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RESEARCH ARTICLE

COMPARISON OF THREE METHODS FOR DETECTION *GIARDIA LAMBLIA* AND SOME RISK FACTORS ASSOCIATED WITH INFECTION IN DIYALA PROVINCE, IRAQ

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ABSTRACT

Giardia lamblia is Giardia lamblia is a single-celled flagellated eukaryote microorganism that infects the gastrointestinal tract of human and a variety of other mammalian hosts (Marangi *et al*., 2010). Many methods was used to diagnose *Giardia* such as which is still being recommended as the reference standard (Gharavi *et al*., 2005), immune methods which have been shown to be sensitive and cost-effective compared with microscopic examination (Gharavi *et al*., 2005) but they still require numerous reagent, washing procedure and incubation steps (Harba *et al*, 2012). A recently Polymerase Chain Reactions (PCR) is developed technique that offers the greatest potential for the detection of a wide range of microorganism including *Giardi* a in stool. In this study, three method including:Direct smear method, iodine stain method and PCR method was used to detected *Giardia* in stool samples. The results show that the Direct smear method revealed 53/ 424 *Giardia* positive cases (12.5%), while iodine stain method diagnosed 55/ 424 cases (12.97%). PCR method detected 62/424 Giardia positive cases (14.62%). More number of males were found to be positive than females and the highest number of positive cases was in the age group <5 years. There was significant difference between infections according to the source of drinking water and the highest rate of infection was in group whose used river as a source of drinking water. The infection was related with the residence of individuals and more number of positive cases were in rural compared with urban. An analysis of number of infection according to number family members showed statistically significant difference among groups and the highest rate of infection were in group with 12 ≥14 members .the recent study concluded that PCR was sensitive method for diagnose of *Giardia* and the poor sanitary and health habits number of family in addition to age group still the risk factors of infection.

INTRODUCTION

Giardia lamblia is a single-celled flagellated eukaryote microorganism that infects the gastrointestinal tract of human and a variety of other mammalian hosts (Marangi *et al*., 2010). It is also infect many species of animals including reptilian andavis (Alam *et al*., 2011). Since it has a fecal-oral transmission cycle and is contracted by ingestion of contaminated water or food or by person to person contact, the highest disease burden is found in areas where sanitary conditions are poor (Ali and Hill, 2003; Younas *et al*., 2008).

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The highest rates of infection are encountered in developing countries, while in developed countries, infections occur mostly among persons living in closed communities, homosexual men, immigrants and increasing importance, travelers returning from highly endemic countries (Huang and White, 2006, Bernawi *et al*., 2013). It is known that some infected persons are asymptomatic whereas other suffer from fulminant diarrhea, abdominal pain, bloating, flatulence, malaise and severe weight loss resulting from malabsorption (El-Nahas *et al*., 2013). This may lead to changes in intestinal epithelial function and microvilli shortening (Mank *et al*., 1997). Conventional microscopy of three stool samples (with or without concentration techniques) for detecting *Giardia* cyst or trophozoite is still being recommended as the reference standard (Gharavi *et al*., 2005). Disappearance of the parasite

cysts from fecal samples will not always mean cure of infected persons, because this parasite has periodic expulsion on alternative days or during various hours of the day (David *et al*., 2011). It also has a short latent time in some patients, and there is always a probability of its being hidden by bile pigments (Satoskar *et al*., 2009). In recent years, new methods have been investigated for the detection *Giardia* spp., including an enzyme-immuno assay and immune fluorescent antibody microscopy test (IFA). These methods have been shown to be sensitive and cost-effective compared with microscopic examination (Gharavi *et al*., 2005) but they still require numerous reagent, washing procedure and incubation steps (Harba *et al*., 2012).

A recently Polymerase Chain Reactions (PCR) is developed technique that offers the greatest potential for the detection of a wide range of microorganism including *Giardia* in stool. PCR methods for detection of *Giardia* in clinical and environmental samples have been described (Nikaeen *et al*., 2003). This study is among the first molecular experiments in Iraq (Diyala) for detection of parasites in feces samples. The aim of this study is to compare conventional microscopy and PCR as diagnostic methods for *Giardia* detection and to assess the efficiency of PCR in such diagnosis.

