# A COMPARATIVE STUDY OF GIARDIA LAMBLIA DETECTION BY USING OF MICROSCOPIC, SEROLOGIC, AND MOLECULAR METHODS, IN SULAIMANI PROVINCE

Safin Hamad Ismail [a] and Shahnaz AbdulKadir Ali [b]

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## ABSTRACT

### *Background*

*Giardia lamblia* is an intestinal flagellated protozoan parasite that infects humans and animals. Giardiasis causing more than 200 million symptomatic infections globally it is one of the most common causes of diarrhea in developing countries, and frequent cause water-born/food-born parasitic diseases.

### *Objectives*

This study was carried out to investigate the prevalence rate of *G. lamblia* in Sulaimani Province hospitals and to identify the best and accurate method for identification.

### *Patients and Methods*

In the present study, 355 fecal specimens were collected from patients in three hospitals (Dr. Jamal A. Rashid Pediatric Teaching Hospitals, Ibrahim Pasha Health Center and Directorate of Preventive Health) in Sulaimani Province during the 1st November 2018 to the 30 April 2019. All samples examined by direct microscopic examination using Saline Wet Mount, and antigen detection by ELISA technique (RIADASCREEN *Giardia* ELISA kit). DNA extracted by used (QIAamp Fast DNA Stool Mini Kit 50), and for PCR amplification, used JW1, the forward primer: 5' GCG CAC CAG GAATGT CTT GT 3' and JW2, the reverse primer 5' TCA CCT ACG GAT ACC TTG TT 3' to amplify a 183-bp region of the 18S *G. lamblia* rRNA gene.

### *Results*

Out of 355 stool samples, fifty samples were positive for *G. lamblia* at a prevalence rate of (14.1%) by Direct wet mount and ELISA, and forty of them were positive (11.4%) by PCR. Males showed a higher (18.1%) prevalence rate than females (5.4%). The highest rate (26.9%) of infection was found in (13-18) age groups, while the lowest rate (2.3%) was fond in (6-12) year of ages. The prevalence rate in rural area was higher than urban area (15.7%), (13.4%) respectively. The sequences alignments were 91.30% and 98.52% similar to M90523.1 and M90524.1 respectively. In comparison to Microscopic exam, both ELISA and PCR recorded the same specificity rate 99.67% as direct wet mount, but were different sensitivity rate 100% and 80% respectively.

### *Conclusion*

Direct wet mount and ELISA was more sensitive than PCR (80%), but they were specific at the same rate (99.67%), and this indicate that there is more than one gene of *G. lamblia* is endemic in Sulaimani Province.

**Keywords:** *Giardia; Direct wet mount; ELISA; PCR; Comparison.*

[a] Ministry of Health, Sulaimani Directorate of Health, Public Health Laboratory, Kurdistan Region, Iraq.
[b] Department of Microbiology, College of Medicine, University of Sulaimani, Kurdistan Region, Iraq.
Correspondences: safinhamadbsc@gmail.com

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## INTRODUCTION

*Giardia lamblia*, synonymous *Giardia doudenalis*, and *Giardia intestinalis*, is an intestinal flagellated protozoan parasite that infects humans and several animal species. *G. lamblia* belonging to the *Giardia* genus that commonly causes diarrheal disease throughout the world [1]. *G. lamblia* is more common in undeveloped countries, and responsible for 500,000 new cases every year in Asia, Africa, and Latin America [2]. About two hundred eighty million are presently estimated to be infected with *Giardia* each year throughout the world [3]. Because of *G. lamblia* high prevalence and major impact on the patient's quality of life, giardiasis has become one of the most important public health problems and can be debilitating for healthy individuals that result in significant morbidity, especially in children [4].

Transmission of *G. lamblia* generally occurs by the fecal-oral route through the ingestion of food, water, or recreational water contaminated with cysts. Also, there have been some waterborne/foodborne outbreaks of *G. lamblia*. It is also transmitted by person to person contact, in areas where poor sanitary conditions are common [5].

