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Genetic variants of *giardia lamblia* isolates from food handlers in Erbil city

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Abstract

Background and objective: *Giardia lamblia* is a flagellated protozoan that lives and proliferates in the small intestine of human and other mammals causing gastrointestinal disorder called giardiasis. Giardiasis mainly spreads in developing countries which lack sanitation and hygiene awareness and persons become infected by ingestion of contaminated food and water with mature cysts. This study was designed to detect the distribution of giardiasis in human and to differentiate between the assemblages of *Giardia lamblia* A and B by using Glutamate dehydrogenase (*gdh*) gene

Methods: A total of 2000 fecal samples were collected from symptomatic and asymptomatic food handler aged ranged between (20-30) and more than 30 years old that regularly attend the central laboratory in Erbil city. Primary diagnosis depends on a direct microscopic examination of the stool. Genotyping was done for Giardia positive samples (n = 39) targeting the Glutamate dehydrogenase gene (*gdh*) using two sets of primers for amplification of 458bp fragment, by RFLP and PCR-sequencing screening methods for *G. lamblia.*

Results: Out of the total 2000 samples screened for *G. lamblia,* 39 (1.95 %) were positive. According to the multiple alignment results, it was found that the isolates belonged to sub-assemblage AII 21 (53.8%), assemblage B 11 (28.2%), sub-assemblage BIII 9 (81.8%) and sub-assemblage BIV2(18.2%) genotypes. No statistically significant result was found between symptomatic and asymptomatic as assemblage and sub-assemblage genotypes.

Conclusion: The present study provides the first data on the assemblages and sub-assemblages of *G. lamblia* in food-handlers in Kurdistan region, Iraq. Identification and confirming of AII, B, BIII, and BIV genotypes in Erbil community indicates the anthroponotic and anthropozoonotic transmission cycle of Giardia infection. Our understanding of the diversity of *G.lamblia* mainly comes from the sequence information of multiple gene loci.

Keywords: *G.lamblia*; Glutamate dehydrogenase gene; Genotype; RFLP PCR; Assemblage and sub-assemblage.

Introduction

Giardia is a small entericprotozoan parasite with the distinction of being among the most common causes of diarrhea in humans and farm animals worldwide, causing giardiasis.(1)

G.lamblia is an obligate parasite that colonizes the intestinal tract of a wide range of hosts, including humans and causes diseases ranging from self-limited diarrhea to more severe clinical manifestations.(2) Approximately 280 million people are reportedly infected with this parasite every year, and the prevalence of infections in humans ranges between 0.4 and 7.5% in high-income countries, and 8–30% instated of in low and middle income countries.(3,4)

Although most infected people experience no or mild clinical signs, *G. duodenalis*

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infection can result in long-term tissue injury sequelae and malnutrition.[2,5,6] The course of *G. duodenalis* infection is often very prolonged, and the host may maintain subclinical infection for a long extended period,[2] which limits the understanding of the true clinical impact of *G. duodenalis*, as the actual frequency and risk of *G. duodenalis* infection may be higher than currently estimated. A recent prospective study found that *G. duodenalis* infection among children in low- and middle -income countries is associated with retarded growth and intestinal injury.[7]

