes showed that there were (250) cases constituted by (132) male which represents (52.8%) and (118) female which characterize (47.2%) suspected as acute gastritis cases. The total infection rate *Helicobacter pylori* was (98) positive samples which characterize (39.2%) and (152) negative samples at the rate of (60.8%).The total infection rate with *Giardia lamblia* was (20) positive samples which represent (8.0%) and (230) negative samples at the rate of (92.0%).

Twenty positive samples of *Giardia lamblia* were successfully amplified for trios phosphate isomerase (tpi)gene by Multiplex-PCR assay and the result showed the presence of assemblage (A)was in 9 samples, assemblage (B) was in 7 samples and 4 samples were mixed assemblages (A+B).

Key words: Phylogenetic tree, *Giardia lamblia*, gastrointestinal patients.

INTRODUCTION

Gastrointestinal diseases are the main causes of morbidity and mortality throughout the world and especially in creating countries. Causes of gastrointestinal disease include a wide variety of bacteria, viruses and parasites [1]. Both *Giardia lamblia* and *Helicobacter pylori* take possession of the upper gastrointestinal tract and as result activating predictable side effects on the functional gastrointestinal infection [2].

Giardia lamblia (otherwise called *Giardia intestinalis* and *Giardia duodenalis*) is a protozoan organism that is a common cause of diarrheal disease in both the creating and industrialized countries [3]. The protozoan parasite is the causative agent of giardiasis in a wide range of vertebrate, including humans [4].It has estimated that about 200 million individuals had infected each year in Africa, Asia and Latin America. In the industrialized countries, overall prevalence rate of giardiasis is 2-5%. However, *Giardia lamblia* infects children early in life with a prevalence rate 15-20% of children younger than 10 years that common [5].

Isolates of *G. lamblia* are classified into eight assemblages (A-H) depend on the characterization of the *glutamate dehydrogenase* (gdh), *small-subunit* (SSU) rRNA, and *triose phosphate isomerase (tpi)* genes [6]. Phylogenetic multi locus analysis utilizing *beta- giardine*, *glutamate dehydrogenase* and *triose phosphate isomerase* genes based molecular methods have been used on representative of each major genetic group to study the relations among genotypes from various hosts [7].

Helicobacter pylori causes one of the most common infections in humans. The infection is widespread all over the world and affects all age groups. It has calculated that the percentage of world population that had affected by *H. pylori* is 50% [8]. Therefore, *Helicobacter pylori* is one of the most common bacterial infectious agents it inhabits the stomach of humans. Internationally, the pervasiveness of *H. pylori* disease in developing countries is noticeably higher than that in other countries. Moreover, the obtaining of *Helicobacter pylori* appears to happen at higher rates in developing countries [9].

Bacterium is a gram-negative that colonized the human gastric mucosa where it might give rise to symptoms such as recurrent peptic ulcers and chronic gastritis, and has also been associated with gastric tumor [10].

The relationship between *Giardia lamblia and Helicobacter pylori* infection might have many explanations such as; that *G. lamblia* and *H pylori* transmitted by the fecal-oral route, and thus, this involvement might show that two microorganisms share this common route of transmission [11]. Also the significance of polymicrobial infections has increased huge consideration of scientists in recent years and some synergistic infections had recognized. In synergistic polymicrobial infections, one microorganism makes a positive environment for another one to more effectively colonize a particular position of their common host [12]. Therefore, gastritis resulting from each organism predisposes the individual to the other one [13]. Additionally, hypochlorhydria be actuated by *H. pylori* increases susceptibility to enteric infections such as salmonellosis, giardiasis, and other infections [14].

MATERIALS AND METHODS

Patients

Two hundred and fifty patients, with the main clinical feature (gastrointestinal disturbances, nausea, bloating and vomiting) were examined and diagnosed by specialist physicians as suspected with acute gastritis. The basic demographic features included: age, gender, education level, water supply, food sources, socioeconomic status, smoking and symptoms.

Detection of *Helicobacter pylori*

***Helicobacter pylori* antibody test [15]**

Blood samples (2 ml) were collected from suspected individuals. The blood specimens were examined by using immune chromatographic test (ICT) for the qualitative detection of *H. pylori* antibody as described by the manufacturer.

