PCR technique for detection of *Giardia lamblia* in microscopically positive stool samples of the patients in Al-Diwaniyah hospital /Iraq.

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Abstract:

Giardia lamblia is one of the most common human parasites and causes a lengthily course of nonbacterial diarrhea. Disease outbreaks due to *Giardia* infection are often attributed to contaminated food and water supplies. A major problem associated with detection for this organism is the lack of sensitive and reliable methods. PCR has the potential to address many of the limitations. We have performed a PCR-based method for sensitive detection of *Giardia*. Because the sensitivity of PCR is a function of the efficiency of DNA extraction from parasite. *Giardia* samples were collected from stool of infected persons and first detected microscopically and the second step used of the PCR technique to ensure the infection,DNA was extracted and used of a 619 bp conserved fragment related to the DNA was extracted ,and the GL50803_17190 Eukaryotic peptide chain release factor subunit 1[*Giardia lamblia* ATCC 50803]gene was used as the target for PCR amplification which rarely used. The results suggest the potential utilities of PCR for sensitive detection of *G.Lamblia* which isolated from stool of infected persons with Giardiasis.

Key words: *Giardia lamblia*, PCR, GL ATCC50803 gene, Giardiasis

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Introduction:

Giardia lamblia is a flagellated protozoan parasite that colonizes and reproduces in the small intestine, causing giardiasis. The parasite attaches to the epithelium by a ventral adhesive disc, and reproduces via binary fission (1). Giardiasis does not spread via the bloodstream, nor does it spread to other parts of the gastrointestinal tract, but remains confined to the lumen of the small intestine (2). *Giardia* trophozoites the stage of organism that split and stained, its characteristic pattern resembles the familiar "smiley face" symbol (3). Chief pathways of human infection include ingestion of untreated sewage, a phenomenon particularly common in many developing countries (4) contamination of natural waters also occurs in water sheds where intensive grazing occurs. *Giardia* infects humans, but is also one of the most common parasites infecting cats, dogs and birds (5). *Giardia intestinalis* consists of seven genetically different assemblages, designated A to G, and an additional genetic variant was recently described in seals (6). All assemblages have similar morphology, and they cannot be differentiated by microscopy. Based on genetic and host-specific data, it has been proposed that assemblages A to G be re-classified as separate *Giardia* species (7). Mammalian hosts also include cattle, beavers, deer, and sheep (2). Intestinal protozoa account for a minority of cases of acute traveler's diarrhea, but they are common pathogens in travelers who experience protracted diarrhea during or after travel. Evaluation of the traveler with chronic diarrhea should include a careful examination for typical infecting organisms, such as *Giardia* and *Entamoeba* species, *Giardia* and the other diplomonads are unique in their possession of two nuclei that are similar in appearance, DNA content, transcription and time of replication (8). There are five chromosomes per the haploid genome. The genome has been sequenced and was published in 2007, although the sequence contains several gaps (9) .

The genome of the eukaryotic protest *Giardia lamblia* is compact in structure and content, contains few introns or mitochondrial relics, and has simplified machinery for DNA replication, transcription, RNA processing, and most metabolic pathways. Protein kinases comprise the single largest protein class and reflect Giardia's requirement for a complex signal transduction network for coordinating differentiation. Lateral gene transfer from bacterial and archaeal donors has shaped Giardia's genome, and previously unknown gene families, for example, cysteine-rich structural proteins, have been discovered. Unexpectedly, the genome shows little evidence of heterozygosity, supporting recent speculations that this organism is sexual. This genome sequence will not only be valuable for investigating the evolution of eukaryotes, but will also be applied to the search for new therapeutics for this parasite, The genome of *G. lamblia* WB clone C6 (ATCC50803) is ~11.7MB in size, distributed on five chromosomes. The edited draft genome sequence contains 306 contigs on 92 scaffolds (Supporting Online Material). The genome is compact. (10).