Since infection with *G. duodenalis* is usually asymptomatic, the hidden burden of giardiasis may not be obvious. Recently, giardiasis has attracted increasing attention due to its zoonotic potential,[8] so there is thus an urgent need to fully understand its mechanism. Giardia is currently divided into nine species[9] based on traditional morphological characteristics and sequence information. Most *Giardia* species and genotypes exhibit reservoir host specificity[10] and can range from highly host-specific, such as those with documented records from one or a few limited hosts, to host generalists, including those with documented records from a dozen or more different host species.[9] Our understanding of the diversity of *Giardia* mainly comes from the sequence information of multiple gene loci.[11,12] Earlier investigations found differences in the symptoms of people infected with *G. duodenalis*, but it was not clear whether these strains were the same assemblages.[13] Several recent reviews have discussed different assemblages (assemblages: refer to different genetic groups or subgroups within the species *G.duodenalis*) of *G. duodenalis*, which may represent the existence of new Giardia species, but further biological and genetic studies are needed before confirming new species names.[2,9,11,14,15] Several studies have suggested that the assemblages are genetically distant from each other, and several genomic analysis results also showed that the isolates of assemblages A, B, and E represent three different genetic populations.[16] Allozymic analysis of 21 cytosolic enzymes (i.e., containing 23 geneloci) showed that *G.duodenalis* assemblages encompass a rich population substructure and that pronounced genetic divergence exists among these sub-assemblages.[13] In accordance with the analysis results, assemblage A was divided into four sub-assemblages (AI-AIV), assemblage B was divided into four sub assemblages (BI-BIV), assemblage C was divided into three sub assemblages (CI-CIII), and assemblage E was divided into three sub-assemblages (EI-EIII). Notably, some of the sub-assemblages showed host preference. The AII, BIII, and BIV assemblages specifically infect humans, BII specifically infects monkeys, EI specifically infects sheep, EII specifically infects cattle, and EIII specifically infects pigs. Alternatively, *G.duodenalis* assemblages or sub-assemblages can be identified by some gene lociβ-giardin (*bg*), glutamate dehydrogenase (*gdh*) and triose-phosphate isomerase (*tpi*) genes. Based on multilocus sequence analysis, several sub-assemblages A (AI, AII, and AIII) have been identified, of which AIII is commonly reported in wild ruminants, and of which AI and AII are most commonly found in animals and humans.[15] Despite the possible presence of two invalid sub-assemblages (BIII and BIV), assemblage B still shows high sequence diversity in the multilocus analysis.[8] Based on the comparison and polymorphisms of glutamate dehydrogenase (*gdh*), the small-subunit of ribosomal RNA (*SSU*), and triosephosphate isomerase (*tpi*) genes, *G.duodenalis* must be considered as a species complex, whose members are classified to at least eight distinct genetic groups (A to H) or assemblages[17,18]

Food-handlers are one of the most important sources of distribution and transmission of intestinal parasitic infections including *G. lamblia*, to humans.

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No previous studies have been conducted on identification of *G.lamblia* assemblages and sub-assemblages in food-handlers, therefore, this present study was designed to determine the assemblage and sub-assemblages of *G.lamblia* isolated from food handlers referred to Erbil health centers.

Methods

Specimen collection & microscopic examination

This cross-sectional study was carried out from July to December 2023 in Erbil city. Overall, 2000 fecal samples were collected from food-handlers that annually visit to health centers for getting a health certification. Fresh fecal specimens were collected in a clean stool cup. Each container was labeled and immediately transferred to the parasitology and molecular units at Erbil public health laboratory for microscopic examination and molecular analysis.

Microscopic examination

This examination is conducted on each sample of stool according to.[19] This method was executed by preparing a clean glass slide and two drops of normal saline were placed on one half slide and a drop of Lugals iodine on the others half, two sticks were taken from stool samples using a wooden stick from different places area of samples (about the size of pinhead). One of them was put on normal saline and mixed well, while the other swab mixed with Lugals iodine then covered by cover slip and examined using the magnification power 400x (40 x 10) to record the presence of trophozoites and/or cysts. Approximately 1 gr of each fresh stool positive samples stored at -20 ˚C without any preservative solution for molecular analysis

Molecular method

DNA extraction

Concentrated cyst was the major source for genomic DNA extraction using FavorPrep stool DNA isolation Mini Kit (50 preps) according to the instructions of the manufacturer protocol with some pre-treatment before using kit. After extracting of DNA from all stool samples, the DNA was stored at -20°C for PCR amplification.

PCR Amplification

The fragment 458 bp of glutamate dehydrogenase (*gdh*) gene was amplified by a single PCR using the forward glutamate dehydrogenase (*gdh*) GDHF (5′-TCAACGTCAACCGCGGCTTCCGT-3′) and reverse glutamate dehydrogenase (*gdh*) GDHR (5′-GTTGTCCTTGCACATCTCC-3′) primers. The identification of the glutamate dehydrogenase (*gdh*) gene (458 bp) by using polymerase chain reaction (PCR) and PCR-RFLP techniques was carried out at the Medical Research center, Hawler Medical University, in Erbil City/ Iraq. Briefly, DNA was isolated from stool samples (by using 50 prep DNA extraction kit (FavorPrep stool DNA isolation Mini Kit (50 preps), and the polymorphic region is amplified by PCR (Boiron/ Genekam Biotechnology ready to use PCR master mix), concentration and volume of the solutions in PCR reaction were:(15µl Master mix, 3µl DNA sample, 2µl Forward primer, 2µl Reveres primer and 26µl ddH2O). The PCR conditions were as follows: an initial denaturation step at 94 °C for 5 min and 35 cycles at 94°C for 45 sec (denaturation), 60°C for 30 sec (annealing), and 72°C for 45 sec (extension) with a final extension step for 5 min at 72°C. Positive sample of *Giardia* DNA and distilled water were used as a positive and negative control. Five µL of each PCR products were separated by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light to evaluate success of the reaction.