***Helicobacter pylori* fecal antigen test [16]**

Stool specimens were examined by using of rapid immune chromatographic test (ICT) for the qualitative estimation of bacterial antigen in fresh stool samples as described by the manufacturer.

General Stool Examination [17]

Wet mount**:** Giardia cysts and trophozoites has identified on wet fecal smears using light microscopy. Each stool sample should be examined for detecting *G .lamblia* stages by using direct double wet preparation of 0.85% of NaCl and 1% of lugols iodine and examined using 10 X and 40 X.

***Giardia lamblia* fecal Ag test [18]**

The stool specimens were examined by the used of rapid immune chromatographic test (ICT) for the qualitative detection of *G. lamblia* antigen in fresh fecal samples as described by

manufacturer. Instructions given by the manufacturer have followed.

Fecal samples collection

Fresh fecal samples were collected by using sterile containers and transported in to a cooled box (temperature approximately 10°C). Then, the samples were transported to the molecular laboratory at College of Health and Medical Technology / Baghdad. And stored immediately at-20°C for molecular analysis (Multiplex -PCR).

Primers [19]

The PCR primers that were used in this study for detection *G. lamblia* genotypes (A,B) based on *triose phosphate isomerase*(tpi) gene were designed in previous study and these primers were provided from (Macrogen Company / Korea)as following tables (1) and (2):

Table 1:Primers of the primary PCR

Genotypes	Primer	Primer Sequence (5-3)	PCR product
Genotype A	TPIA-F1	CGAGACAAGTGTTGAGATG	----------
	TPIA-R1	GGTCAAGAGCTTACAACACG	
Genotype B	TPIB-F1	GTTGCTCCCTCCTTTGTGC	----------
	TPIB-R1	CTCTGCTCATTGGTCTCGC	

Table 2:Primers of the nested PCR

[	Primer	Primer Sequence (5-3)	PCR product
Genotype A	TPIA-F2	CCAAGAAGGCTAAGCGTGC	476pb
	TPIA-R2	GGTCAAGAGCTTACAACACG	
Genotype B	TPIB-F2	GCACAGAACGTGTATCTGG	140pb
	TPIB-R2	CTCTGCTCATTGGTCTCGC	

Polymerase chain reaction (PCR) [20]

The PCR technique was performed for detection *G. lamblia* genotypes based on *triose phosphate isomerase* (tpi) gene from human stool samples. This method was carried out according to method described in previous study as following steps:

Genomic DNA Extraction

Genomic DNA from feces samples were extracted by using stool DNA Extraction Kit (QIAGEN / Germany) and done according to company instructions.

Multiplex PCR master mix preparation

Multiplex primary PCR and nested PCR master mix was prepared by using (Multiplex PCR Premix Kit) and this master mix done according to company instructions.PCR reaction was carried out in a 25µl reaction containing (Green Master Mix, Primers, DNA template) and the volume was completed to 25µl by using nuclease-free water. Then, all the PCR tubes had transferred into spin vortex centrifuge at 3000rpm for 3 minutes and placed in PCR thermo cycler**.**

PCR Thermocycler conditions

Primary and nested PCR thermocycler conditions were done according to primer annealing temperature and PCR technique condition.

Table 3: Primary PCR thermocycler condition

PCR step	Temp.	Time	repeat
Initial Denaturation	94C	5 min	1
Denaturation	94C	30 sec	30 cycle
Annealing	52C	30 sec	30 cycle
Extension	72C	1 min	30 cycle
Final extension	72C	10 min	1
Hold	4C	Forever	-

Conditions for the nested PCR were identical to the primary PCR except for the annealing temperature which was raised from 52C° to 54C°. All other parameters of the amplification cycle were remained unchanged.

PCR product analysis: The PCR products had analyzed by agarose gel electrophoresis and were visualized by ethidum bromide staining following steps as company instruction.

Sequencing [21]

After confirming the amplification of the *triose phosphate isomerase* gene by nested PCR, three purified PCR product, were sequence on both strands for each genotype. 25µl from PCR products had sent to (Macrogene Company / Korea) to determine the DNA sequencing for this gene to construct the Phylogenetic tree in order to assess the extent of genetic diversity within *G. lamblia* isolates, as well as their evolutionary relationship with other *Giardia spp*.

