Correlation between Assemblages A and B Giardia lamblia infection with melatonin, ghrelin, Hsp70 and TFF3 biomarkers in patients

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KEYWORDS

Giardia lamblia, nested, PCR, TPI, biomarker

ABSTRACT

Background: Giardiasis is considered one of the parasitic diseases affecting humans, caused by a flagellate protozoan known as *Giardia lamblia*. It is a common gastrointestinal pathogen that can lead to various clinical and other complications. ***Objective:*** Study aimed to diagnose infection with the *G. lamblia* in children aged (1-12 years) using a direct wet mount, and to determine Assemblages A and B *Giardia lamblia* by targeting the TPI gene using Nested-PCR, in addition to evaluating the relationship of infection of assemblages with (melatonin, ghrelin, Hsp70 and TFF3) using ELISA assay. ***Method***: By examining 238 stool samples for children suffering from diarrhea and some intestinal symptoms visiting some hospitals in Salah al-Din Governorate. ***Results:*** 34 (14.2%) positive samples were recorded by microscopic examination. 100 fecal samples using the Nested-PCR, showed 60 (60%) positive samples for the *G. lamblia*, including 18 (30%) for the assemblage B, 31 (51.66%) samples for assemblage A, as well as 11 (18.33%) mixed infections of A+B. The study of biomarkers revealed a highly significant increase in the levels of (melatonin, ghrelin, Hsp70 and TFF3) in the serum of patients with mixed A + B infection, while the lowest average concentrations of biomarkers were recorded in children infected with group A. ***Conclusion*:** Direct microscopic examination of stool samples It has good sensitivity for detecting *G. lamblia* parasite, and the TPI gene targeted in Nested-PCR technology has high sensitivity and specificity for genotyping detection of the parasite. Group A is the most common among patient samples. Mixed infections (A+B) have the greatest effect.

1. Introduction

Giardia lamblia, also known as (Giardia duodenalis or Giardia intestinalis) is the causative agent of giardiasis [1].G.lamblia caused a widespread illness in both humans and animals that was recognized As opportunistic, affecting healthy people immunocompetent. According to the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), Giardia lamblia is the eleventh parasite that is transmitted by food in terms of priority[2]. Early detection of giardiasis is essential for effectively treating and preventing diseases. The conventional laboratory diagnosis includes microscopic examination of at least three independently collected fecal samples to detect trophozoites or cysts [3]. Moreover, G. lamblia is categorized into 8 genotypes termed by A, B, C, D, E, F, G and H, according to triosephosphate isomerase (tpi), glutamate dehydrogenase (gdh) and small–subunit (SSU) rRNA genes. Assemblage A and B mainly infect humans and a wide range of other hosts like livestock, cats, dogs and wild mammals [4]. Giardiasis presents a significant challenge due to its growing prevalence in the environment. Nevertheless, conventional diagnostic techniques continue to exhibit limited sensitivity. Utilizing genetic testing for identifying human pathogens through copro-PCR proves to be a beneficial approach with satisfactory sensitivity in detecting G. duodenalis in human fecal sample[5]. The chemical characteristics called biomarkers, which are markers of the physiological, pathological, or pharmacological response to therapeutic intervention, can be objectively checked in fluid and tissue samples[6]. Conceptually, biomarkers can have diagnostic, prognostic, or therapeutic value, with Numerous varieties based on type, characteristics, application, genetics and molecular biology methods in addition to its characteristics[7]. Hormone melatonin is mainly produced via the pineal gland, but it can also be found in smaller amounts in the retina, skin, and gastrointestinal tract white blood cells and bone marrow cells [8]. The hormone ghrelin is made from ghrelin Predominantly in the stomach, but also expressed in many other organs including the intestine The pancreas, kidneys, myocardium, hypothalamus, and pituitary gland[9]. Heat shock protein 70 is a protein produced by living cells when exposed to sub-lethal temperatures. It is a protein that causes Stress has been shown to protect the brain from various nervous system injuries [10]. Trefoil factor 3 or TFF3 are formed in intestinal goblet cells in association with mucin [11].

2. Methodology

Study design and population

The present cross-sectional study was conducted between July 2023 and June 2024. A total of 238 children, aged between 1 day and 12 years, visited the outpatient clinic at a hospital in Salah al Din, Iraq, seeking medical advice. These children presented with various gastrointestinal symptoms, such as weight loss, cramping in the abdomen, diarrhea, and gas. Furthermore, several kids displayed no symptoms at all.