Restriction fragment length polymorphism (RFLP)

Thermo Scientific *BspLI* restriction endonucleases enzyme *BspLI* (Nla IV) to distinguish sub-assemblages AI, AII, from assemblages B and *Rsa1*

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(thermo-scientific) was applied to distinguish between BIII and BIV sub-assemblages separately. RFLP analysis was carried out directly on PCR products in a 15 µL reaction volume including 8 µl of PCR product was added to 1X reaction buffer and 1 µL (10 U/ µl) *BspLI* or 1 µL(10 U/µl) *Rsa1*. Digestion took place at 37 °C for 24hours. The restricted fragments were separated and visualized by electrophoresis on 4% agarose gel, stained with ethidium bromide and detected under ultraviolet light. A 100 bp DNA ladder was used as a size marker. The assemblages and sub-assemblages were distinguished according to the restriction patterns (BspII, RsaI).

Ethics consideration:

The study project was approved by research ethics committee (protocol number : 1.00, date 3-9-2022) of medical research center, Hawler Medical University, and permission to collect samples was obtained from the public laboratory, Erbil Governorate, and participant gave verbal consent after being informed of the study's purpose and were assured promised that any information they provided would be kept confidential.

Statistical analysis: Data was analyzed using the statistical package for social science (SPSS statistical for windows, version 25.0. Test of significance included the Pearson's Chi-Squar test (or Fishers Exact test for sparse data). Statistical significance was set as a *P*-value <0.05

Results

Association between *Giardia* and different demographic and clinical characteristics

A total of 2000 participants were included in the study, with 39 individuals testing positive for *Giardia* infection. The distribution of positive cases varied across demographic and clinical characteristics. Among males, 29 cases tested positive compared to 10 cases among females, however, the difference was not statistically significant (*P* >0.346). The prevalence of *Giardia* increased with age. The highest percentage was observed in individuals aged >30 years and the association between age group and *Giardia* infection was highly significant (*P* <0.001) (Table 1).

Positive cases were most prevalent among individuals with a university-level education (3.9%) followed by secondary (2%) and primary education levels (1.2%). The differences across educational levels were statistically significant (*P* = 0.011). The prevalence of *Giardia* infection was higher among individuals living in poor conditions (2.6%) compared to those in medium (1.4%) and good (2.1%) conditions; however, these differences were not statistically significant (Table 1). The prevalence of *Giardia* was comparable between symptomatic participants at 2% and asymptomatic participants at 2%, with no statistically significant difference.

The frequency of assemblage and sub-assemblage genetic variant according to clinical finding is shown in Table (2), among symptomatic cases 9 (56.2%) are categorized as indicated AII and 5 (31.25%) cases showed assemblage B, while 2 (12.5%) had a mixed genotypes AII+B. Statistically no significant correlation between the genotype (assemblage) and clinical presentation. In our study, there was no significant relationship between the variant of genotype BIII, BIV and clinical symptomatic presentation as indicated in Table (3).

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Table 1 Association between *Giardia* and demographic characteristics of the specimen screened