Statistical analysis

The analysis was done by SPSS (Statistical Package for Social Sciences) version 16. For all analysis, statistical significance was considered at highly significant level P-value of <0.01, significant level P-value of <0.05 and non-significant level P-value >0.05.

RESULTS AND DISCUSSION

The total numbers of samples examined were (250) cases suspected as acute gastritis infection. The total infection rate for *H. pylori* was (98) positive samples which represent (39.2%) and (152) negative samples at the rate of (60.8%)**.** The total infection rate with *Giardia lamblia* was (20) positive samples which represent (8.0%) and (230) negative samples at the rate of (92.0%) as shown in table (4).There was significant difference (P. value of <0.05) between distribution of *G. lamblia* infection and *H. pylori* co-infection.

Table 4: Positive and negative percentage of *H. pylori* and *G. lamblia* infection

G. lamblia GSE-Ag	*H. pylori* Ag		Total
	Negative	Positive	
Negative	152	78	230
	60.8%	31.2%	92.0%
Positive*	0	20*	20
	0.0%	8.0%	8.0%
Total	152	98	250
	60.8%	39.2%	100.0%
*p. value <0.05 S			

In developing nation co-infections relating to few unique pathogens commonly occur. A few late cross-sectional studies from various areas had detailed a potential relationship between *Giardia lamblia* and *Helicobacter pylori*, which both organism colonizes the gastrointestinal tract in their human hosts [2]. So the co-infection of *H. pylori* and *G. lamblia* is common for their similar route of transmission and strong correlation to socio-economic levels [22,39].

The results in table (4) showed that the prevalence of infection among patients suffering from gastrointestinal disturbances was 39.2%, which lower than in neighboring countries such as Jordan (82%) and Turkey (63%) [23, 24]. This difference might due to number of samples or life style [25].

The present study showed that the prevalence rate of *G. lamblia* was (8.0%) which lower than in neighboring countries such as Iran (30.0%), Turkey (30.6%), Egypt (10.0%)and Sudan (22.0%) [26,27,28,29].These differences were probably due to varying sample size, geographical locations, personal health education, public sanitary conditions and periods of the studies. There were significant differences (p <0.05) between distribution of *G. lamblia* infection and *H. pylori* co-infection.

Total number of samples examined were 250, (109) of them showed positive result of *H. pylori* when examined by blood antibody test in the rate of (43.6%) and (98) positive result when examined by rapid fecal antigen test in the rate of (39.2%), as shown in table (5) .

Table 5: Percentage of positive and negative *H. pylori* detecting by blood antibody and fecal antigen

Total number of samples examined	*H. pylori* (blood Ab)		%	*H. pylori* (fecal Ag)		%
	+ve	-ve		+ve	-ve	
250	109	141	250	98	152	250
	(43.6%)	(56.4%)	100%	(39.2%)	(60.8%)	100%
	P. value > 0.05 NS					

Fecal antigen tests had detected antigens in stool samples and this test could be done for humans in all age groups and showed rapid results without needing of sophisticated laboratory equipment. The test had a high sensitivity, specificity and accuracy [30]. Table (5) showed the positive specimens of *H. pylori* there was slight difference between *H. pylori* infection and detection methods by blood antibody and fecal antigen. This difference due to the rapid fecal antigen test had several advantages which include high sensitivity, specificity, accuracy and could detect the cured of infection after treatment. While the blood test has used to measure antibodies to *H. pylori* infection but cannot detect current infection or how long the patient had the infection and these because the blood test can be positive for months, even if the infection was cured. [31,32].

The total number of positive *H. pylori* fecal antigen were 98 specimens, (20) of them showed positive result for *G. lamblia* when examined by fecal antigen in the rate of (20.4%) and (9) positive result of *G. lamblia* when examined by GSE in the rate of (9.2%), as shown in table (6).

Table 6 : Positive and negative percentage of *G. lamblia* detecting by GSE and fecal Ag

Positive fecal *H. pylori* Ag 98 (100%)	GSE (*G. lamblia*)		Fecal Ag (*G. lamblia*)	
	+ve	-ve	+ve	-ve
	9 (9.2%)	89(90.8%)	20* (20.4%)	78 (79.6%)
	* P. value <0.05S			

Table (6) showed positive specimens of *G. lamblia* when detected by general stool examination and *G. lamblia* fecal antigen. There were significant differences between *G. lamblia* infection and detection method by fecal antigens. This difference might be due to several factors which include; the potential variations in fecal excretion of cysts, *G. lamblia* can identify in 50-70% of patients after a single stool examination and more than 90% after three stool examinations [33]. While detection of *G. lamblia* by fecal antigens had a rapid and effective method with high sensitivity and specificity and detects *Giardia* antigen in stool specimens even when the count of the parasite is low. Thus, reducing the possibility of missing even the asymptomatic cases [34].