Routine examination of stool specimens collected on consecutive days or even within the recommended 10-day period may not confirm infection with this organism. Only 50% to 70% sensitivity has been reported by this technique (11).The low sensitivity is due to the irregular excretion of cysts or trophozoite and examination of stool samples by unskilled personnel (12).Other methods such as jejunal biopsy and duodenal aspirate examination give better results but they find little utility, as they are invasive. Immune diagnosis of the parasite based on detection of *Giardia* specific antibody in serum and antigens in the stool proved to be better diagnostic methods (13). However, the findings suffered from serious drawbacks pertaining to false positive results due to cross reactivity with other microorganisms. In the last few years, DNA pre based diagnosis of diseases is gaining importance. This system has the advantage over other diagnosis methods for being highly specific and at the same time sensitive. Recently, a number of DNA-based methods for detection of *Giardia* have been described (14).

The sensitivity of PCR detection is greater than the microscopic examination, making it of greater use for detection of low numbers of cysts in stool samples (15).The polymerase chain reaction has been employed for selective amplification of *Giardia* DNA using giardian gene as the amplification target (16). One of more important gens in *Giardia lamblia* named as ATCC 50803 (GL50803_17190 Eukaryotic peptide chain release factor subunit (11). Strain WB clone C6 genome was sequenced to 11.3 coverage using the whole genome shotgun method. It is 12 Mb in size and contains 6488 predicted open reading frames distributed throughout 5 chromosomes (chromosome polymorphism has been identified in different strains) and more than 13,100 predicted proteins. Karyotyping studies found chromosome sizes to range from 1.6 to over 3.8 Mb, This Whole Genome Shotgun sequencing project has been completed and deposited at DDBJ/EMBL/Gen Bank under the project accession AACB02000000 (12).

The number of studies related to the identification of *Giardia lamblia* in Iraq depending on PCR technique are limited. These studies have been conducted in few locations and identification was depended on the cysts morphology, necropsy and / or histopathologi - al findings.

In this study, we aimed to comparison between two techniques to identify the parasite *Giardia lamblia* causing Giardiasis in humans using traditional methods (microscope) and molecular technique based

on the polymerase chain reaction (PCR)**.**

Materials and Methods

1- Stool Sample collection

From April to Oct. 2015 a total of 150 samples of stool were collected from suspected infected patients with Giardiasis attending to the general hospital and clinics were spread in Al- Diwaniyah province, Iraq for the examination and treatment .The sample were isolated from stool of infected persons and collected in Eppendorf tubes and stored in freezing until used in DNA extraction(12).

2-Direct examination method (Microscopic examination):

The first step in the present study is identified the positive samples from whole samples that collected from suspected patients with Giardiasis. examined microscopically to determine the parasite either by direct methods in cases of acute diarrhea in which the samples examined either freshly, or used of a natural formalin buffer solution which concentrated 10% or by staining the samples with Iodine or Giemsa stain to made the internal components more clear.

3-Stool DNA Extraction

Genomic DNA was extracted from stool samples of patients by using AccuPrep® Stool Genomic DNA Extraction Kit (Bioneer, Korea**).**

4-DNA profile

For detection of DNA that extract from stool samples with a Nano drop spectrophotometer (THERMO. USA) for detection and measurement the concentration of nuclear acids (DNA and RNA), Where is detected DNA concentration (ng / µl) and measuring the purity of the DNA by reading the absorbance at a wavelength of between (280-260 nm) (13).

5-PCR- Protocols

The DNA samples which extracted from stool samples would use in thermal cycler machine to amply the DNA by using the sense primer 5- CGGCA AGAACA C CAAGGTTG -3 and TATTC CGAAACA antisense primer 5- GACGCGCT -3 with 619 BP which designed by NCBI site (Primer 3 plus program), according to the PCR program shown in the Table (1).

Which 50 µl of PCR master mix used for amplification of DNA.

Also (5µl) of DNA template that extracted from stool samples was added then 1.5 µl of each type of Primers(forward and reverse)added to the master mix and then blend well using Exispin vortex centrifuge ,then this tubes would transferred to the thermocycler machine .The PCR products were electrophoresed in agarose gel and visualized on UV translluminator and then photographed using photo documination.

PCR technique Table (1): Thermal cycler program of technique.