In developing countries prevalence rate was 10-30% in young children, while in developed countries is 2-3% infections occur mostly in persons living in closed communities, homosexual men, immigrants, and travelers returning from highly endemic countries were 2-5% of symptomatic patients [6]. *G. lamblia* complex is found in the intestine and can infect a wide variety of vertebrate hosts. This complex is morphologically similar, but genetically with some different assemblages. Isolates of *G. lamblia* are classified in to eight assemblages A-H, based on the molecular characterization of *Giardia* such as: glutamate dehydrogenase (gdh), elongation factor 1-alpha (ef1-α), small-subunit ribosomal RNA (ssu rRNA), beta giardin (g), and triose phosphate isomerase (tpi) genes etc. These assemblages include A, B, C, D, E, F, G, and H [7]. Some assemblages of *G. lamblia* infect a wide range of mammal hosts, whereas others are restricted to cats or dogs, and some are known to infect only one host species [8]. *Giardia* has two stages, cysts and trophozoites. The cystic stage is infectious and ingesting a few as 10 cysts is sufficient to acquire the disease. Mainly infections in asymptomatic/carrier, but some clinical symptoms such as vomiting, anorexia, diarrhea, flatulence, abdominal pain, greasy stools, and nausea may occur in acute and chronic gastrointestinal infections [2].

There are different methods used to detect *G. lamblia* cysts and trophozoites. Currently, the microscopic method on fresh and concentrated fecal specimens is still commonly utilized. In addition, immunoenzymatic and molecular methods are also available for routine diagnosis and research training. Antigen detection methods are fast, easy to perform, and more sensitive compared to microscopic exam, but they have some disadvantages such as the need for certain reagents and high cost compared to microscopy [9]. Recently, molecular techniques were developed and shown to be more efficient and sensitive for the detection of cysts and trophozoites. PCR technique is an example of a recent advancement, it is also beneficial for short analysis time and reduce the risk of contamination [2]. This study was designed to compare three methods: direct saline wet mount with a commercially available stool antigen detection enzyme-linked immunosorbent assay (ELISA) kit and (PCR) technique for the detection of *G. lamblia* in human stool specimens, and choose which one is the best and accurate.

## MATERIALS AND METHODS

### *Specimen collection and microscopic examination*

The study was carried out in the research laboratories of the Microbiology department/College of Medicine / Sulaimani University and Central Health laboratory/ Sulaimani. Samples were collected from the 1st November 2018 to the 30 April 2019. A total of 355 fresh stool samples were collected from symptomatic and asymptomatic/carrier, inpatient and outpatients attend the laboratory of three hospitals in Sulaimani Province. A questionnaire list was arranged for information's of each patient before the collection of specimens. Each specimen was divided into three parts for microscopic exam, ELISA, and PCR. The first fresh portion immediately was used to prepare slides for a direct wet mount examination. Slides were screened at 10X and 40 X magnifications for the presence of cysts and trophozoites of *G. lamblia*. Two other stool samples were stored at -20°C without any preservative for performing ELISA and PCR later, each test repeated three times for each specimen.

### *ELISA by RIDASCREEN Giardia test*

Fecal samples were subjected to three rapid freeze-thaw cycles before the application.

The RIDASCREEN® Giardia test (R-Biopharm AG, Darmstadt, Germany) is an enzyme immunoassay based on the detection of antigens of *G. lamblia* cyst and trophozoites in stool specimen. The thawed stool samples (100 mg) were mixed with 1 ml of sample dilution buffer and centrifuged at 5000 rpm for 5 min. The supernatant was taken for further tests. 100 µl of stool suspensions were pipetted in the microwells along with 100 µl each of positive and negative controls provided by the manufacturer and from this step was carried out in an automated ELISA system DiaSorin ETI-max3000. Measure the extinction at (450/620 nm). Values above 0.156 were considered as positive.