Regarding the frequency of the *triose phosphate isomerase* gene in *G. lamblia* positive isolates. It was shown that *triose phosphate isomerase* (tpi-A) genotype represents (9) isolates in the rate of (45%). It was the most frequent genotype followed by *triose phosphate isomerase* (tpi-B) genotype represents (7) isolates in the rate of (55%) and (4) isolates mix genotype(A&B)in the rate of (20%)as appears in table (7).

Table 7: Frequency of *triose phosphate isomerase* genotype in *G. lamblia* positive isolates

Positive fecal Ag (*G. lamblia*) 20 (100%	Genes	*tpi*-A	*tpi*-B	*tpi*A & *tpi*B	Total
	No.	9	7	4	20
	%	45%	35%	20%	100%

Outcomes of molecular analysis for *triose phosphate isomerase* (*tpiB*) gene of *G .lamblia* isolates showed that the PCR products in lanes 1,2,3,4,6,9, 13,16,17,18 and 20 were positive results which shown the migration of DNA band according to molecular weight at 140 bp PCR product sizes and were visualized by ethidum bromide staining. Figure (1).

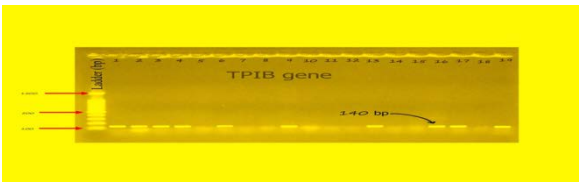

Fig: 1 Agarose gel electrophoresis image of *triose phosphate isomerase* (*tpi*B) gene (2% agarose, 100 V for 90 minutes). Lane M marker; DNA ladder 100-1500bp molecular weight marker.

Results of molecular detection for *triose phosphate isomerase* (*tpi*A) gene of *G. lamblia* isolates showed that the PCR products in lanes 2,3,5,7,8,9,10,11,12,14,15,18 and 19 were positive which shown the migration of DNA band according to molecular weight at 476bp PCR product sizes and were visualized by ethidum bromide staining. Figure (2).

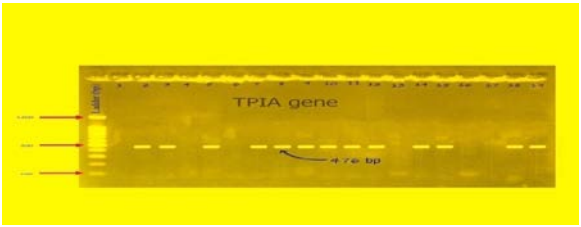

Fig:2 Agarose gel electrophoresis image of *triose phosphate isomerase* (*tpiA*) gene (2% agarose, 100 V for 90 minutes). Lane M marker; DNA ladder 100-1500bp molecular weight marker.

The *trios phosphate isomerase* (*tpi*) gene was successfully amplified in the nested PCR as shown in table (7) and figures (1,2).The results showed the presence of assemblage(A) was in 9(45%) samplesat476,576,1005bp PCR product size and assemblage (B) was in 7(35%) samplesat140bp PCR product size and there were 4(20%) samples had mixed of assemblages (A+B).These results agree with previous study which used the same primers conducted by Turki and others [35] whom mentioned that the percentage of assemblage(A) was 30.14% and assemblage (B) was 26.03% and this meaning the assemblage (A) was predominance and have virulence factors more than assemblage (B). On the other hand, these results disagreement with the study was done by Yahya and others [36] whom reported the genotype (B) was prevalent at percentage 61% while the genotype (A) showed in percentage 39.1%.

Figure (3) comprised of *G. lamblia* (our isolates) for genotype (B) and *G. lamblia* (stander isolates) ID (KY6136606.1) from NCBI. The compatibility between our isolates and stander isolate showed the replacement of Guanine nucleotide to Adenine nucleotide at locus (254).