Step	Temperature and duration	
Initial denaturation	95°C for 4 min	
Denaturation	94°C for 30sec	30 cycles
Annealing	59°C for 30 sec	
Elongation	72°C for 1 min	
Final elongation	72°C for 5 min	

Results

Initial examination of stool (150 samples) that collected from patients suspected infected with Giardiasis by using of traditional techniques, which include clinical, macroscopic and microscopic examination and the results showed 120 (80%) samples were positive The stool samples were collected from both genders and deferent ages as shown in table (2, 3).

Table (2): Microscopic examination of the parasite *G.lamblia* according to gender.

Sex	Negative	positive	Percentage
Male	12	66	44%
Female	18	54	36%
Total	**30**	**120**	**80%**

Table (3): Microscopic examination of the parasite *G.lamblia* according to age.

Age category (years)	*Total number*	*Number of positive Patients*	*Percentage %*
≤ 10	*59*	*44*	*36.6%*
> 10	***91***	***76***	***63.3%***
Total	***150***	***120***	***80%***

Then the 150 stool samples were tested by conventional PCR assay in which the DNA extracted from stool which collected from patients that clinically suspected had Giardiasis, only 135 samples (90%) which appeared positive and identified the species *Giardia lamblia* with 619 bp PCR product of Eukaryotic peptide chain release factor subunit 1 in Al- Diwaniyah province in Iraq (Figure 1).

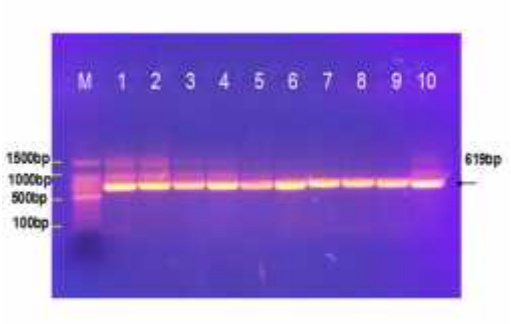

Figure (1): Agarose gel electrophosis show the PCR product results for *Giardia lamblia* of Eukaryotic peptide chain release factor subunit 1 where M: 1500bp ladder, Lane (1-10) are 525 pb positive samples.

Discussion:

The results of current study showed altitude in infection with intestinal parasites in recent years especially with *Giardia lamblia* even there are improvement in diagnostic, therapeutic and preventive methods.

Diagnosis of *Giardia lamblia* by conventional microscopic methods following the application of faecal concentration techniques, especially Zinc sulphate flotation and centrifugation remains a relatively reliable indicator of infection (Zajac *et al*., 2002).The detection of *Giardia* by microscopy or faecal ELISA is of limited epidemiological value. The development of the direct immunefloure- ce microscopy has improved the sensitivity of detecting and quantitating the feacal *Giardia* cysts and more accurate prevalence rate and cysts excretion intensities as compared to the conventional microscopy

(17).

There is need for a sensitive and specific diagnostic procedure for detecting the etiological agent of infectious disease, with *Giardia*, molecular techniques particularly PCR based procedures have greater sensitivity and specificity than the conventional diagnosis that are reliant on microscopy or immune diagnosis (18). One of major advantage of PCR based techniques is the ease of interpretation which usually involves the visualization of small number of bands on a gel , A greater awareness of parasite contamination of the environment and its impact on health has precipitated the development of better detection

methods for water borne pathogens such as *Giardia* (19). In addition, molecular techniques can provide genotypic characterization of the parasites isolated from the faecal sample or water, thus providing valuable data for determining the source of contamination (18).

Many studies about the Giardiasis were done in Iraqi provinces like the study in Baghdad which showed infection percentage 17.7%,in Kerbala 17.8%,in al-Najaf about 14.8%,while the infection with Giardiasis in Babylon 12.72% (20). Also there are many studies were done in neighboring countries like the study in Al- Kuwait showed the percentage of infection with Giardiasis 3.2% (21).While a study was done in Iranian health center about the prevalence of some intestinal parasites and in which the results showed the infection percentage 19.3% (22).

