Evaluation of the Employment of Four Laboratory Diagnostic Methods in Detecting of *Giardia lamblia* among Children in Kirkuk City.

⁎*Yahya Jirjees Salman, ⁎⁎Maha Ismaiel Mussttafa*

⁎ *Department of Microbiology/ College of Medicine /Kirkuk University.*

⁎⁎ *Department of Biology/ College of Science /Kirkuk University.*

Abstract:

Background: Giardiasis is the most common cause of diarrhea among people in Iraq, caused by *Giardia lamblia* parasite. Laboratory diagnosis requires diversity usage of method to reveal the parasite with in different types of specimens.
A*im:* The aim of this study were to determine the prevalence of giardiasis in Kirkuk city, to assess the efficacy of four different methods employee in detecting Giardia parasite and an attempt to extract and amplify DNA of this parasite using mixed primers of Giardia assemblages A and B.
Material and Methods Cross sectional study was carried on a total of 310 stool samples were collected and tested for giardiasis by using direct microscopy, ELISA-corpo-antigen, Lateral immune-chromatography assay (Triage panel) and PCR technique.
Results: The overall rate of parasitic infection was (51.93%); *Giardia lamblia* rate was (20.32%). Giardiasis among males was higher than in females. Traige panel show high efficacy for detecting *Giardia lamblia* than detecting of *Entamoeba histolytica* and cryptosporidium. Statistically the differences among direct microscopy, ELISA and Triage panel were not significant. Application of PCR single step technique show high rate of sensitivity than other methods in detecting giardiasis. Amplified Giardia genome length extended from 280 to 750 bps with mean of 437.6 bps.
Conclusions: Giardiasis among peoples in Kirkuk city was high especially among males. Triage panel and ELISA were simple and easy, but were less sensitive than conventional microscopy methods. PCR technique using k 725 gene (Mixed primers of assemblages A1, A2 and B) loci was performed for the first time in Kirkuk city with high sensitivity and specificity than other laboratory methods.
Key words: Giardiasis, Cryptosporidium, ELISA, Triage panel, PCR, Sensitivity.

Introduction:

Giardia intestinalis (also known as *G. lamblia, G. duodenalis*) is the most commonly diagnosed protozoan worldwide causing non-bacterial diarrhea [(1)]. It is a complex species with similar morphologic characteristics but with phenotypic and genotypic heterogeneity [(2)]. In recent years; genotypic classification has been applied for the identification of this parasite [(3)]. Giardia genotypes A and B have been isolated in human samples and show biological and pathogenic differences [(4)]. In the amplified fecal samples of the isolates obtained, zoonotic genotypes A II and B were detected, the latter being predominant. These results match the global

predominance of genotype B [5]. Children with genotype-B infection were reported to release more cysts than those infected with genotype A [6]. Diagnosis of Giardia by conventional microscopic methods following the application of fecal concentration techniques, especially zinc sulphate flotation and centrifugation remains a relatively reliable indicator of infection [7]. The detection of Giardia by microscopy or fecal ELISA is of limited epidemiological value. The development of the rapid lateral immune-chromatography assay Triage panel improved the sensitivity of detecting and quantitating the fecal Giardia cysts and more accurate prevalence rate and cysts excretion intensities as compared to the conventional microscopy. 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 [8]. One of major advantage of PCR based techniques is the eases of interpretation which usually involves the visualization of small number of bands on a gel [9]. The prevalence of *Giardia duodenalis* genotypes was determined in adult dairy cows. Specimens, cleaned of fecal debris and concentrated using (CsCl) density gradient centrifugation, were subjected to PCR and DNA sequence analysis. *G. duodenalis* infection, the prevalence was ranged from (3%) to (64%), with an average prevalence of (27%). DNA sequence analysis of the 16S rRNA gene revealed the presence of Assemblage A (2%) and Assemblage E (25%) in *G. duodenalis* infection [10].

The goals of this study, first is to evaluate the employee of direct microscopy, lateral immune-chromatography assay, ELISA-corpo-antigen with PCR usage for detecting *Giardia lamblia* in stool samples in Kirkuk city. The second aim is to extract *Giardia lamblia* DNA from stool samples and to detect purity and genomic mass of the Giardia parasite.