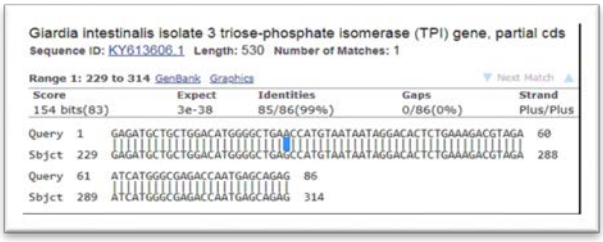

Fig:3 The alignment result between our strains and the reference strain (KY613606.1). Identity was 99%. Amplified region located between nucleotides 229 to 314 of (*tpiB*) genotype.

Isolates had been confirmed by sequencing and the result was *G. lamblia* in identification with (99%). Sequence analysis was determined when comber with the data that obtained from National Center for Biotechnology Information (NCBI). The results of *trios phosphate isomerase* (tpiB)gene sequence analysis had showed that there was a single nucleotide polymorphism as showed in figure (3).

Nucleotide substitution has been in just one site of *trios phosphate isomerase* (tpiB) gene of sequence strain (1_TpiB.ab1). This substitution (point mutation) One mutation was shown at position 254 that changed the Guanine (G) to Adenine (A). Transitional changes (G254A). This alteration in the nucleotide sequence of gene cause mutation at position (85) of *triose phosphate isomerase* (tpiB) genotype that change amino acid Serine to Asparagine. Figure (4).

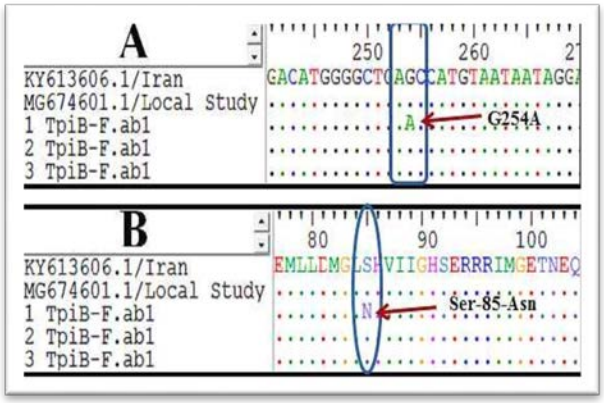

Fig:4 Mutation in *triose phosphate isomerase* (*tpiB*)(A) Nucleotide substitution. (B) Amino acid substitution.

Guanine nucleotide had substitution to Adenine at locus (254). This alteration in the nucleotide sequence of gene cause mutation at position (85)of *trios phosphate isomerase*(*tpiB*) gene that changes amino acid serine to proline figure (4).

The DNA sequencing of *triose phosphate isomerase* (*tpiB*)gene to create the Phylogenetic tree in order to evaluate the degree of genetic diversity within *G. lamblia* isolates, as well as their evolutionary relationship with other *Giardia spp* [21]. Figure (5).

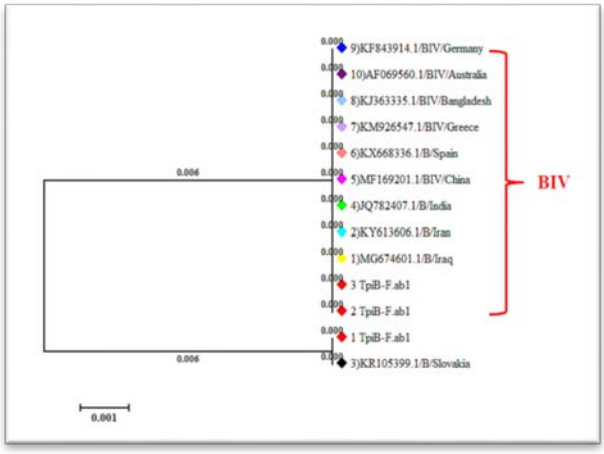

Fig:5 Neighbor-joining trees based on *tpiB* gene sequences. (1-10 Strains)= (Reference strains),1 TpiB.ab1, 2 TpiB.ab1 and 3 TpiB.ab1= (Study Isolates) (Mega v.6).

Phylogenetic tree was constructed using the public neighbor-joining method. Figure (5) shows the genetic similarity between the reference strain and our isolates according to *triose phosphate isomerase* gene. The variability percentage in (tpiB) was (0.001), this means very low variability in 1 nucleotide for each 1000 nucleotides.