MATERIALS AND METHODS

Subjects

This study was conducted in the period from September 2011 to Auguest 2012. Four hundred twenty four patients ranging in age from three month to 70 years as outpatient in Baquba general hospital were enrolled in this study (227 males and 197 females) for gastro intestinal problems. They complained of persistent diarrhea, flatulence, epigastric tenderness, crampy painful abdominal pains and copious light-colored greasy stools.

Conventional microscopy

Stool samples processing

Stool samples were collected from each patients. Each fresh fecal sample (2 gm) was divided into 2 parts. One part (1 gm) was processed immediately by mixing in phosphate buffer saline (PBS). Direct wet mount smear was done by taken drop from the mixing and prepare slide for direct examination and the remained of mixing was centrifuged, followed by formal-ether concentration (Satosker *et al*., 2009). Concentrated specimens (1ml) were used to prepare smears for examined and for staining procedures.

The second part of each sample was processed by mixing in 5ml of PBS, and filtering through cheesecloth. The filtered sample (1.5 ml) was mixed in 7.5 ml heather's suger solution (Satosker *et al*., 2009) and centrifuged at 500 rpm for 5 minutes. The resulting interface and upper layer of liquid were transferred using a disposable pipette to a clean sterile 10 ml tube and kept in 4C for PCR diagnostic procedures.

Staining method

lugol's iodine stain were used. The slides were observed under light microscope using low (x10) and high (x40) power magnifications for *G.lamblia* diagnosis. Three slides were prepared for each patient.

DNA extraction

The specimens were washed twice with sterile phosphate buffer saline and centrifuged for 5minutes at 14.000 rpm. The pellet was kept at -4 C until used. For the QlAamp method the stool pellet was subjected to six freeze – thaw cycles. DNA was extracted using QlAamp DNA stool mini kit (QIAGEN, Germany) according to the manufacturer instructions of kit.

PCR

The DNA was amplified to manufacturer's instructions of Genekam biotechnology Ag, Germany kit to detect *Giardia lamblia.*

Questionnaires

At time of specimen collection, patients and / or their parents complete a written questionnaires to identify risk factors of *Giardialamblia* infection. Standardized questionnaires covering demographic data, source of drinking water, residences of patients and number of family members.

Statistical analysis

Data entry and analysis were performed using SPPS (Ver. 17 for windows) and Midscale software. For each technique, sensitivity, specificity, positive and negative predictive values and accuracy were calculated in comparison to iodine stain as the standard stain for *G. lamblia* cyst. Chi square test was used for presence or absence of association between qualitatively expressed relations P values < 0.05 was statistically significant

RESULTS

This study was conducted to evaluate deferent methods used for detection of *G. lamblia* in human stool samples. Four hundred and twenty four stool samples were examined. Direct smear method revealed 53/ 424 *Giardia* positive cases (12.5%), while iodine stain method diagnosed 55/ 424 cases (12.97%). PCR method detected 62/424 *Giardia* positive cases (14.62%). More number of males were found to be positive than females without significant deferent and the highest number of positive cases was in the age group <5 years and there was significant different (Table 1). Also, there was significant difference between infections according to the source of drinking water as shown in Table (1) and the highest rate of infection was in group whose used river as a source of drinking water. The infection was related with the residence of individuals and more number of positive cases were in rural compared with urban (Table 1).

An analysis of number of infection according to number family members showed statistically significant difference among groups as showed in Table (1) and the highest rate of infection were in group with $12 \geq 14$ members.