Materials and Methods:

In total, 310 fresh stool specimens were tested at the Medical Research laboratory – Kirkuk College of Medicine. The samples were obtained from patients attending the private clinics and medical labs in Kirkuk city who presented with abdominal symptoms, mainly diarrhea. Prior to processing complete information were reported in a special questionnaire prepared for this purpose. One aliquot of each sample was immediately examined using direct wet preparations of lugols iodine (1%) and (0.85%) of NaCl for detecting motility of Giardia trophozoites and other intestinal protozoan parasites. The residue of each specimen was preserved by adding sufficient amount of (2.5%) of potassium dichromate for examination by other laboratory methods [11]. A second aliquot of each stool specimen was immediately frozen and stored at -20 C°. Subsequently, the frozen aliquots were thawed and mixed thoroughly before testing with immune-chromatographic dipstick tests (Triage Micro Parasite Panel) which is an enzyme immunoassay for the detection of *G. lamblia*, *E.histolytica/dispar* and *Cryptosporidium parvum* in fresh or fresh frozen, un-fixed human fecal specimens. The presence of the specific antigens is detected usually by the presence of a purple- black color bar

next to the name printed and the test device. This procedure was done according to [12]. The Giardia C-ELISA (capture enzyme immunoassay) kit is a qualitative in vitro enzyme immunoassay for the detection of *Giardia lamblia* cyst antigen in fecal specimens was used. The procedure was applied according to instruction of manufactured company and according to that used by [13]. Genomic mass of *Giardia lamblia* were detected by using the four following steps:

First: DNA extraction from stool samples, for that, the E.Z.N.A. stool DNA Kit was purchased from Omega bio kit company –German.

Second: DNA purity assessments: Total of 107 extracted DNA elutes in step one were checked for purity using Thermo Scientific Nano-Drop™ 2000c spectrophotometer manual protocol ,that carried on by using a ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. [14].

Third: the amplification of each specimen that done by using conventional Gene-Amp PCR System 9700, Dual 384-Well Sample Block Module .While amplification kit has been manufactured by *GenekamBiotechnology AG*, Germany was used to detect *Giardia lamblia* (in one step). It contains the following: Tube **A** forward primer, which consist of a mixture of assemblage A1, A2 and B). Tube **B** reverse primers for all assemblages. Positive control (tube D1), negative Control (tube D2), DNA Marker (tube E): (max 1000 bp): 100, 150, 200, 300, 400, 500, 600, 700, 800, 900,1000bp and Dye (tube F). Thermo-cycler (Gene Amp® PCR System 9700Dual 384-Well Sample Block Module) was switch on for sample amplification process, and the amplification was done according to manufactured company instruction which included the following cycles: 15 seconds at 95° C, 15 seconds at 60° C and 15 seconds at 72° C. Each temperature degrees were repeated 35 cycles.

Four: Gel Electrophoresis which involve the following procedure; Gel Agarose (2.0%) in TAE (1x) buffer(agarose powder 2.0 gm was dissolved in 100 ml Tris –acid borate buffer which prepared by adding 10 ml of TAE 1x to 100ml of distilled water. Heated gently avoiding boiling, 50µl of ethidium bromide stain solution (0.5µg/ml); was added to agarose solution then poured in to gel tang containing special chambers with standard coombs. After 5 to 10minutes and before the gel completely dry, the combs were up stand hold to permit pores in the gel).About 200ml of 1x TAE buffer was added to gel chamber, 2µl of dye (tube F) was added to each micro-tubes. Amount of 10µl of marker (tube E: 100bp) were inserted in to the first and the last lane of electrophoresis, while other lanes were inserted with amplified samples. The gel electrophoresis instrument was set for 60 min. at 120 Volt. After finishing the electrophoresis, the visualizing of Giardia DNA band was done with wearing UV goggles. The length of giardia genome was measures by using UV standard scale and confirmed with the length of marker bands at the first and last lanes to give out the length of giardia genome /bps.

Statistical analysis:

The following terms and equations were used for detecting the efficacy of laboratory methods in detecting *Giardia lamblia*; TP=True positive, NP=True negative, FP=false positive, FN=false

negative, PPV=positive predictive value and NPV=negative predictive value. Sensitivity=TP/ (TP+FN). Specificity=TN/ (TN+FP), Accuracy= (TN+TP) (TN+TP+FN+FP), PPV=TP (TP+FP) and NPV=TN/ (TN+FN) [15]. All data in the present study were stored in Microsoft Excel program and arranged in tables. Some statistical formulas such as: Chi-square, t-student test, Fisher test and sign test for medium were used to detect variances among parameters in the study at probability 0.05 and.0.01.

