Real-time PCR Assay for Diagnosis of Eukaryotic Peptide Chain Release Factor Subunit 1 Gene in the Stages of *Giardia duodenalis*

Saba Fadhil Ali Mala
Al-Furat AL–Awsat Technical Univ.
saba.fadhl55@yahoo.com

Dhamiaa Maki Hamza
Kerbala Univ., Collage of medicine
dhamiaam@yahoo.com

Abstract

Giardiasis caused by *Giardia duodenalis* is a common intestinal disease. Although this parasitic infection is found in mammals including human, pets and livestock. A total of 250 samples was collected from patients that were suspected the infection with *Giardia duodenalis* during the period from August 2014 to October 2015.

The aim of this study is to investigate the molecular identification of *Giardia duodenalis* in patients suffered from diarrhea depending on a Eukaryotic peptide chain release factor subunit 1 gene to Confirm its existence in both stages of *Giardia dudenalis* (Trophozoit and cyst) and the possibility adopting it as a diagnostic gene.

The results revealed that 200 samples were positive depending on the microscopic examination , 100 samples of stool have been chosen to isolate the cysts from it, while 100 samples of stool were used for trophozoits isolation , which in turn have undergone the third stage of the examination for the purpose of qualitative accurate diagnosis of *Giardia* using of quantitative Real Time PCR assay for amplifying the target region Eukaryotic peptide chain release factor subunit 1 and study the possibility of using the a
diagnostic gene.

Key words :
Giardia duodenalis, Real Time PCR, GL Eukaryotic gene, Trophozoit and Cyst stage.

الخلاصة

داء الجيارديا من الامراض الطفيلية الشائعة الانتشار في جميع انحاء العالم والمسبب له نوع من الطفيليات يسمى طفيلي الجيارديا الاثنا عشرية. بالرغم من إن الإصابة بالطفيلي تشمل العديد من اللبائن ومن ضمنها الانسان والحيوانات الاليفة. جمعت 250 عينة براز من المرضى المشكوك بإصابتهم بداء الجيارديا الاثني عشرية للفترة 2014 –2015.

الهدف من الدراسة الحالية هو اثبات إمكانية استخدام التقنيات الجزيئة لتشخيص طفيلي الجيارديا الاثنا عشرية في المرضى المصابين بالإسهال اعتمادا على الجين Eukaryotic peptide chain release factor، والذي يعد من الجينات المميزة في الكائنات الحية الحقيقية النواة والذي يوجد في طفيلي الجيارديا ولكن الدراسات المتعلقة بهذا الجين قليلة جدا عالميا واتجهت الدراسة الحالية الى اثبات إمكانية اعتماده كجين تشخيصي بأثبات وجوده في الاطوار الحياتية المختلفة لطفيلي الجيارديا.

وظهرت 200 عينة موجبة اعتمادا على الفحص المجهري وقسمت لمجموعتين المجموعة الأولى (100 عينة) استخدمت لعزل الاكياس والمجموعة الثانية (100عينة) استخدمت لتنمية الطور الخضري للجيارديا واستخدمت المجموعتين لعزل الحامض النووي DNA والذي استخدم بدوره كهدف لتضخيم الجين في تقنية RT–PCR ودراسة إمكانية عزله من الطورين المتكيس والخضري.

الكلمات المفتاحية: جيارديا الاثني عشر , فحص السلسلة البوليميرية ذات الوقت الحقيقي ,الطور المتغذي و المتكيس

Introduction

The genome of the eukaryotic protist *Giardia duodenalis*, an important human intestinal parasite, is compact in structure and content, contains few introns or mitochondrial relics. The whole life cycle can be completed in vitro with stimuli that mimic gastrointestinal conditions (Gillin *et al*., 1996). Giardiasis which is caused by

G.duodenalis is a common intestinal disease. Usually *G. duodenalis* is transmitted by contaminated water with cyst (Cacci and Ryan,2008).

infection of the host is initiated by ingestion of cysts, followed by excystations and colonization of the small intestine by the trophozoite form of the parasite, which multiplies by vegetative growth in the intestine.

Trophozoites, like most intestinal parasitic protozoa, undergo dramatic biological changes to survive outside the intestine of their host by differentiating into resistant cysts (encystation) (Gillin *et al*., 1996).

The pathophysiology of giardiasis depends directly upon the ability of the trophozoite form to replicate in the host upper small intestine *G.duodenalis* is also a serious zoonotic parasite that infects many mammals, including dogs and cats. Infection in human can lead to abdominal cramps, acute or chronic diarrhea, and Malabsorption (Thompson, 2004). in developing countries the prevalence may reach as high as 20–60%. In humans, the infection produced by *G.duodenalis* (giardiasis) may produce several symptoms such as diarrhea, abdominal discomfort, distension, flatulence,and vomiting. In addition, giardiasis may present with severe damage of the small intestine (Silvia *et al*, 2002).