### *DNA extraction and PCR Amplification*

DNA was extracted from frozen stool samples of about 180-220 mg using the QiaAmp Fast DNA stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. DNA (elute) samples were stored at -20°C until PCR was performed. The sequences of the oligonucleotides used as primers to amplify a 183-bp region of the 18s *G. lamblia* rRNA gene are JW1, the forward primer: 5' GCGCACCAGGAATGTCTTGT 3' and JW2, the reverse primer: 5' TCACCTACGGATACCTTGTT 3' [(10)]. Amplification was conducted in a reaction volume of 20µ as manufacturers' instructions (macrogen, Korea). For each sample 2µ DNA template, 1µ forward and 1µ reverse primer with 10µ master mix with ddH2O was added to make up the volume (Genet Bio). DNA amplification carried out using thermal cycler (Techne, USA). The cycling parameters for the reactions were as followed by 50 cycles: 95°C for 5 minutes (initial denaturation), of 95°C for 1 minute (denaturation), 58°C for 1 minute (annealing), 72°C for 1 minute (extension) and then a final extension at 72°C for 5 minute followed by a cooling period of 30s at 40°C.

### Statistical analysis

Data were analyzed by using Statistical Package for the Social Sciences (SPSS) (version 23.0). Cross sectional analysis and frequency calculations have been done for the variables. Pearson's Chi-squared ($\chi$ 2) and Fisher's Exact Test were used to assess the significance of the response and P-value < 0.05 was considered statistically significant.

## RESULTS

The results of the study showed that out of 355 samples only 50 were positive with *G. lamblia* at a rate of infection 14.1% as shown in (Table 1) .

It was clear that 44 males were positive at a prevalence rate of 18.1%, while 6 females were positive at a prevalence rate of 5.4%, with a significant difference between both sexes ($P < 0.05$), (Table 2).

The prevalence rate among (13-18 years) age group recorded the highest rate (26.9%) while the age group of (6-12 years) was the lowest (2.3%), with a significant difference between the age groups ($P < 0.05$), Table 3.

According to locations there was no significant difference between the rate of infection among the patient of urban areas (13.7%) and the patient of rural areas (15.4 %) ($P > 0.05$), Table 4.

### Microscopic Examination

Using direct wet mount by saline detected the cysts that appear as a large oval shape transparent with some thread like granules and a basal body and dark points resembles the nucleus of a *G. lamblia*, (Figure 1).

### Immunological identification of *G. lamblia* by used ELISA technique

ELISA test cleared that the positive samples appeared as deep yellow while the negatives were colorless, and equivocal samples as pale yellow see according to Calculating the Cut-off such as this formula: Cut-off = extinction for the negative control + 0.15, (Figure 2).

### Molecular identification of *G. lamblia* isolates from stool samples

From the result of examined samples of *G. lamblia* isolates by PCR, forty samples were successfully amplified as *G. lamblia* at band size 183 base pairs (Figure 3).

The results of using the three methods for detection showed that the higher rate of infection was 14.1% recorded by both direct wet mount examination and ELISA technique, while by PCR technique was 11.2% less than the other methods used. (Table 5)

### *Sequencing results*

The results of the PCR sequencing product of *G. lamblia* which obtained 183bp were sequenced in both

forward and reverse direction in Macrogene Company. Then they were blasted in NCBI (National Center for Biotechnology Information) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that there were different mutation sites noticed in the sequenced samples. The sequences obtained in the present study alignment were 91.30% and 98.52% similar to M90523.1 and M90524.1 respectively.

### *Sensitivity and Specificity*

The comparison between the results of using three tests revealed that direct wet mount and ELISA method recorded the highest sensitivity (100%), while PCR recorded the lowest sensitivity (80.00%), all methods has the same specificity (99.67%) ,Table 6.