Fecal samples collection

The sterile cup was used to collect a single stool sample chosen randomly. Both macroscopic and microscopic analysis of the samples were conducted right away.All samples were placed in designated tubes without preservatives and stored at −20 °C until molecular analysis was performed.

Serum samples collection

90 Serum samples (60 positive for parasite+30 negative controls) were collected from pateints and suspected of being infected with *Giardia*, during the collection of stool samples, and stored at -20 °C for later use with the ELISA test.

Microscopic examination

This method was executed by preparing clean glass slide and two drops of N.S. was put on one half slide and a drop of Lugals iodine on the other half, two swabs are taken from stool samples by wood stick from different places of samples (about pin head in size). 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 400 (40 x 10) to record the presence of trophozoites and/or cysts [4].

Molecular technique

DNA Isolation

DNA was isolated from stool samples Using a (Stool DNA Extraction) kit prepared by the Taiwanese company Geneaid. The concentration and purity of genomic DNA extracted by Nanodrop (Napi, Korea) was measured by reading the absorbance at (260/280 nm) and stored at − 20°C.

Giardia lamblia DNA amplification by nested PCR

The molecular characterization of *Giardia lamblia* loci, specifically the triose phosphate isomerase gene (tpi), is identified through nested PCR. This gene, tpi, is relatively short in length. Consistency is observed in the primer design used across various research studies. The amplification of the tpi gene was carried out using specific primers, detailed in Table 1 [5].

PCR for nested amplification

A PCR that consists of two cycles is called nested. First denaturation is necessary for the PCR's first round. This thermocycler was set for four minutes at 94 °C, a temperature that is necessary to break DNA bonds. Denaturation was then carried out for 30 seconds at 94 °C. The perfect primer annealing temperature for the TPI gene is 52 °C for 30 seconds. The final step was to extension at 72 °C for 30 seconds. Finally, a four minute extension at 72 °C is required. The second round of nested PCR had the same phases as the first. Nonetheless, the second round of nested PCR requires annealing at 62°C, which differs from the first round. Gel electrophoresis to separate amplified DNA, a 2% agarose gel was utilized, and the DNA was stained with DNA safe dye. The DNA ladder was employed on 110 volts for 60 minutes and viewed by used gel documentation [5].

Table 1. The primer sequence utilized

Primer	Sequence	Size	Reference

tpi gene	Tpi F 5-AAATIATGCCTGCTCGTCG-3 Tpi R 5-CAAACCTTITCCGCAAACC-3	605 bp	[5]
Assemblage A	AF 5-CGCCGTACACCTGTCA-3 AR 5-AGCAATGACAACCTCCTTCC-3	332 bp	
Assemblage B	AssBF 5- GTTGTTGTTGCTCCCTCCTTT-3 AssBR 5- CCGGCTCATAGGCAATTACA-3	400 bp	

Human Melatonin, Ghrelin, Heat Shock Protein70 and Trefoil factor 3 protein ELISA test

90 Serum samples (60 positive +30 negative control) were used to assess Melatonin hormone, Ghrelin hormone, Heat Shock Protein70, Trefoil factor 3 protein using the ELISA kits (China-BT LAB). Human antibodies percolated through the ELISA plates. We added the samples to the plates and correlated the color development in the substrate solution with the levels of Melatonin hormone, Ghrelin hormone, Heat Shock Protein70, Trefoil factor 3 protein. The process was stopped by adding a stop solution, and absorbance was measured at 450 nm.

Statistical analysis

Statistics were tested by IBM SPSS 26.0 and GraphPad Prism (version 8, GraphPad Software Inc., CA, USA). The data was expressed as mean ± SD, and significant results were determined using the T-test ($P < 0.05$).

Ethics approval and consent to participate

In accordance with the mandates of the Scientific Committee of the Salah al-Din Health Department and the Scientific Committee of the College of Science at Tikrit University, authorization for the research study was issued on 9 July 2023, following the guidelines outlined in administrative directive 3625. Subsequent to informing the parents about the study, a questionnaire was utilized to obtain samples from the children, with parental consent obtained prior to gathering information on each child, including their name, address, and prevalent symptoms.

3. Result and Discussion

Diagnosis of *G. lamblia* by direct Examination

The total number of this study was 238, 34 sample (14.285%) test result was positive for microscopic light infection of *Giardia*, Uninfected samples (85.714%). As in Table 2 and Figure 1.