Infection with *G.duodenalis* leads to acute/chronic diarrhea in some individuals but remains asymptomatic in others. Several studies indicate that severe or symptomatic giardiasis may cause malabsorption of nutrients and affect the nutritional status of the host. However, few studies reported the potential effect of moderate or asymptomatic giardiasis, thus the effects on the nutritional status of the host (Silvia *et al*,2002). Although this parasitic infection is found in mammals including human, pets and livestock, few species within the genus *Giardia* can infect humans (Akram , 2000).

Giardia has two sets of genes whose expression is uniquely regulated. Variant surface proteins (VSP) are expressed one at a time on the surface of trophozoites, whereas the encystation-specific genes are expressed only as trophozoites differentiated are into cysts. Thus, the methods of genetic recombination and transcriptional regulation in this primitive eukaryote are of interest (Adem, 2001).

The parasites are continuously exposed to environmental toxins act to pressure the host immunity, which could affect the stability of the genome and it gives away of mechanical repair the genetic material of some of these objects have not been studied in detail so far (Singh *et al*., 2013).

The Homologous Recombination is an important process to repair the damages caused as it's important to find the genetic diversity of parasite to escape from host immune response (Conway *et al*., 2002). In addition Davis-Hayman & Nash noted that the Giardia parasite has two sets of genes are modified genetic expressions are exceptionally. In order to address such important topics as DNA content, gene rearrangement and complete deletion of a gene in Giardia, markers that are more selectable must be identified. In addition, the differences in homologous recombination and expression of transfected DNA among Giardia groups are critical areas that should be addressed (Homan *et a*l.,1998).

Giardia duodenalis have seven complex genotypes termed (A-H), Genotypes A and B are the main and only causes of human infections (Silvia *et al*, 2002, Akram,2000).

The succession is about 12 million base pairs and contains about 5000 protein-coding genes (Adem, 2001). The GC content is 46%. Trophozoites have four of a ploidy and the ploidy of cysts is eight, which in turn raises the question of how *Giardia* maintains homogeneity between the chromosomes of the same and opposite nuclei. Modern sequencing technologies have been used to resequencing different strains

(Helmy *et al*.,2009) .Giardia trophozoites have a sufficient chance to pick up genes from bacteria and to hunt products of host and bacterial metabolism, Like that of both Trichomonas and *Entamoeba* protozoa .

Giardia's genome contains many lateral gene transfer (LGT) candidates, indicating that LGT has played an important role in shaping Giardia's genome and metabolic pathways, while LGT from bacterial and archaeal donors has shaped Giardia's genome, and previously unknown gene families, for example, cysteine-rich structural proteins, havebeen discovered.Unexpectedly,the genome shows little evidenceof heterozygosity, supporting recent speculations that this organism is sexual(Morrison *et al*., 2007).

This genome sequence will not only be precious for investigating the evolution of eukaryotes, but will also be applied to the search for new therapeutics for this parasite. Trophozoites get hold of most energy from anaerobic glycolysis or metabolism of arginine and many metabolic genes and pathways most closely resemble bacterial homologs (Conway *et al*., 2002).

The current study was conducted to determine both stages of *G. duodenalis* in Babylon by molecular technique, the RT-PCR method was performed based on a Eukaryotic peptide chain release factor subunit 1. As this is a suitable technique for direct diagnosis of *G. duodenalis* in crude specimens. The main purpose of this study was to evaluate the correlation between the infection with Giardiasis and patients with diarrhea depending on the GLEukaryotic gene and to improve it if this type of gene could be considered as a diagnostic gene.

Materials and methods:

1. Sample collection

Two hundred stool samples from microscopically positive *G.Duodenalis* were collected from clinical laboratories in Babylon province between August 2014 and October 2015. After direct analysis by microscope, the samples without any preservation were kept at 4°C.

2. Conventional Microscopy

One drop of fecal suspension was transferred to a microscope slide with a cover slip. Each slide was then examined as a direct mount at 40_ magnification and the presence or the absence of *G. duodenalis* cysts was recorded. For further confirmation, formal–ether concentration technique was performed (Hala *et al*., 2013)

3.Purification of *Giardia duodenalis* cysts

Zinc sulfate flotation method was used for cysts purification in which small amount of feces 3 mg was mixed with distilled water in a clean cup by wood stick to make suspension so that cysts would be free from the feces. Suspension filtered through gauze was centrifuged for 5 minutes at 1000 rpm. Moreover, the supernatant poured off. Zinc sulfate solution was added and the contents were centrifuged again at the same rate and time. The surface layer which contained the cysts was transferred to another tube with distilled water by using pasture pipette. Cysts were concentrated by centrifugation at 1000 rpm for 5 minutes and 2 ml of phosphate buffer saline were added to the sediment and stored at $4C^0$ until use (Helmy *et a*l., 2009)

4. In Vitro Cultivation of *Giardia duodenalis*

G.duodenalis trophozoites (strain WB, clone C6, ATCC 50803) were cultured in modified TYI-S-33 medium with bovine bile [Keister DB], supplemented with bile and antibiotics at the following concentrations.
penicillin, 200 IU/ml; streptomycin, 200 ,ug/ml; Corresponding author. vancomycin, 20 ktg/ml; and clindamycin, 20 ,ug/ml. The last two antibiotics were required to eliminate contamination with oropharyngeal flora and were omitted after the initial isolation phase. In some cases, we also used stoppered vials of lyophilized modified Diamond TPS-1 medium which was rehydrated immediately before use, as it is described by (Gordts *et al*., 1985 , Gordts *et al*. ,1984).