Characteristics	Positive Giardia No. (%)	Negative Giardia No. (%)	Total No. (%)	*P*-value
Sex				
Male	29 (2.2)	1291 (97.8)	1320 (100)	0.346
Female	10 (1.5)	670 (98.5)	680 (100)	
Total	39 (1.95)	1961 (98.05)	2000 (100)	
Age group (years)				
< 20	7 (1.1)	643 (98.9)	650 (100)	< 0.001
20-30	10 (1.1)	886 (98.9)	896 (100)	
> 30	22 (4.8)	432 (95.2)	454 (100)	
Educational level				
Primary	10 (1.2)	837 (98.8)	847 (100)	0.011
Secondary	17 (2.0)	832 (98.0)	849 (100)	
University	12 (3.9)	292 (96.1)	304 (100)	
Living condition				
Poor	20 (2.6)	739 (97.4)	759 (100)	0.156
Medium	13 (1.4)	948 (98.6)	961 (100)	
Good	6 (2.1)	274 (97.9)	280 (100)	
Residency				
Urban	27 (2.0)	1359 (98.0)	1386 (100)	1.000
Rural	12 (2.0)	602 (98.0)	614 (100)	
Clinical presentation				
Symptomatic	11 (2.0)	560 (98.0)	571 (100)	1.000
Asymptomatic	28 (2.0)	1401 (98.0)	1429 (100)	

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Table 2 Frequencies of assemblage and sub-assemblage genetic variant according to clinical finding

Clinical presentation (diarrhea)	Sub-assemblage AII No. (%)	Assemblage B No. (%)	Assemblage AII+B No. (%)	Total No. (%)	*P -value*
Symptomatic	9 (56.25)	5 (31.25)	2 (12.5)	16 (100)	
Asymptomatic	12 (52.2)	6 (26.1)	5 (21.7)	23 (100)	0.753
Total	21 (53.8)	11 (28.2)	7 (18.0)	39 (100)	

Table 3 Frequencies of assemblage B and sub-assemblages' genetic variant according to clinical findings

Clinical presentation (diarrhea)	Sub-assemblage BIII No. (%)	Sub-assemblage BIV No. (%)	Total No. (%)	*P -value*
Symptomatic	3 (60.0)	2 (40.0)	5 (100)	0.354*
Asymptomatic	6 (100)	0 (0)	6 (100)	
Total	9 (81.8)	2 (18.2)	11 (100)	

* By Chi square test.

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RFLP Genotyping analysis of the gdh gene

The 458 bp *Giardiagdh* locus was successfully amplified using conventional PCR.

Figure 1 and 2 present a representative gel electrophoresis image of the PCR products obtained with *gdh* primers. BspL1 and RsaI restriction enzymes digested PCR products to identify and characterize *G.lamblia* genetic sub-assemblages (Figure 2 and 3). Different digestions produce different fragment sizes (Table 4). Digestion of the 458 bp amplified fragment with BspL1 revealed that 53.8% of the 39 isolates belonged to sub-assemblage AII, 28.2% were recognized as assemblage B, and 17.9% had a mixed genetic profile, displaying traits of both AII and B. No samples were identified as sub-assemblage AI. 23% were classified as sub-assemblage BIII, while 5.12% were labeled as sub-assemblage BIV.

Table 4 Expected and diagnostic fragment sizes following digestion of a 458 bp PCR result from the *Giardia* gdh gene

Enzyme	Fragment		Subtype
	Expected	Diagnostic	
BspLI	16,39,47,87,123,146	87,123,146	AI
	16,39,47,69,77,87,123	69,77,87,123	AII
	47,123,288	47,123,288	B
RsaI	30,131,298	131,298	BIII
	30,428	30,428	BIV

Figure 1 Amplification of gdh gene for DNA of *G.lamblia* from the infected human. Molecular weight marker (100 bp ladder), Lanes (2,5,8-10) are positive samples for *gdh* gene at 458bp, Lane (3,4,6 and 7) are negative and Lane 4-10 *G. lamblia* isolates from patients. Running conditions: Agarose gel (1.5%), 5v/cm for 1 hour, stained with ethidium bromide.