Table 1. Potential risk factors and *G. lamblia* infection

Risk factors	No. of infection	Percentage %	P. value	Chi-squares
Sex				
Males	30/424	7.075		
Females	25/424	5.896	0.289	0.486
Age groups				
≤ 5	31/424	7.311		
≥ 5 ≤ 18	16/424	3.773	0.000	15.545
≥ 18	8/424	1.886		
Source of drinking water				
River	3/424	0.707		
Tap water	48/424	11.320		
Well	1/424	0.235	0.000	117.764
Tank	3/424	0.707		
Residence of individuals				
Rural	42/424	9.905		
Urbn	13/424	3.066	0.000	16.351
Number of family members				
4-7	4/424	0.943		
8-11	17/424	4.009	0.000	25.807
12 ≥ 14	34/424	8.018		

PCR detection method was done to investigate for *Giardia lamblia* DNA in stool. The results showed more numbers of infection than (many of negative samples in conventional method was positive in pcr methods) conventional microscopy method as shown in Fig. (1)

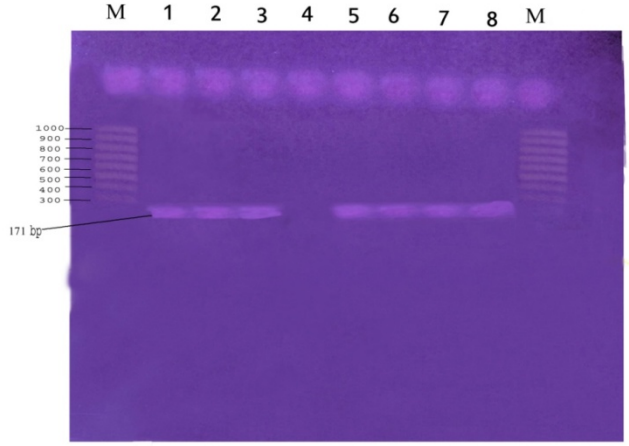

Fig.1. Electrophoresis for pcrdetection of *Giardia lamblia*

M:Lada 4: negative sample 1,2,3,5,6,7,8 :positive samples
Gel Conc.:3%, 80 voltage for 40 min.

Table 2. Sensitivity of PCR method in comparison to iodine stain method for detection giardiasis

Giardiasis Test	PCR	Tp PCR	Fp PCR	Total
Positive	55	7		62
Negative	3	355		55+7(FN+TN)
Total	55+3(Tp+FN)	355+7(TN+Fp)		55+7+3+355 (Tp+Fp+TN+FN)

Tp: True positive, TN, True negative, FN: False negative, FpFals positive
Sensitivity of test is its ability to detect people who have disease

The sensitivity of PCR method in comparison to iodine method fordetection giardiasis was shown in Table (2):

The sensitivity was 94.82 %, specificity was 98.066% positive predictive value (PPV) was 88.709% and negative predictive value (NPV) was 99.162% as shown in formulas bellow:

Formula to calculate sensitivity

$$\text{sensitivity} = \times \frac{TP}{TP + FN} 100$$

$$\text{sensitivity} = \times \frac{55}{55 + 3} 100 = 94.827$$

Specificity is the ability of test to detect people who do not have disease.
Formula to calculate specificity

$$\text{Specificity} = \frac{TN}{TN + FP} \times 100$$

$$\text{Specificity} = \times \frac{355}{355 + 7} 100 = 98.066$$

Formula to calculate PPV

$$PPV = \times \frac{TP}{TP + FP} 100$$

$$PPV = \times \frac{55}{55 + 7} 100 = 88.709$$

Formula to calculate NPV

$$NPV = \times \frac{TN}{TN + FN} 100$$

NPV = 355

355 + 3 = 358

$$\times = \frac{355}{355 + 3} 100 = 99.162$$

The sensitivity of PCR method in comparison to direct wet smear for giardiasis is shown in table 3 and formals bellow:

Table 3. Sensitivity of PCR method in comparison to direct wet smear for giardiasis detection

Formula to calculate sensitivity

	Giardiasis		
Test PCR	True Positive PCR	False Positive PCR	Total
Positive	53	9	62
Negative	FN-ve 1	TN-ve 361	FN+TN
Total	TP+FN 53 + 1= 54	TN+FP 361+9	TP+FP+TN+FN 53+9+1+361

TP= True positive, TN= True negative, FN= False negative, FP= False positive Sensitivity of test is ability to detect people who have disease.

$$\text{Sensitivity} = \times \frac{TP}{TP + FN} 100$$

$$\text{Sensitivity} = \times \frac{53}{53+1} 100 = 98.148$$

$$\text{Specificity} = \frac{TN}{TN + FP} \times 100$$

$$\text{Specificity} = \frac{361}{361 + 9} = 97.567$$

$$\text{PPV} = \times \frac{TP}{TP + FP} 100$$

$$= \frac{53}{53 + 9} = 85.483$$

$$\text{NPV} = \frac{TN}{TN + FN} \times 100$$

$$\text{NPV} = \frac{361}{361 + 1} = 99.723$$

The sensitivity was 98.148%, specificity was 97.567%, PPV was 85.483% and NPV was 99.723%.