**Table 1. *G. lamblia* infection in Sulaimani hospitals by Direct wet mount examination.**

| No of examined sample | Positive samples | |
|---|---|---|
| | No. | % |
| 355 | 50 | 14.1 |

**Table 2. The rate of *G. lamblia* infection between the genders.**

| Gender | Examined samples | The result of DWM test | | P. Value |
|---|---|---|---|---|
| | | Positive for *G. lamblia* | | |
| | No. | No. | % | |
| **Male** | 243 | 44 | 18.1 | < 0.05 |
| **Female** | 112 | 6 | 5.4 | |
| **Total** | 355 | 50 | 14.1 | |

**Table 3. Infection rate of *G. lamblia* according to the age groups.**

| Age (years) | | Frequency | The result of DWM test | | P value |
|---|---|---|---|---|---|
| | | | Positive for *G. lamblia* | | |
| | | | No. | % | |
| **1** | **< 5** | 64 | 3 | 6.3 | < 0.05 |
| **2** | **6-12** | 42 | 1 | 2.3 | |
| **3** | **13-18** | 19 | 7 | 26.9 | |
| **4** | **19-25** | 73 | 20 | 21.5 | |
| **5** | **26-35** | 60 | 10 | 14.3 | |
| **6** | **> 35** | 47 | 9 | 16.1 | |
| **Total** | | 355 | 50 | 14.1 | |

**Table 4. *G. lamblia* infection rate related to the patient's places.**

| Place | Frequency | The result of DWM test Positive for *G. lamblia* | | P value |
|---|---|---|---|---|
| | | No. | % | |
| **Urban** | 277 | 38 | 13.7 | > 0.05 |
| **Rural** | 78 | 12 | 15.4 | |
| **Total** | 355 | 50 | 14.1 | |