5.DNA extraction

Genomic DNA was extracted from cysts and trophozoites. Those isolations that didn't have suitable DNA were excluded from the study.The trophozoite's DNA was extracted using AccuPrep® Stool DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instruction with some modification and used as template for RT-PCR assay. Extraction of genomic DNA from *Giardia's* cysts was carried out according to our previous published paper .(Pestehchian *et al.*, 2012)

6. RT-PCR amplification

One set of primers was designed to be used against the coding region of the Eukaryotic peptide chain release factor subunit 1 gene of *G. duodenalis* (trophozoites and cysts) a 127bp fragment was amplified using the forward primer GLCPNR1 (5'-CGAACAGCCTTTTCATCAGCTC-3'and the reverse primer GLCPNF1 (5'-AACGCTGGTCAAAGATGGC -3') To the DNA is amplified by the use of the forward and reverse primers which are designed by NCBI site (Primer 3 plus program)
.

7. RT-PCR protocols

Real-Time PCR was preformed for detection of both stages of *G.duodenalis* by using the primers and TaqMan probe specific for GL. Eukaryotic peptide chain release factor subunit 1 In the DNA technique was carried out according to method described by (Tungtrongchitr *et al*. , 2010)

8. Real-Time PCR master mix preparation

Real-Time PCR master mix was prepared by one step Reverse Transcription and Real-Time PCR detection kit (AccuPower® 2X GreenStar™ qPCR Master Mix, Bioneer. Korea), and done according to company instructions as it is shown in the following Table (1):

Table (1): RT-PCR component for gene amplification

RT-PCR Master mix	**Volume**
2X Green star master mix	25 µL
DNA template	5µL
forward primer (10pmol)	1µL
reverse primer (10pmol)	1µL
DEPC water	18µL
Total	**50µL**

The RT-PCR master mix reaction components that mentioned in (table 1) were added into standard PCR tube(containing 8 wells strips tubes), Then all strips tubes vortexing for mixing the components and centrifuge for 3000 rpm for 3 minutes in Exispin centrifuge, after that transferred into Exicycler Real-Time PCR thermocycler (Life Technologies Corporation , 2012).

9. Real-Time PCR Thermocycler conditions

Real-Time PCR thermocycler conditions were set up according to primer annealing temperature and RT-PCR Taq Man kit instructions as it is shown in Table (2):

Table (2): Amplification protocol for detection of gene (thermal cycler program)

Step	Condition	Cycle
Reverse transcriptase	95 °C 15 min	1
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	45
Annealing/Extension	60 °C 30 sec	

Thermal cycles were applied to inspect the Real-Time PCR by relying on instructions Accu Power® 2X Green Star TM qPCR Master Mix and aslo by calculating the degree Tm prefixes used the apparatus Mini Opticon Real-Time PCR system BioRad / USA.

10-Real-Time PCR Data analysis

RT-PCR data analysis was performed by calculation of the threshold cycle number (CT value) that presented the positive amplification of gene in Real-time cycle number (Bibby-scientific Inc, 2014).

Results

This study included 200 stool samples collected from patients infected with Giardiasis and the results showed comprising 109 (54%) males and 91 (45%) females. The prevalence elevated (78%) among older children aged six to ten years, but (52%) among children aged one to five years, and was higher among males (60%) than the it was among females(52%) (Table 3).

Table (3) : Classification of children according to age and gender.

Age	Female	Male	Total
1-5	24(46.2%)	28(53.8%)	52(40%)
6-10	33(42.3%)	45(57.7%)	78(60%)
Total	57(43.8%)	73(56.2%)	130 (100%)

The Symptomatic patients were stunted, gastrointestinal symptoms reported included diarrhea, vomiting and dehydration. The average duration and frequency of diarrhea were 3-9 days and 4-6 times a day, while the average duration and frequency of vomiting were 2-3 days and 2-7 times a day respectively (Tables 4, 5).The consistency of the stool samples were submitted by the symptomatic patients ranged from loose, watery and mucoid , Abdominal pain was observed in 90% (180/200 patients) of patients. Other manifestations, such as flatulence 38% (76/200), anorexia and nausea 31.5% (63/200).

Table (4): Clinical characteristics of the study patients that infected by trophozoiote of *G. duodenalis* .

