مجلة المثنى للعلوم الصرفة
AL-Muthanna Journal of Pure Sciences (MJPS)
VOL.(3), NO.(2), 2016

First isolation of *G. lamblia* genotyping by Multiplex-PCR assay for detection of mixed infection in Human Stool in AL-Muthanna province–Iraq

Nabaa Munther Turki, Muhammed Oda Mallah, and Yassir Dakheel Kremsh Alasadiy
Corresponding author: E mail: nabaa.munther@yahoo.com
Received 17-5-2016, Accepted 30-10-2016, Published 7-11-2016

Abstract

This study was carried out in AL-Muthanna Province during the period from October 2014 to March 2015 for detect the mixed genotyping of *G. lamblia*. A total of 200 stool samples (male and female), were taken from human aged (>1-40>) years who suffering from acute or persistent diarrhea. The samples examined by Multiplex-PCR assay, the results of the PCR assay showed the presence of mixed Assemblages of *G. lamblia* in 17 positive samples and high percentage of mixed assemblages EF was 41.18% and lower percentage in assemblages ABEF, AE, BE, ABF, ABE were 5.88% with significant differences at level $P \leq 0.05$.

Keyword: Giardia lamblia ,Genotyping ,Human ,Stool ,Iraq .

1-Introduction:

The flagellated protozoan Giardia lamblia (synonymous with Giardia duodenalis, Giardia intestinalis) is an intestinal parasite that can infect many species in the animal kingdom including mammalian, domestic animals and humans(Thompson, 2004). In human, G. lamblia can cause gastrointestinal infections ranging from mild to severe as well as chronic disease. Infection occurs by fecal oral route transmission, either by direct contact or by ingestion of contaminated food or water(Monis and Thompson, 2003). Isolates of G. lamblia are classified into eight assemblages (genotypes) (A-H) depend on the characterization of the glutamate dehydrogenase (gdh), small-subunit (SSU) rRNA, and triose phosphate isomerase (Tpi) genes (Molina and Basualdo, 2013). Phylogenetic multi locus analysis using (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 (Caccio, et al.,2005).

2-Material and Methods:

2-1.Patients:

This study was carried out in AL Muthanna province in the period from October 2014 to March 2015, to determine prevalence and genotypes of *G. lamblia*, a total of 200 stool samples (male and female), were taken from Human aged (>1-40>) years who suffering from acute or chronic diarrhea in the Educational- AL-Hussein hospital in Samawa, General AL-Rumaiytha hospital, Feminine and Children's hospital in Samawah and Rumaiytha health centers, then it examined by Flotation and Conventional-PCR assay.

2-2.Fecal samples collection:

Fresh fecal samples were collected by using a sterile containers and transported in to a cooled box (temperature approximately 10°C). Then, the samples were transported to the laboratory at College of Science- AL-Muthanna University, at the laboratory the fecal samples were divided into two portions, one portion was for the microscopic examination of parasites while the other portion was stored immediately at-20°C for molecular analysis(Multiplex -PCR).

2-3- Primers

The PCR primers were using in this study for detection *G. lamblia* genotypes A, B, E, and F based on *triosephosphate isomerase* (*Tpi*) gene, *G. lamblia* A and B genotypes were designed by (Minvielle, *et al*., 2008) Whereas, *Giardia lamblia* E and F genotypes (were designed in this study by using NCBI-GenBank data base and primer3 plus *G. intestinalis* Genotype E isolate 15 *triosephosphate isomerase* (*TPI*) gene, partial cds GenBank: AY228646.1 (Sulaiman, *et al*., 2003) and *G. intestinalis* Genotype F isolate 15 *triosephosphate isomerase* (*TPI*), partial cds, isolate: C5 GenBank: AB569391.1 (Suzuki, *et al*., 2011), and these primers was provided from Bioneer company, Korea as following table (2-1):

Table (2-1): Primers of *G. lamblia* genotypes (A,B,E,F).