Table 2. diagnosis of Giardia by Microscopic Examination

	Microscopic examination	
Number of positive cases	34	14.285 %
Number of negative cases	204	85.714 %
The total number	238	

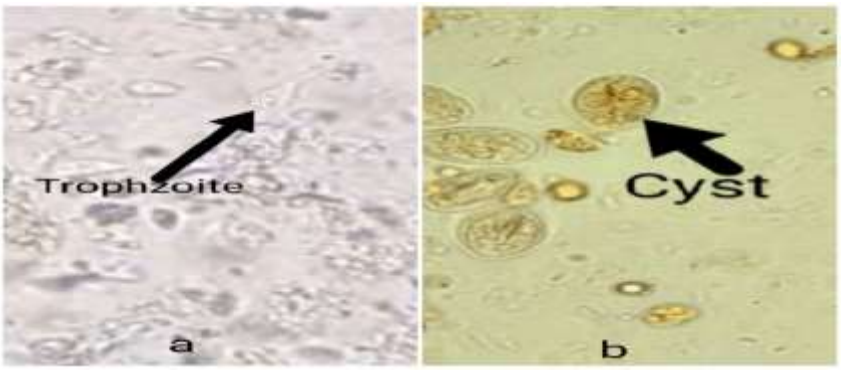

Figure 1 (a) show Trophozoite of by wet smear normal saline (0.9%) 40X (b) cyst by Lugol's iodine (1%) 40X

Diagnosis of *G. lamblia* specie by Nested PCR assay

Nested PCR diagnosis was performed on 100 samples, of which 34 samples were diagnosed by light microscopy, and 66 samples were negative by light microscopy. only 60(60%) positive samples for DNA from the specific TPI gen. As in Table 3 and Figure 2 (a).

Table 3. diagnosis of *Giardia lamblia* by PCR assay

	PCR assay fore TPI gene	
Number of positive cases	60	60%
Number of negative cases	40	40%
The total number	100	100%

Diagnosis of assemblages A and B Giardia lamblia by Nested PCR assay

Nested PCR diagnosis was performed on 60 samples, using the PCR product as a template for the diagnosis of Giardia lamblia assemblages. As in Table 4 and Figure 2 (b)and(c).

Table 4. PCR results for assemblages

	PCR assay fore assemblages	
assemblage A	31	51.666%
assemblage B	18	30%
Mixed infections of assemblage A and B	11	18.333%
The total number	60	

Figure 2 (a) PCR product is subjected to electrophoresis in a gel made of 2% agarose. M: DNA size marker (100 bp ladder) is used to denote the size of DNA. Bands in wells (1-10) indicate the presence of *Giardia lamblia***. (b).** Nested PCR analysis revealed the presence of Giardia assemblage A products, which exhibited positive samples by show bands at 332 bp. The wells (1-10) on the gel corresponded to the positive samples(c) The nested PCR analysis revealed the presence of Giardia assemblage B products, which exhibited positive bands at 400 bp. The wells (1-12) on the gel corresponded to the positive samples.M:marker lader 100 bp.

Biomarkers study Human ELISA test

Melatonin hormone

The results of the research revealed a statistically significant rise in the mean melatonin concentration in patients with assemblages A+B, reaching 980.41±34.30 ng/L, while the lowest average melatonin concentration was recorded in patients with assemblage A, reaching 650.70±28.02 ng/L. As in Table 5

Table 5 Melatonin concentrations by assemblages in patients with giardiasis

Assemblages of Giardia lamblia	The number	Mean ± Std. Error ng/L	P.value
A	31	c650.70±28.02	0.0001
B	18	b840.41±18.26	
A+B	11	a980.41±34.30	

Ghrelin hormone

The findings of the present study demonstrated a significant rise in the average. ghrelin concentration in patients with assemblages A+B, reaching 12.5835±0.63 ng/ml, while the lowest average ghrelin concentration was recorded in patients with assemblage A, reaching 7.5594±0.37 ng/ml. As in Table 6

Table 6. Ghrelin concentrations according to assemblages in patients with giardiasis

Assemblages of Giardia lamblia	the number	Mean ± Std. Error ng/ml	P.value
A	31	c 7.5594±0.37	0.0002
B	18	b10.9426±0.41	
A+B	11	a12.5835±0.63	

Heat shock protein70 Hsp70

The results of the current study showed a highly significant increase in the average Hsp70 concentration in patients with assemblages A+B, reaching 55.25±1.78 ng/ml, while the lowest average Hsp70 concentration was recorded in patients with assemblage A, reaching 33.88±1.18ng/ml. As in Table 7.