Figure (6) comprised of *G. lamblia* (our isolates) for genotype (A) and *G. lamblia* (stander isolates) ID (L02120.1) from NCBI. The compatibility between our isolates and stander isolate showed the replacement of:

1- Cytosine (C) at position 926 changed to Thymine (T).
2- Cytosine (C) at position 1094 changed to Adenine (A)
3- Thymine (T) at position 1202 changed to Cytosine (C).
4- Adenine (A) at position 1284 changed to Guanine (G).
5- Adenine (A) at position 1304 changed to Guanine (G).

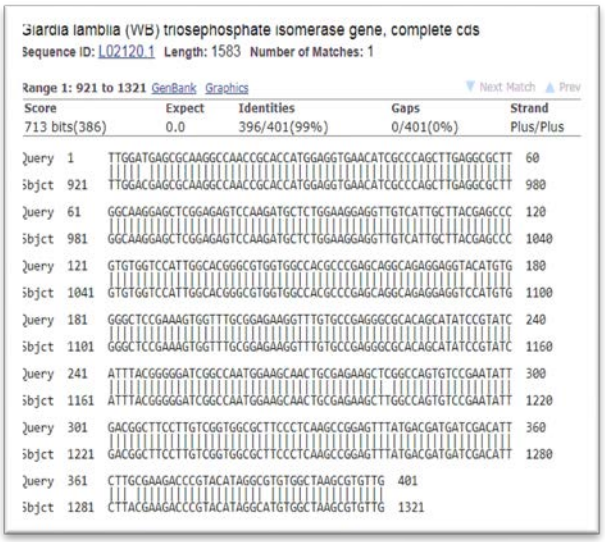

Fig:6 The alignment result between our strains and the reference strain (L02120.1). Identity was 99% and E-value was 0.0. *Amplified region located between nucleotides 921 to 1321 of (*tpiA*) genotype.

Nucleotide substitutions were seen in five sites of (tpiA)genotype for all sequence strains of *Giardia lamblia* (2 TpiA.ab1, 12 TpiA.ab1 and 18 TpiA.ab1). Five mutations were shown at positions (926, 1094, 1202, 1284, and 1304). Four mutations from them were transitional changes (C926T, T1202C, A1284G and A1304G) and one transversional change (C1094A). Figure(7)

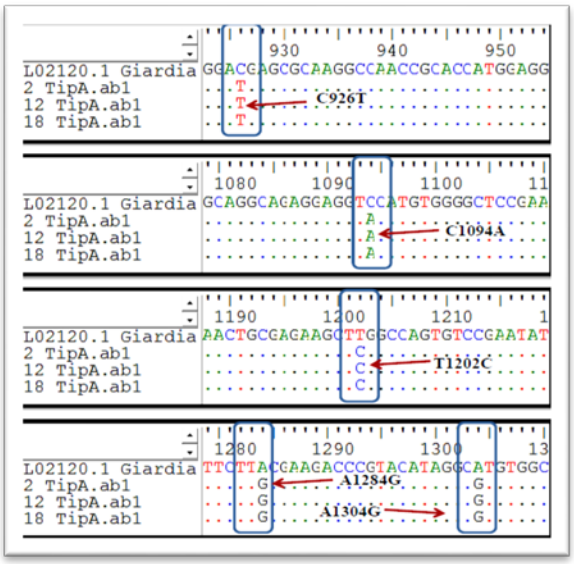

Fig :7 Nucleotide substitutions (Mutations) for (*tpiA*) gene.

The alterations in nucleotide sequence of gene cause four mutations of(tpiA)gene that change amino acids (Threonine at 309 changed to Methionine, Serine at 365 changed to Tyrosine, Isoleucine at 401changed to Serine and Histidine at 435 changed to Arginine) and one silent mutation (Leucine at 428 remain Leucine). Figure (8).