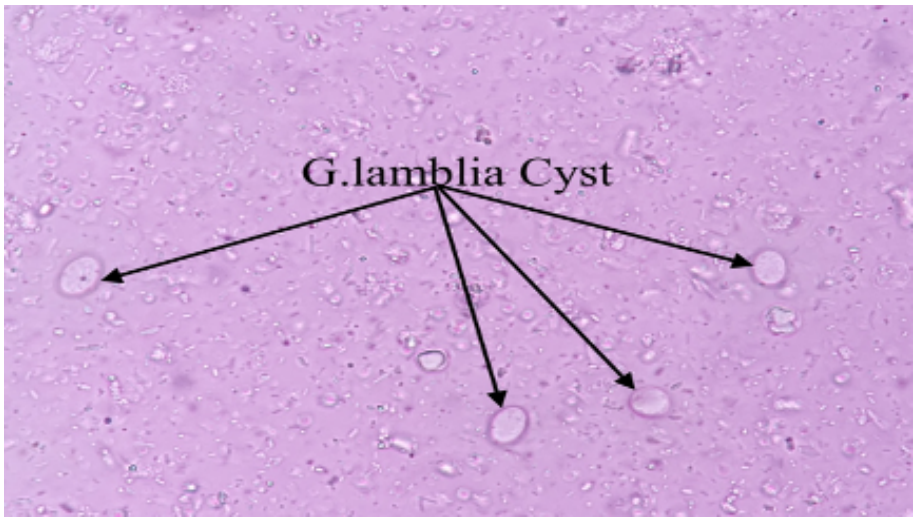


**Figure 1. *G. lamblia* cyst by direct saline wet mount examination with 40X magnification**

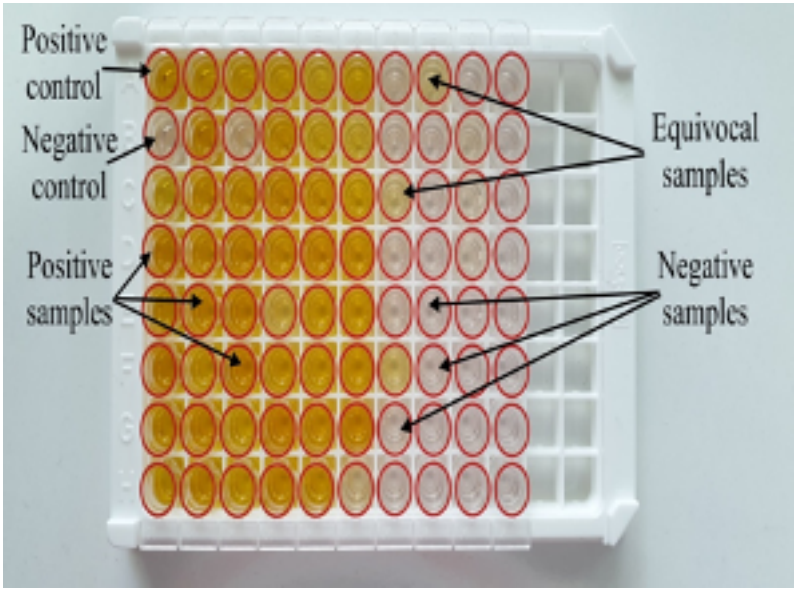


**Figure 2.Enzyme linked immunosorbent assay (ELISA) test detected *G. lamblia* antigen.**

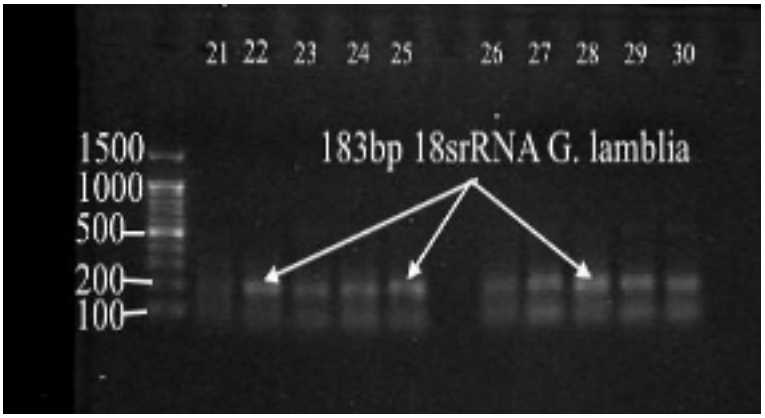


**Figure 3 An ethidium bromide-stained agarose gel (2%) electrophoregram of amplified PCR products for the identification of G. lamblia. (Left Lane is the molecular weight marker [1500bp ladder], and samples.**

**Table 5. Comparison between efficacies of different methods for diagnosis of *G.lamblia* infection in Sulaimani Hospitals.**

| Test methods | No. Of examined samples | The results No. Of positives | |
|---|---|---|---|
| | | No. | % |
| **Direct wet mount** | 355 | 50 | 14.1 |
| **Elisa technique** | 355 | 50 | 14.1 |
| **PCR technique** | 355 | 40 | 11.2 |

**Table 6. Specificity, sensitivity, positive and negative predictive value of methods used in diagnosis.**

| Test | Sensitivity % | Specificity % | Positive predictive value % | Negative predictive value % |
|---|---|---|---|---|
| **Direct Wet Mount** | 100 | 99.67 | 98.04 | 100 |
| **ELISA Test** | 100 | 99.67 | 98.04 | 100 |
| **Polymerase Chain Reactions** | 80.00 | 99.67 | 97.56 | 96.90 |

## DISCUSSION

*Giardia lamblia* is one of the most common human parasites that cause giardiasis. The infection is cosmopolitan and endemic in areas where there are lower morals of personal hygiene and lacking good sanitation. Transmission is either direct (fecal-oral route) or indirect (ingestion of contaminated water or food) [11].

The results of the current study showed that the total prevalence rate of *G. lamblia* in patients attending the Hospital in Sulaimani Province was (14.1%). This indicates that Sulaimani Province is endemic with this parasite; the endemicity may be due to the distribution of this parasite through contaminated food and water with the cystic stage of this parasite. This result was higher than those recorded in Duhok city (9.5%), Erbil city (5.7%) and in Kirkuk city (9.35%) respectively [12, 13].

Most of the patients in this study were asymptomatic/ carrier, and this may be because of several samples were collected in the cold and rainy seasons. The data of the present study recorded a significant difference ($P < 0.05$) between males (18.1%) and females (6.4%) prevalence rate, and this may be due to that males possibly are more exposed to *G. lamblia* cysts because of occupation and dealing with contaminated food and water outside home than females. This result agrees with the result [14], and disagree with [15, 16].