Table 7. Hsp70 concentrations by assemblages in patients with giardiasis

Assemblages of Giardia lamblia	the number	Mean ± Std. Error ng/ml	P.value
A	31	c33.88±1.18	0.0006
B	18	b41.77±1.43	
A+B	11	a55.25±1.78	

Trefoil factor3 TFF3

The findings of this study indicated a pronounced and highly significant rise in the mean TFF3 concentration in patients with assemblages A+B, reaching 256.0673±10.06 ng/ml, while the lowest average TFF3 concentration was recorded in patients with assemblage A, reaching 166.4603±6.31 ng/ml. As in Table 8.

Table 8. TFF3 concentrations by assemblages in patients with giardiasis

Assemblages of Giardia lamblia	the number	Mean ± Std. Error ng/ml	P. value
A	31	c166.4603±6.31	0.0003
B	18	b198.0239±7.17	
A+B	11	a256.0673±10.06	

DISCUSSION

Giardiasis represents the predominant etiology of protozoan infections and diarrheal afflictions on a

global scale.Among several demographic categories, children appear as the most vulnerable cohort to Giardia infection [12, 13]. Within the scope of this research, Feces specimens were obtained of 238 pateints, revealing the incidence of giardiasis of 14.285% by microscopic examination. This percentage is in close agreement with previous prevalence estimates [14] documented in the vicinity of Tikrit, where a prevalence of 14.30% was reported. However, notable inconsistencies exist when compared to the results of disparate investigations, such as investigations conducted in Mosul [15]. These studies determined *G. lamblia* infection 1.04%. The variations observed can be attributed to differences in sample size, disparities in environmental circumstances across the studied regions, and various factors including personal hygiene, population density, sanitation standards, geographical positioning, climatic conditions, and socioeconomic standing. Furthermore, the discrepancies are influenced by the level of precision, laboratory expertise, and methodologies employed in the analysis of stool samples. Several investigations have focused on various genetic loci within the genome of *G. lamblia* for molecular characterization, exhibiting differing levels of sensitivity and specificity. The utilization of the TPI gene in this research for the DNA identification of *G. lamblia* was based on its notable genetic homogeneity and polymorphism[16, 17]. The presence of G. lamblia genomic DNA was detected in 60 samples out of 100(60%), with 34 samples confirmed positive by microscopic examination. This study is similar to previous studies, In Iraq, Baghdad Governorate, the TPI gene was duplicated in 4 of 7 samples, representing 57.14% [18], and in the city of Diwaniyah, the gene doubled in 27 of 50 samples, a rate of 54% [19]. The current study differed from other studies. In Tikrit, the gene was amplified in 6 of 17 samples, at a rate of 35.29% [20]. In Najaf, the gene was amplified in 80 of 100 samples, at a rate of 80% [21]. These occurrences are attributed to various factors that could impact DNA isolation, including the presence of some PCR inhibitors in feces, conditions related to Specimen preservation and the particular technique or model of DNA isolation kit used. Furthermore, discrepancies in amplification conditions and the targeted gene may also play a role, alongside the existence of single nucleotide polymorphisms, deletion insertions, and the diversity of Giardia species, all contributing to the generation of inaccurate negative findings. The elevated prevalence of parasite infection is likely a consequence of the substandard economic and residential conditions prevalent in certain regions of the nation. This situation arises due to habitation in inadequately developed areas owing to the absence of healthcare facilities, high population density, and inadequate access to clean water. Furthermore, various areas within Salah al-Din Governorate face service deficiencies resulting from population growth and insufficient provision of health and educational support for the residents.
The molecular epidemiology of *G. lamblia* has been explored in various global regions to unveil potential links between the genetic variability of the parasite and its clinical manifestations, resistance to drugs, and patterns of environmental transmission[22, 23]. In our investigation, we employed primers specific to the species to profile *G. lamblia* populations obtained from children in Saladin, Iraq. In this study, 51.666% and 30% of the isolates belonged to Giardia assemblages A, B, respectively. These findings align with prior research demonstrating the frequent association of both assemblages with human Giardia, as evidenced by The investigation of several human isolates from various geographical areas [24]. *G. lamblia* genotype A emerges as the predominant assemblage within the present investigation. The former is predominantly linked to zoonotic transmission, contrasting with assemblage B, which exhibits a stronger association with human-to-human transmission[25, 26]. Nevertheless, both categories have been identified in both human and domestic animal hosts including dogs, cats, and livestock [27]. Consequently, ascertaining the origin of the infection might prove challenging, particularly in the absence of subpopulation scrutiny. Nonetheless, in this particular analysis, the prevalence of assemblage A could potentially be clarified by the notable correlation between the rural habitation of the subjects under scrutiny and the heightened occurrence of assemblage A in fecal specimens. In rural regions, the contamination of public water supplies along with close encounters with domesticated animals offer ideal circumstances for interspecies transmission[28, 29]. Previous studies have documented a higher prevalence of assemblage A compared to assemblage B in various countries globally [30, 31]. Consistent findings are reflected in our study, which are in line with research conducted in Egypt, Iran and Iraq where a marked predominance of Among all giardiasis patients, assemblage A was noticed[5, 32-34]. Conversely, there have been conflicting reports