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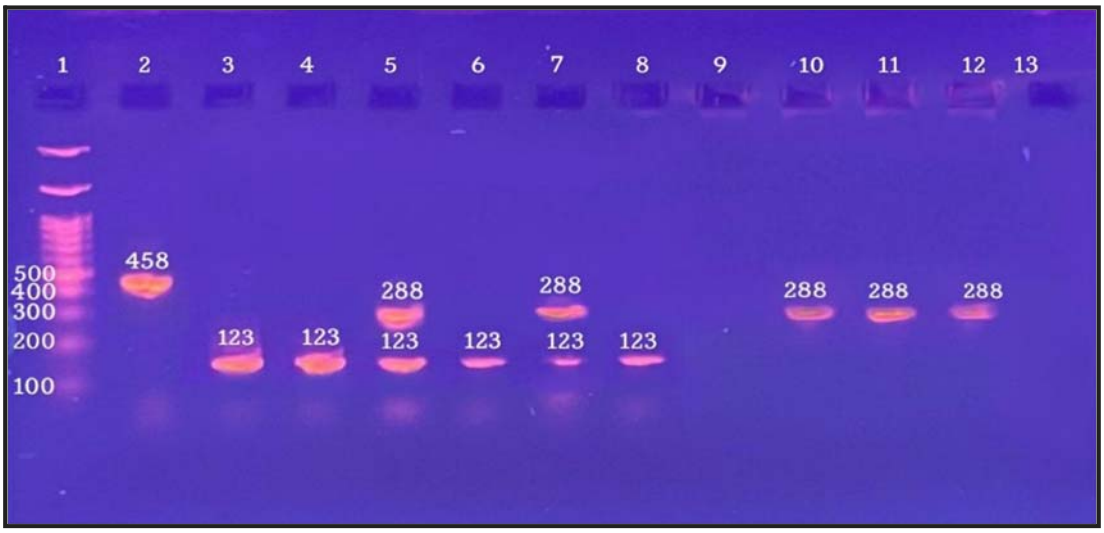

Figure 2 NlaIV (BspLI) enzyme digestion of single PCR product for *gdh* gene of *G. lamblia* and identification of genotype AII and B by RFLP analysis separated on 1.5% Agarose gel stained with ethidium bromide. 100-bp DNA ladder as a size reference in Lane 1. The undigested PCR result (458 bp) in Lane 2. Assemblage AII in Lanes 3-8. Assemblage B in Lanes 5, 7, and 9-13 are negative.

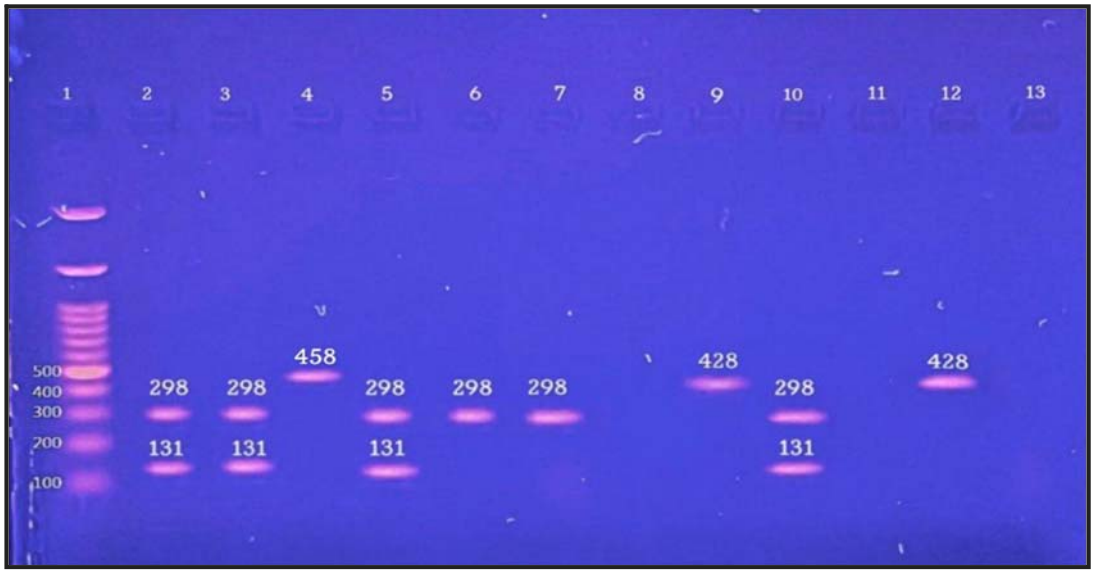

Figure 3 Ras1 enzyme digesion of PCR product of *gdh* of *G.lamblia* and identification of genotype BIII and BIV by RFLP analysis Lane 1; 100 bp DNA marker, 458 bp undigested product, 4-5 assemblage BIII, 9 and 12 assemblage BIV, and 2-4 lane electrophoresis on agarose gel

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Discussion

Giardia lamblia is an intestinal protozoan, widely prevalent, causing serious public health issues in developing countries including Iraq, especially in rural communities. Despite the high prevalence of giardiasis in some regions of Iraq, there have been few molecular studies of the distribution of *Giardia* assemblages and sub-assemblages in Iraq.