Results:

From the examination of total 310 stool samples, the all rate of parasitic infections was (51.93%), this rate consist of high rate of intestinal protozoan infection (42.58%) that involved (20.32%) for *Giardia lamblia* and (22.25%) of other intestinal parasites. while intestinal helminthic rate was (9.35%). Relationship between intestinal protozoan and helminthic distribution was statistically significant, P <0.05. (Table1). According to gender, the rate of giardiasis was higher in males (18.01%) than in females (9.04%), P< 0.05.Giardia co-exist infection rate with other intestinal protozoan parasite was (7.05%) compare to (0.96%) for Giardia co-existing helminthic parasitic infections. More common protozoan coexisted were *Giardia +Cryptosporidium parvum* (2.3%) and *Giardia +Blastocystis hominis* (1.28%), while Common helminthic coexistence with Giardia were *Hymenolepis nana, Enterobius vermicularis* and *Ancylostoma duodenali* (0.32%) for all helminthes equally. Triple protozoan infections were recorded with *Giardia + Cryptosporidium + Ent.coli, Giardia+Blastocysits+Ent.coli* and Giardia+*Iodamoeba+Balantidium coli*, for each the rate was (0.32%) respectively, (table 2). (Table 3) showing the efficacy of three laboratory methods for demonstrating *Giardia, E. histolytica* and *cryptosporidium*, high rate of Giardiasis (20.23%) and (19.35%) were recorded by using direct microscopy(double preparation) and ELISA, compare to (14.51%) by using Triage panel, P<0.05. *Cryptosporidium* and *E. histolytica* detecting rates were (6.45%) and (2.6%) using direct microscopy compare to (4.5%) and (3.5%) using Triage panel respectively, P>0.05. Triage usage for three parasites perform high efficacy for giardiasis (14.51%) followed by (4.5%) and (3.5%) for *Cryptosporidium* and *E. histolytica* respectively, P<0.05.For assessments the efficacy of four employee methods in detecting *Giardia lamblia*, the application of sensitivity, specificity and accuracy of laboratory methods: High rate of giardiasis (21.29%) was reported by using by PCR technique, followed (20.32%) and (19.35 and 14.51)% by using direct wet preparation technique, ELISA and Triage panel respectively P<0.05 .. Statistical analyses reveal low efficacy and sensitivity of Triage panel in detecting giardiasis. While specificity and accuracy of all methods were not significant. Negative predictive values NPV in relation to type of laboratory methods were high, but statistically were not significant, controversy to positive predictive values PPV that showed significances, P<0.05. (Table 4). The molecular study of *Giardia lamblia* by using the extract of DNA from 107 stool samples positive for giardia, reveal (1.7%) of genome purity and 437.56 bps genomic mass or density in 80 extract. While the extract of Giardia positive with other protozoa;

purity and mean genomic mass were (1.56%) and 439.89 bps respectively. Controversy to Giardia positive with intestinal helminthes that show (1.49%) of genome purity and 443.33 bps. Statistical analysis exerts no significance among purity rates and genomic mass of giardia parasite. The use of PCR kit, K725, the amplificated genomes reveal bands migration during electrophoresis process ranged from 280 bps to 715 bps, but the majority of giardia genomes were detected between 350bps to 441bps. (Table 5) and (figure 1).

Table (1): Positive number and percentages of parasitic infections.

Types of parasitic infection	No. Positive	% Positive
Other intestinal protoza	69	22.25
Giardia lamblia	63	20.32
Total protozoan infections	132	42.58 *
Helminthic infections	29	9.35
Total	161	51.93

Total No. exam=310 *P<0.05

Table (2): *Giardia lamblia* Co-infection rate with other intestinal parasites.