The data of age group infection showed a highly significant difference among the age groups in this study. The higher infection rate was found in the (13-18) age group (26.9%) this may be because of that this group works along the day in the restaurants and cafés and contacts with a different group of people, usually, this group maybe asymptomatic/carrier patients, while the lowest rate (2.3%) was recorded in age group (6-12) with significant difference between the age groups ($p< 0.05$). This result disagrees with the results found in Egypt by [17] , and in Portugal [18].

The present study revealed that adults and children over 12 years old (13 to 35 years old) were significantly associated with higher *Giardia* infection rates when compared to children under 13 years old, and this is in disagreement with the study of [15].

There was no significant difference between the patients from urban area (13.7%) and the patients from rural area (15.4%) as this study revealed ($P > 0.05$), this may be due to that both populations of both locations use the good water supplies and of increasing awareness in preventing themselves from being infected. This result agrees of {[14], [19] and [20]}.

Also, the results of using direct wet mount by saline showed a high rate of infection (14.1%), this may be because of its most easily recognized intestinal protozoan in both trophozoite and cystic stages. In most of the positive cases, cysts were more detected than trophozoite this maybe returns to that most of the patients were asymptomatic/carrier. Traditionally, wet mount microscopy used for routine analysis and it's used as the first choice due to it is cheap and easy to perform. This result was similar to the result recorded in Kirkuk city [12].

Our study revealed that using enzyme-linked immunosorbent assay (ELISA) recorded (14.1%) prevalence rate, and this was similar to the rate found by a direct wet mount with high sensitivity and specificity. Generally, this technique provides over 90% sensitivity and specificity. It is practical when numerous samples should be screened. Because it is more practical to analyze large numbers of samples in one run and for the reason the ELISA technique could replace microscopic examination when giardiasis is the most likely diagnosis [21].

Because of the similarity of the results recorded by direct wet mount and ELISA with no false negative and false positive by ELISA, this result disagrees with the results obtained in Duhok City-Iraq by [22], and disagrees with the result given in Turkey by [2].

The results of using PCR recorded a lower rate of infection 11.2% as compared with both direct wet mount and ELISA method. The reasons for this result could be due to the nature of DNA extracted and PCR as well, or maybe to the amount of stool used for DNA extraction that was less than 250 mg which may be in some cases do not contain enough cysts or trophozoites. In addition, the freezing and thawing process may cause parasite DNA degradation or completely lysed which couldn't be detected in this stage, probably the freezing for long period of time may lead to degradation of the DNA of *G. lamblia*, or to that there was more than one type of genes for *G. lamblia*. This result was similar to the result recorded by [17], and disagree with the result found by [14].

In addition to the type of gene used for amplification (18S rRNA) gene, also stool samples may contain PCR

inhibitors, such as bilirubin, bile salts, hemoglobin, phenolic compounds, and complex polysaccharides, which are co-purified together during DNA extraction, it also has enormous quantity of non-specific DNA and the potential for low numbers of cysts [23]. Moreover, the DNA region between the PCR primers and the genomic sequences could be degraded or there were nucleotide mismatches that may cause a strong reduction or even a lack of amplification. It may have needed another type of gene for these ten negative samples to give positive results.

From comparing the results of the sequences gained from the samples with other sequences from the gene bank using the NCBI Blast Local Alignment Search Tool (BLAST) there was similarities between the gene of locally isolated sequences and other sequences recorded in NCBI. The sequences obtained in the present study alignment were 91.30% and 98.52% similar to M90523.1 and M90524.1 respectively [10].

In conclusion, the use of different diagnostic techniques together would increase the chances of obtaining true positives. ELISA test recorded a higher sensitivity rate compared to using direct wet mount as a standard method then followed by PCR. Also the results of comparing the three methods used in this study showed that the three methods were specific at the same level, but they were different in sensitivity level.

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