Timely diagnosis by appropriate technique is a prerequisite for quick and effective therapy of *G. lamblia* was diagnosed conventionally disease. by micro- scopic examination or by antigen or antibody detection. Both the methods revealed its limitations. In recent years, DNA based diagnosis and especially, amplification of functional genes by polymerase chain reaction has revolu- tionized the fields of biological and medical sciences because of its power to produce many copies of a desired, previously undetectable nucleic acid target. However, before that the technique must be optimized for the extraction and amplification of template DNA from heterogeneous human body fluids and especially from stool (8). Atlas in 1991 used the polymerase chain reaction to selectively amplify the giardian gene for detection of *G. lamblia* from water. However, the test was not found highly sensitive, probably due to low copy number of giardian gene (23).

In the last few years the rRNA gene has been selected as the target for detection by PCR in many studies related with diagnosis of *G.lamblia* over the world, in which the SSrRNA gene of *Giardia* has been used as the target for detection (24). In contrast, sequence variability at the intergenic regions of rDNA has been demonstrated among the inter and intraspe- cies (25).

Recently, different groups successfully used the intragenic regions of rRNA gene to distinguish different isolates of a number of prokaryotes and eukaryotes.also,a research was done to show the relation between the genotyping of Giardia and diarrhea in Iranian population depending on PCR techniques and for genotyping of *G. lamblia* are based on polymorphic genes encoding 18S rRNA, glutamate dehydrog- enase (gdh), elongation factor 1-alpha (ef1-α), triose phosphate isomerase (tpi), and β-giardin (8).

Several studies was done about the *Giardia lamblia* one of them the study in China which depend on PCR technique to detection of three zoonotic parasites *Ancylostoma ceylanicum*, *A. caninum*, and *Giardia lamblia* Assemblage A , Three pairs of specific primers were designed based on ITS sequence of *A. ceylanicum* and *A. caninum* and TPI gene of *G. lamblia*, It is concluded that the established multiplex PCR assay is a convenient, rapid, cost-effective, and high-efficiency method for molecular detection and epidemiologic- al investigation of three zoonotic parasites (26).

Here, we used the unusual target for PCR detection known as GL50803_17190 Eukaryotic peptide chain release factor subunit 1 as target of PCR technique to detect the *G.lamblia* in the patients were suspected infected with Giardiasis and the PCR product identified at 619bp.

In our study out of 150 samples were collected from suspected Giardiasis only 120 samples were positive and our study showed no significant different between ages of patients and no different between males and females.

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استخدام تقنية فحص السلسلة البوليميرية في الكشف عن طفيلي الجيارديا لامبليا في عينات البراز الإيجابية مجهريا المعزولة من المرضى في مستشفى الديوانية /العراق.

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الخلاصة:

يعد طفيلي الجيارديا لامبليا واحد من أكثر الطفيليات المعوية انتشارا واحد المسببات الغير البكتيرية للإسهال. الإصابة بهذا الطفيلي ناجمة غالبا من شرب المياه وتناول الغذاء الملوث بالأطوار المكيسة لهذا الطفيلي. المشكلة الأساسية التي تواجه المختصين هي غياب او ضعف الطرق التشخيصية الدقيقة والحساسة للكشف عن الإصابة بطفيلي الجيارديا. تعد تقنية تفاعل السلسلة البوليميرية (PCR) واحدة من أكثر التقنيات الحديثة دقة وحساسية للكشف عن الإصابة بطفيلي الجيارديا لامبليا اعتمادا على استخلاص دنا الطفيلي. جمعت عينات البراز من المرضى المشكوك بإصابتهم بالجيارديا وفحصت مجهريا ثم استخدمت عينات البراز في نوع اخر من التقنيات وهي تقنية تفاعل السلسلة البوليميرية حيث استخلص دنا الجيارديا واستخدم الجين GL ATCC 50803 كهدف للتضخيم بتقنية PCR. واثبتت الدراسة الحالية ان تقنية PCR أكثر دقة وحساسية مقارنة مع الفحص المجهري.

الكلمات المفتاحية

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