The current investigation discovered that 39 people were positive for *G.lamblia* cysts in a total of 2000 stool samples tested for intestinal parasites. Despite the significant prevalence of giardiasis in the Kurdistan region, little is known about *G.lamblia* genetic diversity. In Iraq, just a few investigations have been conducted using human samples. In the present study, the highest rate of infection in age group > 30 years with percentage 4.8% with significant differences, this results was dis-agreement with other study that detected this age group as a high risk group for giardiasis.[20] This result was also in agreement with worldwide report suggesting that giardiasis is one of the serious health problems among population of younger age groups.[21]

The susceptibility could be related to the practicing inappropriate personal hygiene. Lacking in effective immunity has also been postulated to explain this age specific manner. As far as host specificity, AI and AIII are mainly restricted to infected animals species, meanwhile AII, BIII and BIV are classified as zoonotic genotypes.[9,22,23] In human, AII ,BIV are the main genetic variant circulating among the host.

The digested 458 bp amplified fragment using BspLI revealed that (53.8%) of the 39 isolates of infected food-handlers had sub-assemblage AII, (28.2%) had assemblages B, and (17.9%) had a mixed pattern of AII and B. (Table 2). Regarding sub-assemblage AI, it was not detected in this study. The samples that identified as assemblage B were further processed using the RsaI restriction enzyme that revealed (23%) isolates were sub-assemblage BIII (5.12%) were sub-assemblage BIV (Table 3). Other studies reported that 54.5% of infected food-handlers had sub-assemblage AII and 25% had mixed infection to AII and B assemblages. Only 4.6% with BIII sub assemblage had zoonotic potential. Sub assemblage AII is specific for human.[24] The higher rate of sub-assemblage AII demonstrated an anthroponotic origin of the infection, so the infected food-handles can directly transmit this protozoanto consumers via contaminated food and water. Understanding the transmission patterns and distribution of *Giardia* is crucial for effective prevention and control measures. The samples that identified as assemblage B were further processed using the RsaI restriction enzyme that revealed 9 (81.8%) isolates were sub-assemblage BIII 2 (18.2 %) were sub-assemblage BIV (Figure 3).

The present study provides the first data on the assemblages and sub-assemblages of *G.lamblia* in food handlers in Kurdistan Iraq. Research on the diversity of *Giardia*has important practical significance for the formulation of preventive and control measures for giardiasis. Molecular biological technology can help us correctly to understand the characteristics of *Giardia* and provide information on its population genetic differences, genotype classification, distribution and epidemiological differences between animals and livestock. The analysis of genetic variation between and within different populations provides information on the evolutionary and genetic differentiation of *Giardia* and provides information on its transmission dynamics, which may aid efforts to control the infection of this parasite. This enables researchers to trace the source of infection or pollution, assess the public health risk of *Giardia* in the environment, determine the main *Giardia* assemblages during an outbreak, and track the transmission route. Genotyping is considered to be the most

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important tool for identifying *Giardia* species, analyzing genetic diversity, and understanding population structure.[11,12,25-28] A continuously and rapidly growing body of data indicates that *Giardia* species assemblages/ sub-assemblages (Sub-assemblages of *G. duodenalis* refer to further subdivisions within the assemblages of the species *G. duodenalis*) appear to be preferentially harbored by a specific genus of different host categories.[10,22]

Conclusion

Determining the genetic background of *G.intestinal* is a useful approach to understand the pathoaetiology of infection and thus control of giardiasis effectively. On the clinical ground the linkage between a given assemblage and sub-assemblage with or without diarrhea revealed no association. Sub-assemblage AII in particular be one of the most important genotype in transmission of pathogenic *Giardia* to human. *G.intestinalis* positive samples were categorized as genotypes A (AII) and B (BII) with A(AII) genotype as the dominant one in Kurdistan region. Future studies on the epidemiology of human and animal giardiasis, mainly risk elements correlated with genotyping, would assist to comprehend the role of giardiasis.

Competing interests

The author declares that he has no competing interests.

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