Parameter	Total N (%)	Symptomatic N (%)	A Symptomatic N (%)
Stool consistency			
Firm	13**(13%)**	9 **(9%)**	4**(4%)**
Loose	6 **(6%)**	57**(57%)**	9**(9%)**
Mucoid	21**(21%)**	10**(10%)**	11**(11%)**
Male	54 (54 %)	33 (33 %)	21(21 %)
Female	46 (46 %)	24(24 %)	22(22 %)
Gastrointestinal symptoms			
Diarrhea	88**(88%)**	88**(88%)**	0
Vomiting &nausea	78**(78%)**	74**(74%)**	4**(4%)**
Dehydration	59**(59%)**	50**(50%)**	9**(9%)**
flatulence	48**(48%)**	41**(41%)**	7**(7%)**
Anorexia	33**(33%)**	15**(15%)**	18**(18%)**
abdominal cramps	93**(93%)**	45**(45%)**	48**(48%)**

Table (5): Clinical characteristics of the study patients that infected by cyst of *G.duodenalis*

Parameter	Total N (%)	Symptomatic N (%)	Symptomatic A N (%)
Stool consistency			
Firm	20**(20%)**	8**(8%)**	12**(12%)**
Loose	38**(38%)**	18**(18%)**	20**(20%)**
Mucoid	42**(42%)**	20**(20%)**	24**(24%)**
Male	55(55 %)	30 (30 %)	25(25 %)
Female	45(45 %)	21(21 %)	24(24 %)
Gastrointestinal symptoms			
Diarrhea	78**(78%)**	66**(66%)**	12**(12%)**
Vomiting & nausea	45**(45%)**	20**(20%)**	25**(25%)**
Dehydration	39**(39%)**	19**(19%)**	20**(20%)**
Flatulence	28**(28%)**	10**(10%)**	18**(18%)**
Anorexia	30**(30%)**	11**(11%)**	19**(19%)**
abdominal cramps	89**(89%)**	41**(41%)**	48**(48%)**

A total of 200 human *Giardia duodenalis* microscopically positive samples were selected for genomic DNA extraction. the total DNA extraction kit provided by (Bioner/Korea) was used.

DNA extraction was successfully observed from all samples by agarose electrophoresis (0.5%). This DNA was used as a template for Real-Time PCR assay.
The use of RT-PCR techniques in the specific detection of *G.duodenalis* was showed a fluorescence of SYBER Green dye was appeared very clear through the form of amplification plot to the positive samples during the cycle 30 as its shows in fig.(1).

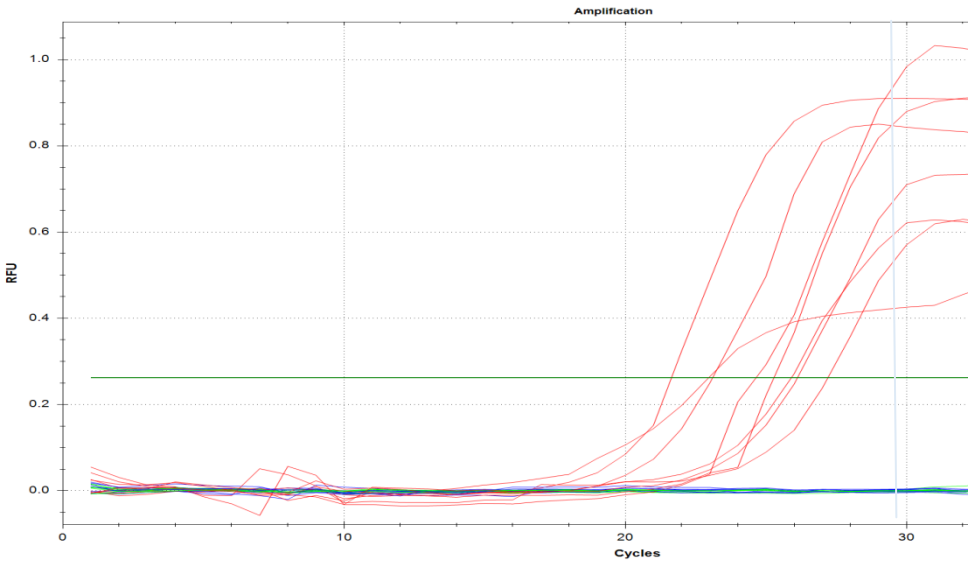

Fig.1: Amplification Plot of region GLE, in which the fluorescence of the dye SYBER Green represent amplificated DNA that was isolated from Trophozoites which located above the Threshold .while under the threshold there was no amplificated DNA that was isolated from the cysts.