Type of parasitic infection Protozoan Mixed infections	Male No.,(%)+ve	Female No., (%) +ve	Total No., (%) +ve	T- student and *P* values
Giardia + cryptosporidium	4 3.6 *	3 1.5**	7 2.3	* p<0.05
Giardia + Blastocystis hominis	2 1.8	2 1.05	4 1.28	**P<0.05
Giardia + Iodamoeba butschlii	1 0.9	2 1.05	3 0.96	
Giardia + Entamoeba coli	1 0.9	2 1.05	3 0.96	
Giardia + Entamoeba. histolytica	1 0.9	0 0	1 0.32	
Giardia + Crypto + E.histolytica	0 0	1 0.5	1 0.32	
Giardia + Crypto + E.coli	1 0.9	0 0	1 0.32	
Giardia + Blastocysits + E.coli	0 0	1 0.5	1 0.32	
Giardia + Iodamoeba + Bal.coli	0 0	1 0.5	1 0.32	
Total	10 9.0	12 6.03	22 7.09	
Helminthic mixed infections				
Giardia+Ancylostoma dueodenale	1 0.9	0 0	1 0.32	
Giardia+ Hymenolepis.nana	0 0	1 0.5	1 0.32	
Giardia+Enterobius vermicularis	0 0	1 0.5	1 0.32	
Total	1 0.9	2 1.0	3 0.96	
Total mixed parasitic infections	11 9.99	14 7.03	25 8.065	
Giardia lamblia (pure)	20 18.01 ***	18 9.04	38 12.25	***P<0.05
Total *Giardia lamblia* infection	31 10.00	32 10.32	63 20.32	

Total number of male = 111 Total number of female= 199 Total No. exam=310

Table (3): Relationship between lab method and distribution of the intestinal parasites.

parasites	Direct wet Preparation No, and % +ve	ELISA corpo antigen No, and % +ve	Triage Cassette No, and % +ve
Giardia lamblia	63 20.23	60 19.35	45 14.51 *a
Cryptosporidium	18 6.45	Not applied	14 4.5 *b
Entamoebahistolytica	8 2.6	Not applied	11 3.5 *c

*a P<0.05, * b P>0.05, * c P>0.05

Table (4): Distribution of *Giardia lamblia* according to laboratory methods.

Lab methods	No.of +ve	% +ve	Sensitivity %*	Specificity %	Accuracy %	PDV	NDV	T-value and P
PCR	66	21.29 a*	82.5 a*	93.75 b*	94.51 c*	82.5 d*	98.36	a*T=19.25 P<0.05
Direct wet preparation	63	20.32	78.75	93.56	92.53	78.75	97.16	b*T=42.51 P<0.05
ELISA	60	19.35	77.20	92.59	91.98	75	96.00	C*t=110.48 P<0.05
Triage	45	14.51	69.56	88.33	85.15	56.25	90.56	d*t=85.48 P<0.05

PDV=Positive predictive value NDV=Negative predictive value.

Table (5): Determination of parasites genome mass and genomes purity.

Parameters Parasites	**Genome mass /bp ***	**Genome purity % ***	**Number**
Pure *Giardia lamblia*	437.56	1.705	80
In males	436.11	1.82	36
In females	443.99	1.59	44
Protozoa genomes mass			
Giardia+ Cryptosporidium pavrum	430.40	1.35	12
Giardia +Blastocystishominis	394.00	1.41	4
Giardia + Entamoeba coli	490.05	2.47	3
Giardia + Entamoebahistolytica	335.00	1.35	1
Giardia+Crptosporidum+Entamoeba coli	550.00	1.25	1
Total	**439.89**	**1.56**	**23**
Helminthes genome mass			
Giardia+ Hymenolepis nana	500.00	1.72	1
Giardia +Enterobiusvermicularis	480.00	1.41	1
Giardia+ Ancylostomaduedenali	350.00	1.35	1
Total	443.33	1.49	1
Total Giardia lamblia genomic mass	**440.26**	**1.54**	**107**