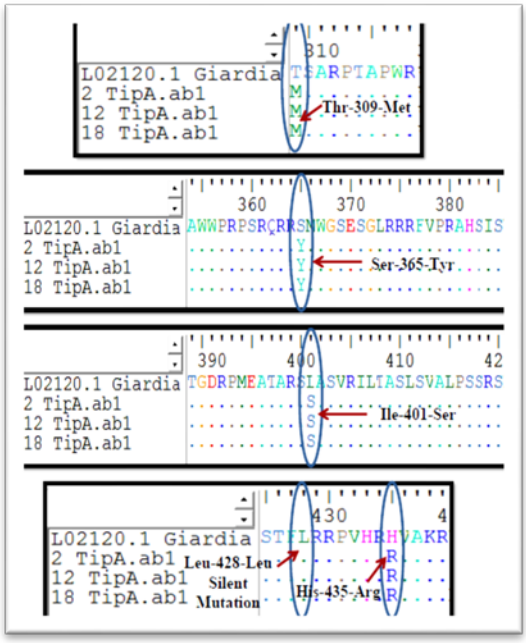

Fig:8 Amino acid alterations (Mutations) for (*tpiA*) gene.

Compatibility between the isolates for triose phosphate isomerase (tpiA) gene and stander isolate ID (L02120.1) from NCBI showed four mutations of them were transitional changes (C926T, T1202C, A1284G and A1304G) and one transversional change (C1094A). Figure (6 and 7). Alterations in nucleotide sequence of gene cause four point mutations of**(**tpiA)gene that change amino acids (Threonine at 309 changed to Methionine, Serine at 365 changed to Tyrosine, Isoleucine at 401changed to Serine and Histidine at 435 changed to Arginine) and one silent mutation (Leucine at 428 remain Leucine). Figure (8).

Finally, the results indicated a point mutation in both genotypes (A and B), so this sort of mutation was an irregular single nucleotide polymorphism mutation in the (DNA) that happens randomly at one point and usually takes place throughout DNA replication. Furthermore, point mutation was some of the time caused by mutations that spontaneously occur amid DNA replication or exposed to mutagens such as environmental factors or certain chemicals that can change DNA of organism [37]. Furthermore, there is one silent mutation in genotype (A) and this type of mutation might happen when a nucleotide was substituted but the same amino acid was produced. This could be occurring because multiple codons were coding for the same amino acid as showed in result of present study [37]. Or silent mutation did not result in a change to the amino acid sequence of a protein and not alter function of protein in which case there was no change in phenotype of the microorganism because silent variation could occur in non-coding regions (outside of genes or within introns), or they might occur within exons [38].

The DNA sequencing of *triose phosphate isomerase* (tpiA) gene to create the Phylogenetic tree in order to evaluate the degree of genetic diversity within *G. lamblia* isolates, as well as their evolutionary relationship with other Giardia spp [21]. Figure (9).

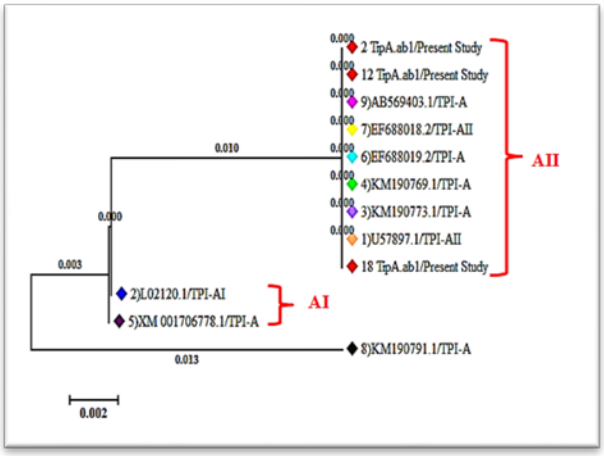

Fig:9 Neighbor-joining trees based on *tpiA* gene sequences.(1-9 Strains)= (Reference strains),2 TpiA.ab1, 12 TpiA.ab1 and 18 TpiA.ab1= (Study Isolates) (Mega v.6).

Phylogenetic tree was constructed using the public neighbor-joining method. Figure (9) shows the genetic similarity between the reference strain and our isolates according to *triose phosphate isomerase* gene. The variability percentage in (tpiA) was (0.002), this means very low variability in 2 nucleotide for each 1000 nucleotides.

CONCLUSION

Twenty positive samples of *G. lamblia* were successfully amplified for *trios phosphate isomerase (tpi)*gene by Multiplex-PCR assay and the result showed the presence of assemblage (A) was in 9 samples, assemblage (B) was in 7 samples and 4 samples were mixed assemblages (A+B).

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