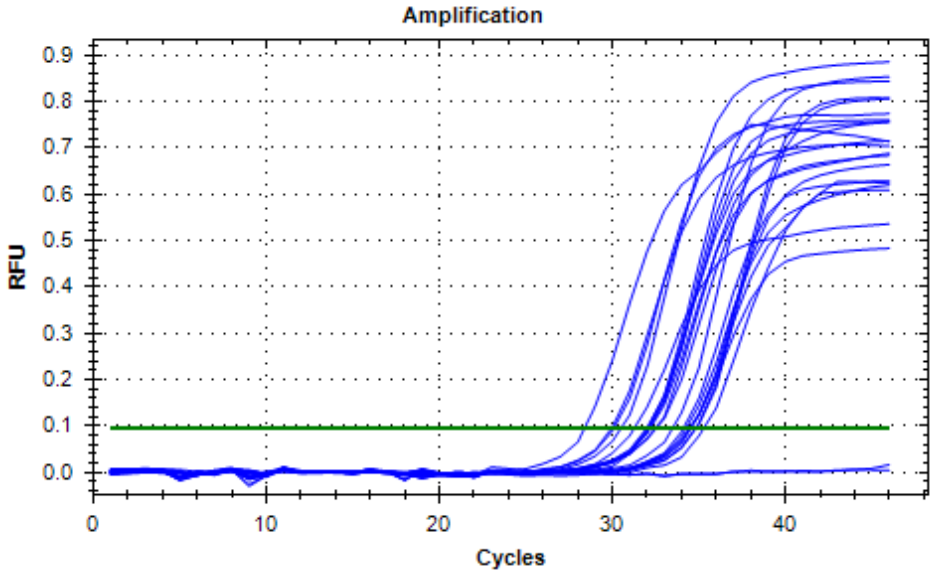

Fig.2: Amplification Plot of region GLE, in which the fluorescence of the dye SYBER Green represent samples of trophozoites DNA which located above theThreshold line while under the threshold there was no amplificated DNA that was isolated from the cysts.

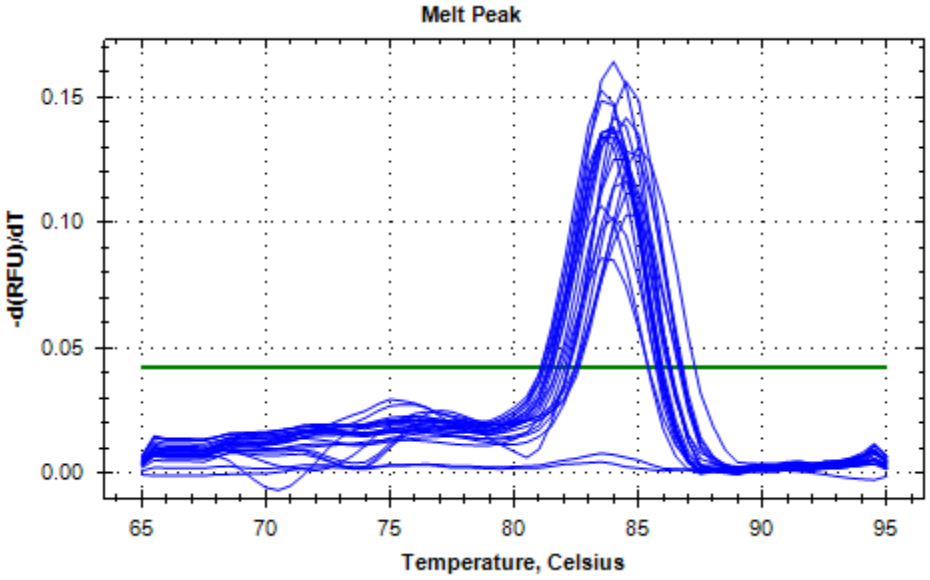

Fig.(3): Melt peak curves from real-time PCR amplification of GL E. (Positive samples shown above of Threshold, and negative samples shown under the threshold), are indicated in the curves.

Discussion

Giardiasis is one of the most common enteric parasites infections in human that have been reported worldwide. In America, Asia, and Africa about 200 million people diagnosed have symptomatic giardiasis, with at least 500,000 new infections mentioned each year (Stark *et al*. , 2007).

This study estimated the prevalence of *G. duodenalis* infections in children in Babylon and the highest rate of infection was among the males than the females.

Most epidemiological researches studies on *G. duodenalis* infection use traditional microscopic detection, because its low cost , furthermore decrease sensitivity of this test may result in the disregard of the correct prevalence of the parasite. In current our study, we used both classic microscope detection and one of molecular techniques (RT-PCR).

Hokelek & Nissen found highest prevalence of giardiasis in childhood. In the present research , the highest number of positive cases was found in the age group of 6-10 years and this finding is similar to a study done by Dawson, (2005) who showed that greater number of reported cases occurred among children aged 1-9 years. In Iran, the highly prevalence was showed in children and it recorded 26.2%.

Moreover, children are less bearing in mind some practice are very important personal hygiene practices such as washing of hand before eating and after using the toilets. Another reason focused on the habit of children to buy food streets whose

sellers some of whom do not care about proper personal hygiene and may be carried of infection of *Giardia* (Ayeh-Kumi *et al*., 2009). immune deficient of children also plays a role for elevated infection rate, it has been showed that children acquire immunity after the primary infection in early life, which effected in some protection in late life (Heresi *et al.* , 2000).