Genotypes	Primer	Primer Sequence(5' –'3)	PCR product
Genotype A	TPIA-F	CGAGACAAGTGTTGAGATG	576 bp
	TPIA-R	GGTCAAGAGCTTACAACACG	
Genotype B	TPIB-F	GTTGCTCCCTCCTTTGTGC	208 bp
	TPIB-R	CTCTGCTCATTGGTCTCGC	
Genotype E	TPIE-F	GACGTTGTTGTTGCCCCTTC	416bp
	TPIE-R	CCAATGGACCATACGGGCTC	
Genotype F	TBIE-F	AACGGCTCGCTCGACTTTAT	323bp

	TPIF-R	ATCCCCTTTTCTAGAGCGCG	

2-4 Polymerase chain reaction (PCR):

The PCR technique was performed for detection *G. lamblia* genotypes based on *triosephosphate isomerase (Tpi)* gene from human stool samples. This method was carried out according to method described by (Minvielle, *et al*., 2008) as following steps:

2-4-1. Genomic DNA Extraction:

Genomic DNA from feces samples were extracted by using AccuPrep® stool DNA Extraction Kit, Bioneer. Korea, and done according to company instructions (www.Bioneer.com).

2-4-2. Genomic DNA Estimation:

The extracted genomic DNA was checked by using Nano drop (THERMO. USA), which measured concentration and purity of the DNA by reading the absorbance in (260/280 nm).

2-4-3. Multiplex PCR master mix preparation:

Multiplex PCR master mix was prepared by using (AccuPower® Gold Multiplex PCR PreMix Kit) and this master mix done according to company instructions as following table (2-2).

Table (2-2): Multiplex PCR master mix preparation.

Multiplex PCR Master mix		Volume
DNA template		5µL
Forward primer (10pmol)	G.A	1µL
	G.B	1µL
	G.E	1µL
	G.F	1µL
Reverse primer (10pmol)	G.A	1µL
	G.B	1µL
	G.E	1µL
	G.F	1µL
PCR water		7 µL

Total volume	20µL

After that, these PCR master mix component that mentioned above placed in standard **AccuPower® Gold Multiplex PCR PreMix Kit** that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (MyGene. Bioneer. Korea).

2-4-4. PCR Thermo cycler Conditions:

PCR and multiplex PCR thermocycler conditions were done according primer annealing temperature and PCR kit instructions following table (2-3).

Table (2-3) PCR Thermo cycler Conditions.

PCR step	Temp.	Time	repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	30 cycle
Annealing	55C	30sec	
Extension	72C	1min	
Final extension	72C	5min	1
Hold	4C	Forever	-

2-4-5. PCR product analysis

The PCR products was analyzed by agarose gel electrophoresis following steps:

1- 1.5% Agarose gel was prepared in using (100ml) 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µl of ethidium bromide stain were added into agarose gel solution.

3-Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.

5- PCR products were visualized by using UV Transilluminator.

3-5.Statistical analysis: Chi-Square (X2) was used for detect statistical difference of data prevalence of disease and effect of other factors at significant differences $P \geq 0.05$ (Al-Rawi,2000).

3-Results:

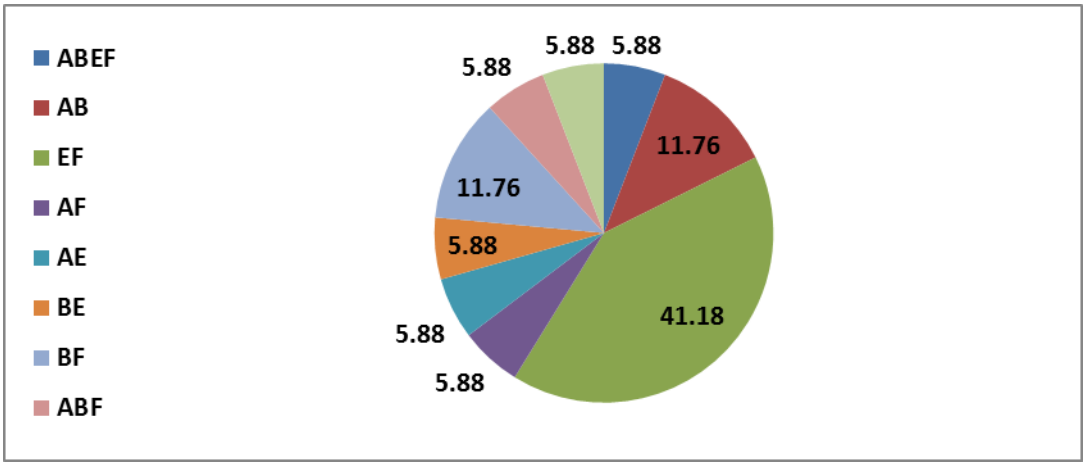
3-1. Mixed Genotyping (Assemblages A,B,E,F) of *Giardia lamblia*

In table (3-1) and figure (3-1), the results of the PCR assay showed the presence mixed assemblages of *G. lamblia* in 17 positive samples that showed ABEF in percentage ratio 5.88%, AB (11.76%), EF (41.18%), AF (5.88%) AE (5.88%), BE (5.88%), BF(11.76)%, ABF (5.88)% and ABE(5.88%) respectively, also the results showed that that high percentage ratio of mixed assemblages EF was 41.18% and lower percentage ratio in assemblages ABEF,AE,BE,ABF,ABE were 5.88% with significant differences at level $P \leq 0.05$.

Table (4-1): Percentage of Mixed genotyping of *Giardia lamblia* infection.

Genotypes	Number of samples	Percentage %	Chi- square
ABEF	1	5.88	X^2.Calculated=18.397
AB	2	11.76	X^2.Table=15.507
EF	7	41.18*	P-Value 0.05=0.018
AF	1	5.88	df=8
AE	1	5.88	
BE	1	5.88	
BF	2	11.76	
ABF	1	5.88	
ABE	1	5.88	
Total	17	100	

***Significant differences at(P≤ 0.05).**



Figure(3-1):Percentage of Mixed genotyping of *Giardia lamblia* Infection.

3-2. Mixed Genotyping Giardia lamblia:

In figure (3-2),the results showed detection mixed *Giardia lamblia* genotype (Assemblage A and Assemblage B) that the lane (1,3,7,8 and 10) showed some positive samples isolates as Assemblage A and B at 576bp and 208bp PCR product size respectively.