indicating the dissemination of Giardia assemblage B among human patients, Both in Iraq and on a global scale[14, 35-37] Nevertheless, it was observed by some individuals that the two groups exhibited a nearly identical distribution among affected individuals[38, 39]. It should be noted that patients infected with assemblages A and B were detected in 18.333% Agree with [33,34] and disagree with [37,19]. The complexity and dynamism of the parasite in the ecosystem are emphasized by these results, revealing that some water and food sources are contaminated by genetically distinctive Giardia cysts with mixed sequences [40, 41]. In addition, the occurrence of infection with multiple genotypes results in persistent transmission and a higher occurrence of mixed genotypes in human giardiasis [42]. The use of assembly-specific primers in the current research enabled the identification of co-infection with mixed populations[43-45].

The relationship between the biomarkers under study and assemblage of the parasite. Studies and research that shed light on this matter noted that there is a relationship and difference between infection with assemblage A or B of the parasite and the severity of clinical signs and disease symptoms, as one study recorded an increase in the level of Fecal calprotectin In patients infected with assemblage B more than assemblage A, there is therefore a possible relationship between the parasite genotype and inflammatory markers or biomarkers[46]. Other studies have shed light on the relationship between the genotype of the parasite and the severity of clinical symptoms and signs, They recorded a higher intensity and severity of the clinical signs represented by pain, abdominal bloating, diarrhea, lack of growth and failure, and various gastrointestinal disorders in patients infected with assemblage B more than assemblage A, which indicates the presence of Difference in virulence factors between assemblages A and B[47]. Variant-specific surface proteins (VSPs) for example VSP5G8 and VSPH7 are potential indicators of acute giardiasis, The presence of VSP5G8 was observed in assemblage B while VSPH7 was found in both assemblages A+B, as is the case with the giardin proteins α-1-giardin, α-7.1- giardin and α-7.3- giardin, considered potential indicators of acute giardiasis, α-1- giardin was observed and recorded with both A+B, while α-7.1- giardin and α-7.3- giardin were specific for the assemblage A [48]. Infection with assemblage B may lead to pro-inflammatory intestinal responses This is observed through inflammatory microscopic examination of the duodenum and an increase in the level of some biomarkers in some patients [49]. Infection with assemblage A may reduce or reduce the chemotaxis of polymorphonuclear leukocytes and reduce the filtration of white granulocytes, chemokines, and cytokines that contribute to attracting white defensive cells to the site of infection and thus have less effect on the epithelial cells in the intestinal lining [49].

Discrepancies in research methodology, participant selection criteria, and limited sample size could elucidate the conflicting findings observed in our investigation and might constrain its generalizability. Furthermore, there was an ignore in assessing the presence of concurrent virus and intestinal microbial infections, which might have influenced the results, inducing clinical presentations and obscuring the authentic clinical repercussions of *G. lamblia* infection. More extensive researches on a large-scale population is needed to confirm the connection between assemblage and biomarkers. Subsequent molecular epidemiological investigations across extensive geographical regions are vital for investigating virulence factors, parasite genotypes, and infection sources in the environment. Certainly, conducting genotyping studies at the subpopulation level is indispensable for determining the prevalence and scope of zoonotic transmission occurrences and the relationship between assemblage and biomarkers.

4. Conclusion and future scope

Giardiasis remains to be a common zoonotic sickness among youngsters in Salah al-Din Governorate, Iraq. The results of our study indicate a high prevalence of Giardia assemblage A and the occurrence of mixed infections in a portion of the population being studied, and There has been a notable rise in the average concentration of biomarkers in patients infected with assemblages A+B, while the lowest average concentration was recorded in patients infected with assemblage A. These results suggest interspecies transmission within our region, rather than just human transmission. It is necessary to

strengthen surveillance measures and increase community awareness regarding disease transmission, particularly in rural regions. This research explores molecular epidemiology and evaluates the efficacy and dependability of species-specific direct primers for genotyping Giardia spp. The recognition of mixed infections highlights the necessity for further genetic investigations focused on clarifying possible associations between the genetic diversity of parasites and biomarkers

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