The pet animals like cats, dogs and different animals represent a possible source for infection as children used to play with them (Al-Mekhlafi *et al.*, 2005). The Prevalence rates of parasites among children in different countries are usually 2-5% but are up to 20-30% in the third world countries (Adem,2001). The parasite are much more widespread in children than in adults, especially in children under 10 years of age, the infection frequency is 2-7 times greater than in adults (Akram , 2000).

In consonance with other studies (Meyer, 1990 ; Hoge *et al.*, 1995 ; Klaus *et al.* , 2007), a high prevalence of *Giardia duodenalis* in asymptomatic patients appeared in the current study. So this study concluded that *Giardia duodenalis* infection presents sub-clinically or the parasites have limited pathogenicity. It could also imply that the majority of the persons in the district are asymptomatic carriers of a non-pathogenic strain. Even though they look identical when viewed through the microscope, there are different genotypes of *Giardia*. These genotypes were referred to as Assemblages.

In Bangladesh observed that zoonotic genotypes of *G. duodenalis* infection (predominantly Assemblage A) was correlated with diarrhea whilst the non-zoonotic genotypes *(*Assemblage B) infection was associated with asymptomatic infection (Haque *et al.*, 2005), they found that *Giardia duodenalis* assemblage B occurred at a significantly higher rate compared to the assemblage A. This study did not spectate between the *Giardia duodenalis* assemblages.

Giardia duodenalis is a common source of epidemic and endemic diarrhea in all world. Infection with *G.duodenalis* includes asymptomatic cyst pathway, acute self-limited diarrhea, and a chronic disorder including diarrhea, Lack of absorption and weight loss. Most cases are asymptomatic, but symptomatic infections involving repeated intestinal symptoms may interfere the normal growth and development in affected children .

Microscopy was consider the gold standard for detection of *Giardia duodenalis* infection. However, this technique is not very sensitive and time-consuming, During recent years, there have been significant advances in the development of molecular-diagnostic tools like the Polymerase chain Reactions(PCR) and the Real-time PCR, to comparison between both of them : the RT- PCR are more advantage: numerical results which are much easier to distinguish than the visualization of a stained gel in the conventional PCR ,there is no need to post-amplification analysis which reduces risks of contamination and lead to better differentiate of *Giardia*_ infections (Franzén *et al*. ,2009) .

In the current study We used a real-time PCR technique based on the aGL50803_17190 Eukaryotic of *G.duodenalis* for the specific recognition *G.duodenalis* stages and diagnosed the DNA in feces samples and proofed the possibility used this gene as diagnostic gene

The present study showed the value of melting temperature about 84 Celsius and these temperature referred to the DNA content from the base pairs (G,C content) ,whenever the G,C content was higher than the melting temperature elevated.

The real-time PCR was negative in all 100 fecal samples in which *G. duodenalis* cysts while *G. duodenalis* trophozoites contain positive in all of 100 fecal samples were detected with microscopic examination of concentrated fecal samples.

There are many studies were done in all world about the best technique for detection of *Giardia duodenalis* one of these study was the study in Egypt which designed to compared upon usual microscopy, direct immunofluorescence assay (DIF) and flow cytometry (FC) for the diagnosis of Giardia cyst in fecal samples**.** He was concluded that direct microscopic examination is benefit in the diagnosis of Giardia as a first choice test. DIF is an excellent technique in clinically suspected cases after negative microscopy. FC found to be less sensitive to obtain exacted organisms' count but it could be an effective alternative method for the detection of Giardia cysts, especially for comprehensive epidemiological studies or extensive surveillance programs as it has the beneficial attribute of rapidity and do not depend on microscope witness. (Etamadi *et al*. , 2011; El-Nahas *et al.* , 2013).

Another study was done by the researcher (Maha and Kevin , 2008) for the detection of the cyst and the oocyst of water born protozoa like the *G.duodenalis* by different techniques ones of them was the RT-PCR and it concluded that the RT-PCR remains the gold standard for the identification of many waterborne pathogens since it delivered specificity and sensitivity in detection (Moshira *et al*. , 2009).

Also there is a research was done in Egypt to diagnosed the *Giardia duodenalis* genotypes in human isolates with diarrhea by used of RT-PCR for selecting the triose phosphate isomerase (tpi) gene and the results showed that genotype *G. duodenalis* isolated from fresh fecal samples in Egypt, in infections commonly presented with intermittent diarrhea, the most prevalent genotype was assemblage A group I. The most fragility age group from ten to twenty year-old individuals (Tahmina *et al*., 2014).

Another study were occurred in Bangladesh to detection the *G.duodenalis* in children by used of different procedures including the microscope, a Real Time PCR and ELISA, the results showed the detection by microscopy is neither sensitive nor specific. In general, pathological Laboratories, detection usually done by simple microscopy where maximum negative results are reported. However, ELISA and PCR are time consumed methods and expensive; they definitely give more accurate results on sensitivity and specificity of the diarrheal parasites. A greater knowledge of parasitic contamination of the surroundings and its impact on health has required developing better-diagnosed methods for *G. duodenalis* (El-Nahas *et al.* , 2013).