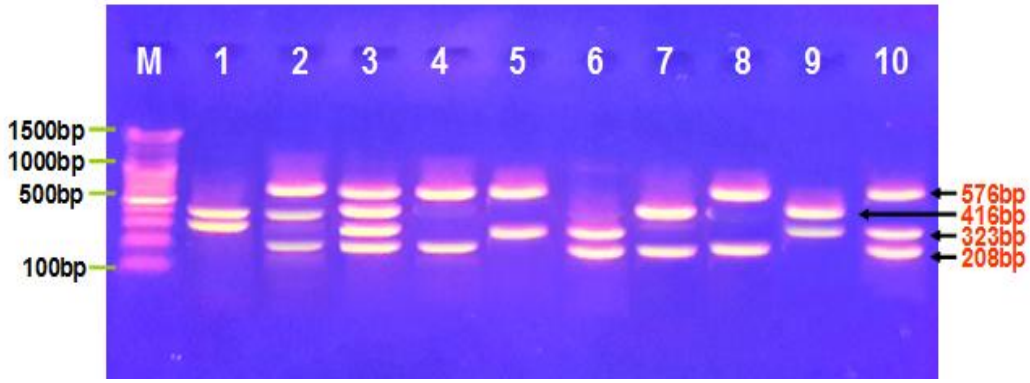


Figure (3-2): Agarose gel electrophoresis image that shown the PCR product of *triosephosphate isomerase (TPI)* gene that using in detection mixed *Giardia lamblia* genotype (Assemblage A, B, E, and F genotype). Where M: Marker (2000-100bp), lane (1) positive isolates as Assemblage E and F, lane (2) positive isolates as Assemblage A, B and E, lane (3) positive isolates as Assemblage A , B, E and F, lane (4) positive isolates as Assemblage A and B, lane (5) positive isolates as Assemblage A and F, lane (6) positive isolates as Assemblage B and F, lane (7) positive isolates as Assemblage B and E, lane (8) positive isolates as Assemblage A and B, lane (9) positive isolates as Assemblage E and F, and lane (10) positive isolates as Assemblage A and B, and F. at PCR products size 576bp, 208bp, 416bp,and 323bp for Assemblage A, B, E and F respectively.

4-Discussion:

4-1.Mixed Genotyping (Assemblages A, B, E, F) of *Giardia lamblia*.

Molecular characterization and phylogenetic analysis of *G. lamblia* isolates from different hosts revealed existence of seven major genotypes assemblage A and B have the widest host ranges encompassing human and a variety of other animals (Hsu *et al.*,2007). The results of the PCR assay showed the presence mixed assemblages of *G. lamblia* in 17 positive samples that showed ABEF in percentage ratio 5.88%, AB 11.76%, EF 41.18%, AF 5.88%, AE 5.88%, BE 5.88%, BF11.76%, ABF 5.88% and ABE 5.88% respectively, also the results showed that that high percentage ratio of mixed assemblages EF was 41.18% and lower percentage ratio in assemblages ABEF, AE, BE, ABF, ABE were 5.88% with significant differences at level $P \leq 0.05$. The occurrence of mixed infections by several assemblages/subtypes of *G. lamblia* due to exposure of humans to multiple sources in the environment as well as direct contact of human and animals (Gelanew *et al.*,2007). In Egypt, the prevalence of mixed infection was reported to be 16% infected individuals (El-Shazly *et al.*, 2004). Also the current study agree with a study conducted in the United Kingdom on 33 infected with *Giardia* parasite and found 9% of patients have a mixed infection of types A+B (Thompson *et al.*, 2004). As the results are agree with Gelanew *et al.*

(2007) in Ethiopia was observed 25% of isolates was mixed infections by PCR assay, specifically, A+ F in seven isolates and A+B in eight isolates. Babaei *et al*.(2008) in Iran, proved that the mixed infection of genotype A and B were only in two samples by rate 5.2%, from total 38 sample. In Nepal, Anjana *et al*. (2009) found that 6% (2 of 35) of isolates was mixed assemblages A+B. Also agreed with Elham *et al*. (2013) in south west of Iran that indicated there were mixed infections of assemblages A+B and by rate 74% (37/50) of samples isolated.

5-References

Thompson RCA (2004). The zoonotic significance and molecular epidemiology of *Giardia* and giardiasis. *Vet. Parasitol*. 126: 15-35.

Monis PT and RCA Thompson (2003). *Cryptosporidium and Giardia*-zoonosis: fact or fiction? Inf. Gen. Evol. 3: 233-244.

Moline, N.B. and Basualdo, j. A. (2013). *Giardia duodenalis*: new insights on an ancient parasite. *Int. j. of parasitol. research Microbiology and parasitol.*5(1). pp 122-131.

Caccio, S.M.; Thompson, R.C.A.; McLauchlin, J. and Smith, H.V. (2005). Unravelling *Cryptosporidium* and *Giardia* epidemiology. *Trend Parasitol*. 21(9): 431-437.

Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H, Trout, J.M, Schantz, P.M., Das, P., Lal, A.A. and Xiao, L. (2003). *Triosephosphate isomerase gene* characterization and potential zoonotic transmission of *Giardia intestinalis*. Emerging infect. Dis.9 (11):1444-1452.

Suzuki, J., Murata, R., Kobayashi, S., Sadamasu, K., Kai, A and Takeuchi, T.(2011). Risk of human infection with *Giardia duodenalis* from cats in Japan and genotyping of the isolates to assess the route of infection in cats. *Parasitol.* 138(4).493-500.

Minvielle, M.C.; Molina, N.B.; Polverino, D. and Basualdo, J.A. (2008). First genotyping of *Giardia lamblia* from human and animal feces in Argentina, South America. Mem. Inst. Oswaldo. Cruz. Rio de Janeiro, 103(1): 98–103.

Al-Rawi, K.(2000). Introduction for Biostatic, Al-Mousul University.

Hsu, BM.; Wan, HY. and Hsu, PC. (2007). Prevalence and genotyping of *Giardia* in husbandry systemsin Taiwan. Parasitol Res. 275-280.

Gelanew, T., Lalle, M., Hailu, A., Pozio, E. and Caccio` S. (2007). Molecular characterization of human isolates of *Giardia duodenalis* from Ethiopia. Acta Tropica, 102, 92–99.

El-Shazly, A.M., Mowafy, N., Soliman, M., El-Bendary, M., Morsy, A.T., Ramadan, N.I., Arafa, W.A., (2004). Egyptian genotyping of *Giardia lamblia. J. Egypt Soc. Parasitol*. 34, 265–280.

Thompson, R.C , Read, C.M. and Monis, P.T. (2004). Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. Infect. Genet. Evol; 4(2):125-130.

Babaei, Z. Oormazdi, H. Akhlaghi, L. Rezaie, S. Razmjou, E. and Soltani Arab shahi SK. (2008).Molecular characterization of the Iranian isolates of *Giardia lamblia*: applicationofthe glutamate dehydrogenase gene. *Iranian J. Publ. Health,*. (*37*), *2, pp.75-82*

Anjana, S. Lalitha, J. Petri, W.A., Jr. and Eric R. Houpt. (2009).*Giardia intestinalis* Assemblages A and B Infections in Nepal. *Am. J. Trop. Med. Hyg.*, 81(3), , pp. 538–539.

Elham S. R, Abdollah. R, Ali. R, Ali. A, Ahmad. S, Mahdi, P.B. (2013).Genotype analysis of *Giardia lamblia* isolated from children in Ahvas south west of Iran, *Jundishapur J Microbiol.*;6(3).

العزل الاول للأنواع الجينية لطفيلي الجياردیا لامبليا بواسطة تقنية تفاعل سلسلة البلمرة متعددة الجينات لاكتشاف الاصابة المختلطة في براز الانسان في محافظة المثنى- العراق

نبأ منذر تركي **محمد عودة ملاح** **ياسر دخيل كريمش**

الخلاصة

أجريت هذه الدراسة في محافظة المثنى خلال الفترة من تشرين الاول 2014 ولغاية مارس 2015 لغرض اكتشاف الانواع الجينية لطفيلي الجياردیا لامبليا. تم فحص 200 عينة براز (ذكور واناث) وبأعمار تتراوح من (<40-1>) سنة ومن الذين يعانون من الاسهال الحاد والمستمر وتم فحص البراز بواسطة تقنية تفاعل سلسلة البلمرة متعددة الجينات. أظهرت النتائج 17 عينة موجبة للفحص وكانت اعلى نسبة للجينات المختلطة هي EF وبنسبة 41.18% وأوطأ نسبة كانت للجينات ABE,ABF,BE,AE,ABEF وبنسبة 5.88% وبفرق احصائي على مستوى $P\leq 0.05$.

الكلمات المفتاحية: طفيلي الجياردیا لامبليا ,الجينات ,الانسان ,البراز, العراق.