The sensitivity of iodine and saline method in comparison with PCR were 88.709% and 85.483%, respectively. The sensitivity was 98.148%, specificity was 97.567%, PPV was 85.483% and NPV was 99.723%. The sensitivity of iodine and saline method in comparison with PCR were 88.709% and 85.483%, respectively.

DISCUSSION

Giardiasis is one of the most common pathogenic intestinal protozoan worldwide (Harba *et al*., 2012). Five million new cases were reported annually and about 200 million people have developed symptomatic giardiasis (Pereira *et al*., 2007). This parasite is also considered as one of the most main non-varial causes of diarrhea in developed countries (Nkrumah and Nguah, 2011). Prevalence of *Giardia lamblia* infection in Iraqi provinces were 34.5%, 38.5% and 23.7% in each of Al-Anbar, Dohuk and Thi-Qar respectively (Al-Joudi and Ghazal, 2005, Al-saeed and Issa, 2006; Hussein, 2010). The present study carried out in Baqubacity, Diyala province in Iraq. The total rate of infection in this study was 12.73% prevalence of infection in this study was lower than in Al-Anbar , Dohak and Thi-Qar province (Al-Joudi and Ghazal, 2005; Al-saeed and Issa, 2006; Hussein, 2010) while the rate of infection was higher than the rate of infection in Al-khalis Town, Diyala province (9.3%) as recorded by Al-Bayati (2000). The fluctuation with other studies due to different in implementing experiment, seasons, socioeconomic status for subjected persons at the time of study. The infection may be more common in warm climates than cool once.

Many risk factors were studied such as sex, age source of water, residence of individuals and numbers of family members. The result showed that there was more positive cases among males compared with females in this may due to the more activities of males and they were more contact with environment than females (Saman *et al*., 2011). There was significant differences among age groups and the highest rate of infection was in age group ≥5 perhaps because they were so young and they were more contact with land and its contaminators (Dib *et al*., 2008) whereas progressively declined in adult indicated that effective immunity towards this parasite might have acquired (Saman *et al*., 2011). The highest rate of infection was in group that used tap water as a source of drink water (11.32%). The explanation of this result may be due to use insufficient concentration of Cl to sterilize water or to contamination of tap water with sewage (Obiukuru *et al*., 2008). There was more positive cases in rural than in urban this may be due to the activities related with agriculture and other outdoor activities which may lead to *Giardia* transmission (Pereira *et al*., 2007). The rate of infection was highest in large families may obtain the effect of crowded as effective transmission factor (Shakkory and Wandy, 2005). Although PCR requires the more costly requirements but the high sensitivity and specificity makes it ideal for confirming the diagnosis when the infection is suspected clinically but the causative agent that that PCR has a high PPV (88.709%) when used to test human samples, which indicates that this test can be use to diagnose or exclude *Giardia* infection in normal members of community instead of the three consecutive stool samples required to improve the diagnostic sensitivity of microscopic examination . This agree with many studies that referred to PCR as a sensitive method to diagnose *Giardia* (latifah *et al*., 2005; Schuurman *et al*., 2007; Tashima *et al*., 2009). PCR method was found to be 94.82% sensitivity and to

be 98.066% specificity compared with iodine method while the sensitivity of PCR was 98.147and the specificity was 97.567% compared with direct wet smear these findings supported that PCR method was good technique for detection *Giardia* in suspected samples. False-positive and false-negative cases are reported even by experienced microscopes (Baig *et al*., 2012). In this study, it was found three false negative and seven false – positive cases resulted by iodine method in comparison to PCR and there were one false-negative and nine false cases by direct wet smear to PCR. This shows that PCR is more superior and effective diagnostic tool that two other method used in this study for identification of *Giardia*.

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