In our study we depend on different gene GL50803_17190 Eukaryotic about it which the researches was rare ,so we used this gene to improve if It could be adopted as a diagnostic gene so we searched and used this gene as a target of RT-PCR in both stages (Trophozoites and cysts) and the results showed a high sensitive of this technique to detect this gene in trophozoite stage while it can not this gene be identified in cystic stage .

References

Adam, RD.(2001). Biology of Giardia duodenalis. Clin Microbiol Rev ; 14:447-475.

Akram, D.S. (2000).*Giardiasis* malabsorption and its effect on child health. Pak Pediatr J;24:155-60.

Al-Mekhlafi, H.M.; Azlin, M.B.; Aini, U.N.; Shaik, A.; Saiah, A. and Fatmah, M.S. (2005). Protein energy malnutrition and soil transmitted helminthiases among Organ Asli children in Selangor, Malaysia. Asta Pac J Clin;14:188-94.

Ayeh-Kumi, P. ; Quarcoo S.; Kwakye-Nuako, G.1; Kretchy, J.P1.; Osafo-Kantanka, A.1.; Mortu, S.(2009). Prevalence of Intestinal Parasitic Infections among Food Vendors in Accra, Ghana. J Trop Med Parasitol. 32:1-8.

Bibby-scientific Inc. (2014). Dissociation Curve Analysis, Technical Note T08-003A , bibby-scientific Inc.

Cacci,S and Ryan, U.(2008). Molecular epidemiology of giardiasis. Mol Biochem Parasitol. 160:75–80.

Conway, C.; Proudfoot, C.; Burton, P.; Barry, J. and McCulloch, R. (2002). Two pathways of homologous recombination in Trypanosoma brucei. Mol Microbiol 45: 1687–1700.

Davis-Hayman, S. and Nash,T. (2002). Review: Genetic transformation of parasitic organisms: Genetic manipulation of *Giardia lamblia*. Molecular & Biochemical Parasitology. 122(34) : 1-7

Etamadi, S. ; Zia-Ali, N.; Babai, Z.; Fasihi Harandi, M.; Zia-Ali, A.; Salari, Z. and Kamyabi, H.(2011). The correlation between clinical signs and genotypes of Giardia duodenalis isolated from patients with Giardiasis in Kerman city. J Kerman Univ Medi Sci. 18:330–338.

El-Nahas, H.A.; Salem, D.A.; El-Henawy, A.A.; El-Nimr, H.I.; Abdel-Ghaffar, H.A.; El-Meadawy, A.M.(2013).Giardia diagnostic methods in human fecal samples:a comparative study.Cytometry B Clin Cytom. 84(1):44-49.

Franzén, O.; Jerlström-Hultqvist, J.; Castro, E. (2009). Petri, William, ed. "Draft Genome Sequencing of *Giardia duodenalis* Assemblage B Isolate GS: Is Human Giardiasis Caused by Two Different Species?". PLoS Pathogens 5 (8): e1000560.

Gillin, F.D. ; Reiner, D.S. and McCaffery, J. M. (1996). Cell biology of the primitive eukaryote *Giardia lamblia*. Annu. Rev. Microbiol. 50, 679-705.

Gordts, B.; W.Hemelhof, K;Van Tilborgh, P.; Retoré, S.; Cadranel,S and J.-P. Butzler. (1985). Evaluation of a new method for routine in vitro cultivation of *Giardia duodenalis* from human duodenal fluid. J. Clin. Microbiol. 22:702-704.

Gordts, B. ; P. Retoré, S.; Cadranel, W. ;Hemelhof, M. Rahman and J.-P. Butzler. (1984). Routine culture of *Giardia duodenalis* trophozoites from human duodenal aspirates. Lancet ii:137-138.

Hala, A.; El-Nahas,D.; Dalia, A. ;Salem,G.; Abeer, A. ; El-Henawy,k.; Hosam ,I. ; El-Nimr,D.; Hasan, A.; Abdel-Ghaffar,B and Ali M. El-Meadawy.(2013). Giardia Diagnostic Methods in Human Fecal Samples: A Comparative Study. Cytometry Part B (Clinical Cytometry) 84B:44- 49.

Haque ,R; Roy, S.; Kabir, M.; Stroup, S.E.; Mondal, D.; Houpt, E.R. (2005).*Giardia* assemblage A infection and diarrhea in Bangladesh. J Infect Dis. 192:2171–2173.

Heresi ,G.P. ; Murphy ,J.R. ; Cleary, T.G. (2000). Giardiasis. *Seminars in Pediatric Infectious Diseases*, 11, 189-95

Heidari, A. and Rokni, M.B. (2003). Prevalence of intestinal parasites among children in day-care centers in damghan-Iran. Iran. J. Pub. Health, 32: 31-34

Helmy, M.M.; Abdel-Fattah, H.S. and Rashed,H. (2009). Real-time PCR/RFLP assay to detect *Giardia duodenalis* genotypes in human isolates with diarrhea in Egypt. J Parasitol. 95(4):1000-4.