*P>0.05

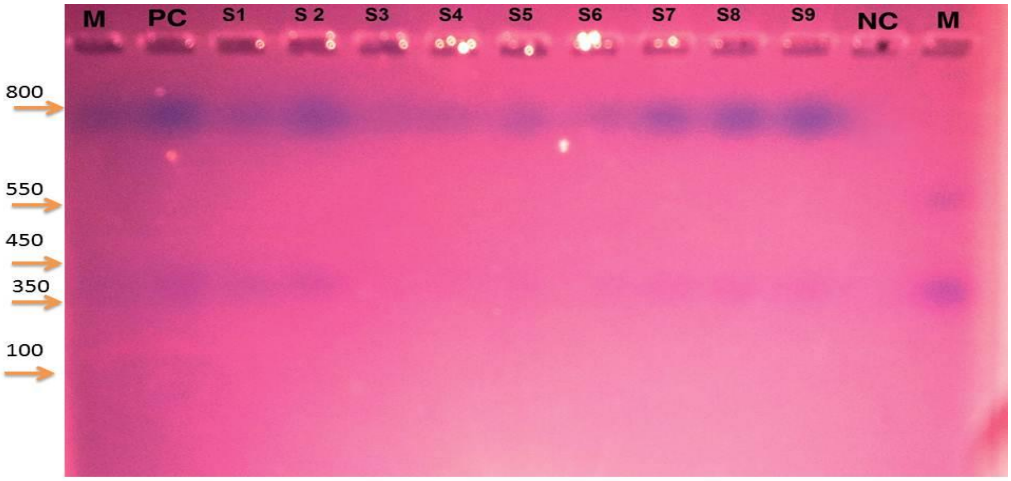

Fig (1). PCR kit :K725-*Giardia lamblia* product on an ethidium bromide-stained 1% agarose gel. Lane M, molecular weight marker (1000 bp); lane NC:negative control; lane PC:positive control; lanes S1 to S9: PCR products from clinical samples.

Discussion:

The all rate of intestinal infection (51.93%) and *Giardia lamblia* rate (20.32%) in the present study were high when compared to those (0.90%, 9.3%, 13.13%, 13.7%, 14.41% and 15.8%) In Kirkuk, Al-Kerbala, Kirkuk, Al-Najaf, Kirkuk and Babylon recorded by [16, 17, 18, 19, 20, 21]. Also it was not agreed with those (11.4%) and (17.1%) recorded in Libya and Brazil respectively by [22, 23]. The rate of *Giardia lamblia* (20.32%) was lower than those (44.59%), (35.89%), and (62.2%) recorded in Kirkuk, Erbil in Iraq and in Egypt by [24, 25, 26]. High prevalence of parasitic infection reflects: lower educational level to health hygiene among children, poor experience in toilet use, overcrowded families, water contamination with Giardia parasite, and lack of insecticides that had role in mechanical transmission of the infective stages of intestinal parasites. The variance of Giardia rates from one region to another might be due to nature of residence survey, level of personal hygiene and sanitation, safety of water consumption from water supplies. In addition to type of diagnostic techniques, size of samples. The rate of infection in males was higher than in females. This might be due to that males are mostly outside their houses and are mostly exposed to feces transmitted parasites. This finding was not agreed with those reported in two studies done among different localities of Kirkuk governorate [19, 20] and with that recorded by Kadir and al-Barzanji in Arbil [24, 27] and with that recorded by Al-Hanoon in Mosul [28] whom they did not find significant difference in the rate of infection between males and females. These differences were probably due to the different in technique used, or could be due to socioeconomic status [24]. For diagnosis of Giardia infections; PCR single step and direct wet preparation microscopy detection of Giardia parasite provided the best results, with sensitivities ranging from (82.5%) and (78.75%). In contrast, the Giardia-Triage panel that reveal low sensitivity (69.56%). Our finding was agree with that recorded by Sharpe, et.al.2001 [12] Lower sensitivity of triage panel might be due that three types of antigens were immobilized on chromatography paper holding three types of specific antibodies [30] or due to high rate of giardia co-infection in the present study. The sensitivity of ELISA test (77.20%) was close with (76.4 %) that recorded in Duhok province [31]. Similar results have been found in Egypt [32], the United States [33] and Germany [34]. The ELISA copro-antigen and Triage panel assays were less time-consuming and easier to perform, but were less sensitive than conventional microscopy methods. While PCR technique remain high sensitive, specific and accurate than other methods, but it is not easy to performed and costly, it can be used in researches or incase when giardia persist in patient in spite of treatment, Thus, these tests might be a useful addition to stool examination for parasites including *Giardia lamblia*, but not a substitute for microscopical methods in the diagnosis of giardiasis. The giardia genome extraction in current study was accurate and precise, because the genomic purity (1.705%) was close to standardized mean (1.6 to 1.8)%. Also *Giardia lamblia* mass mean 437.6bps was very close to 432 bps fragment recorded in Baghdad by Kader and baker, in2011[35] whom they use gdh gene locus amplified in the PCR using primers

GDHiF and GDHiR. Genomic mass extension within study from 280 to 750bps was not agree with that recorded by [36] whom they were show *Giardia duodenalis* genomic extract from sewages ranged from 530 to 750 bps.

Conclusion:

Giardiais in Kirkuk province was high especially among males. PCR technique was highly sensitive for detecting giardiasis in stool samples. ELISA and triage panel were simple and easily performed, but microscopy diagnosis remain high sensitive method. Mean genomic mass of *Giardia lamblia* pure was 437.6 bps with genome extension range from 280 to 750bps.

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