Homan, W.L.; Gilsing, M.; Bentala, H.; Limper,L. and Van-Knapen, F.(1998). Characterization of *Giardia duodenalis* by polymerase chain reaction fingerprinting. Parasitol. Res; 84: 707-714.

Hoge, C.W.; Echeverria, P. and Rajah, R. (1995) Prevalence of Cyclospora species and other enteric pathogens among children less than 5 years of age in Nepal. Journal of Clinical Microbiology 33: 3058 -3060

Hokelek, M. and Nissen MD (2006). Giardiasis. eMedicine Last update May 2006. P : 1-14. Available from: http//giardia\emedicine. [cited in 2006].

Klaus, R.; Ignatius, R. ; Weitzel ,T. ; Seidu-Korkor, A. ;Anyidoho, L. ; Saad, E. ; Djie-Maletz, A.; Ziniel, P.; Amoo-Sakyi, F. ; Danikuu ,F. ; Danour, S. ; Otchwemah, R.N. ; Schreier, E.; Bienzle, U. ;Stark, K. and Mockenhaupt F.P. (2007) Acute childhood diarrhoea in northern Ghana:epidemiological, clinical and microbiological characteristics *BMC Infectious Diseases* 7, 1471-2334.

Life Technologies Corporation (2012). Real-time PCR handbook ,Life Technologies Corporation , PP:67. http://www. lifetechnologies.

Maha Bouzid, Dietmar Steverding and Kevin M Tyler.(2008) Detection and surveillance of waterborne protozoan parasites.Current Opinion in Biotechnology, 19:1–5.

Meyer, E. A. (1990). Introduction, p. 1-9. *In* E. A. Meyer (ed.), Giardiasis. Elsevier Publishing Co., New York, N.Y.

Morrison, HG ; McArthur, A.G.; Gillin, F.D.; Aley , S.B.; Adam, R.D.; Olsen G.J.; Best,A.A.; Cande,W.Z.; Chen,F; Cipriano,M.J.; Davids,B.J.; Dawson,C; Elm endorf ,H.G.; Hehl ,A.B., Holder, M.E.; Huse, S.M.; Kim, U.U., Lasek-Nesselquist E; Manning, G.; Nigam, A.; Nixon, J.E.; Palm, D.; Passamaneck, N.E.; Prabhu A; Reich, C.I.; Reiner, D.S.; Samuelson, J.; Svard, S.G.; Sogin M .(2007). Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*, Science. 28;317(5846):1921-6.

Moshira, M. F.; Helmy,C.; Hisham ,S. ; Abdel-Fattah, and Laila Rashed . (2009). REAL-TIME PCR/RFLP assay to detect *Giardia duodenalis* genotypes in human isolates with diarrhea in Egypt. J. Parasitol., 95(4), pp. 1–5.

Pestehchian, N. ; Rasekh, H.; Babaei, Z. ; Yousefi, H.A. ; Eskandarian, A.A; Kazemi ,M.and Akbari, M. (2012) Identification of genotypes of *Giardia duodenalis* human isolates in Isfahan, Iran, using polymerase chain reaction Restriction Fragment Length polymorphism. Adv. Biomed Res.

Silvia,Y.; Moya-Camarena, N. Sotelo and Mauro E. Vlencia.(2002). Effects of asymptomatic *Giardia duodenalis* infection on carbohydrate absorbtion in well-nourished Mexican children. *Am. J. Trop. Med. Hyg.*, 66(3), pp. 255–259.

Singh,N. ; Bhattacharya,A. and hattacharya, S. (2013). Homologous Recombination Occurs in Entamoeba and Is Enhanced during Growth Stress and Stage Conversion. Plos One, September 2013, Volume 8 , Issue 9, www.plosone.org .

Stark D; van Hal S; Marriott D; Ellis J; Harkness J (2007).Irritable bowel syndrome: a review on the role of intestinal protozoa and the importance of their detection and diagnosis.Int.J.Parasitol.37(1):11–20.doi:10.1016/j.ijpara.2006.09.009. PMID 17070814.

Tahmina Ahmed; Hamedh Khanum; Priyanka Barua; Tuhinur Arjui1; Muhammed Salah and Rashidul Haque.(2014).Detection of *Giardia duodenalis* in children by microscopy,ELISA and REAL TIME PCR assay. Dhaka Univ. J. Biol. Sci. 23(2): 197-204.

Thompson, R.(2004).The zoonotic significance and molecular epidemiology of Giardia and giardiasis. Vet Parasitol. 126:15–35.

Tungtrongchitr, A.; Sookrung, N.; Indrawattana ,N.; Kwangsi, S.; Ongrotchanakun J, Chaicumpa W.(2010). *Giardia duodenalis* in Thailand: Identification of genotypes. J Health Popul Nutr.28:42–42.