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Comparative Analysis of Various Techniques for Giardia lamblia Detection and Association with E coil and Shigella Among Children Attending AL-Imamin AL-Kadhimin Medical City

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Abstract

Diarrhea is an important public health problem worldwide, several causes associated with diarrhea especially in population live under poverty and unsafe water use. Different methods are available and use in diagnosis. This study was carried out to compare of various techniques for *Giardia lamblia* detection and study the association with *E coil* and *Shigella* in patients with diarrhea. A total of 100 children with diarrhoea were enrolled into the study, 57 were males and 43 were females, aged from 2 months -16 years were attendant to AL-Imamin AL-Kadhimin Medical City, during the period from May 2014 to February 2015. Stool samples were collected and analysed for *Giardia lamblia* presence by used light microscopy, enzyme linked immunosorbant assay and polymerase chain reaction as well as used bacterial culture and one-step colored chromatographic immunoassay for *E coil* and *Shigella* detection. Socio-demographic features of the study subjects were also included. Parasitic infection was the most common than bacterial infection. Most intestinal infection was recorded in age group 5-10 years and among males. Comparative analysis of various techniques for *Giardia lamblia* detection show that microscopy detected only 24 cases, while enzyme linked immunosorbant assay detected 32 cases. However, polymerase chain reaction assay detected 42 cases. Statistical analysis showed significant differences. The sensitivities were 57.14% for microscopy and 76.19% for enzyme linked immunosorbant assay, whereas polymerase chain reaction assay had sensitivity of 100% (42/42) and specificity was100%. Bacterial culture and immunochromatography assay show positive result for *E. coli* (12%), and *Shigella* (6%). Co-infection between three microorganisms which revealed that 5 patients with *Giardia lamblia* positive test had co-infection with *E. coli* and 4 patients with *Giardia lamblia* positive test had co-infection with *Shigella*. Polymerase chain reaction highly sensitive and specific than other methods for *Giardia lamblia* detection, direct examination exhibited many false positive and negative results with parasitic infection.

Key word: Diarrhea, giardiasis, *E. coil*, *Shigella*, immunoassay, polymerase chain reaction.

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1. Introduction

Despite that mortality caused by diarrhea has sharply decreased in the past three decades [1]. Gastroenteritis is still the most common health problem worldwide. About 63 % of all diarrhea cases globally occur in children below five years of age [2]. The most common cause is an infection of the intestines due to either a virus, bacteria, or parasite; a condition known as gastroenteritis. These infections are often acquired from food or water that has been contaminated by stool, or directly from another person who is infected. It may be divided into three types: short duration watery diarrhea, short duration bloody diarrhea, and if it lasts for more than two weeks, persistent diarrhea [3].

Protozoan parasites are more prevalent in developing countries epically *Giardia lamblia* constitutes the higher percent followed by *Entamoeba histolytica* and *Cryptosporidium parvum* [4]. *Giardia lamblia* is a flagellated protozoan parasite that colonizes and reproduces in the small intestine, causing giardiasis [5].

Escherichia coli is a gram-negative, facultatively anaerobic, rod-shaped bacterium of the genus Escherichia that is commonly found in the lower intestine of warm-blooded organisms [6]. Diarrheagenic Escherichia coli (DEC) is frequently associated with diarrhea [7]. Diarrheagenic *Escherichia coli* is classified into several subtypes based on pathogenic mechanisms and clinical features are divided into enteropathogenic *E. coli*(EPEC), enterotoxigenic *E. coli* (ETEC), Vero toxin-producing/Shiga toxin-producing *E. coli*(VTEC/STEC) which include its well-known subgroup enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) [8].

Shigella is a genus of gram-negative, facultative anaerobic, non-spore-forming, nonmotile, rod-shaped bacteria genetically closely related to E. coli and one of the leading bacterial causes of diarrhea worldwide [9]. The number of deaths it causes each year is estimated at between 74,000 and 600,000 deaths [10].

Enteric pathogen co-infections play an important role in gastroenteritis, but most research efforts have only focused on a small range of species belonging to a few pathogen groups [11].

During microscopical examination of faecal samples has several disadvantages, (i) correct identification depends greatly on the experience and skills of the microscopist; (ii) sensitivity is low, and therefore examination of multiple samples is needed; (iii) in settings with relatively large numbers of negative results [12]. Some researchers which revealed that enzyme-linked immunosorbent assay (ELISA), direct fluorescent-antibody assay (DFA) and polymerase chain reaction (PCR) are more specific and sensitive alternative methods than direct microscopic examination [13]. So this study aims to compare of various techniques for *Giardia lamblia* detection and study the association with *E coil* and *Shigella* in patients with diarrhea.

2. Materials and Methods

Study population

We collect one hundred stool samples from children in a clean, dry, tight fit cover, (57) were males and (43) were females, aged from 2 months -16 years, who attended in AL-Imamin AL-Kadhimin Medical City during the period from May 2014 to February 2015.

Macroscopic and Microscopic Examination of *Giardia lamblia*

Each specimen examined within half hour in microbiology laboratory in AL-Imamin AL-Kadhimin Medical City according to WHO [14]. So macroscopic examination were used as

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well as microscopic examination done by direct wet mount methods with normal saline and lugols iodine for the detection trophozoite and cyst stage of *G. lamblia* , the other portions of fecal sample were preserved as -20ºC frozen form for ELISA tests and PCR tests, in the same day of collection.

Detection of *Giardia lamblia* antigen by RIDASCREEN® Giardia

Enzyme immunoassay for the qualitative determination of *G. lamblia* in fecal samples was done according to The RIDASCREEN® Giardia test.

Extraction of *Giardia lamblia* DNA from Stool

The DNA extraction was performed by using AccuPrep® Stool DNA extraction Kit for stool according to the manufactures instructions, then extracted DNA was measured by NanoDrop 1000 spectrophotometer instrument, 3µl were aspirated using special tips and inserted in specified socket in the machine, DNA was quantified by the refractive index using the wave length 260nm, 280nm. DNA concentration was calculated with the OD_{260nm}. The purity was estimated with the OD_{260nm}/OD_{280nm} ratio, a ratio of 1.8-2 was generally accepted as pure for DNA. DNA extraction was successfully observed from samples by agarose electrophoresis (1.5%). Then it was used as a template for PCR assay.

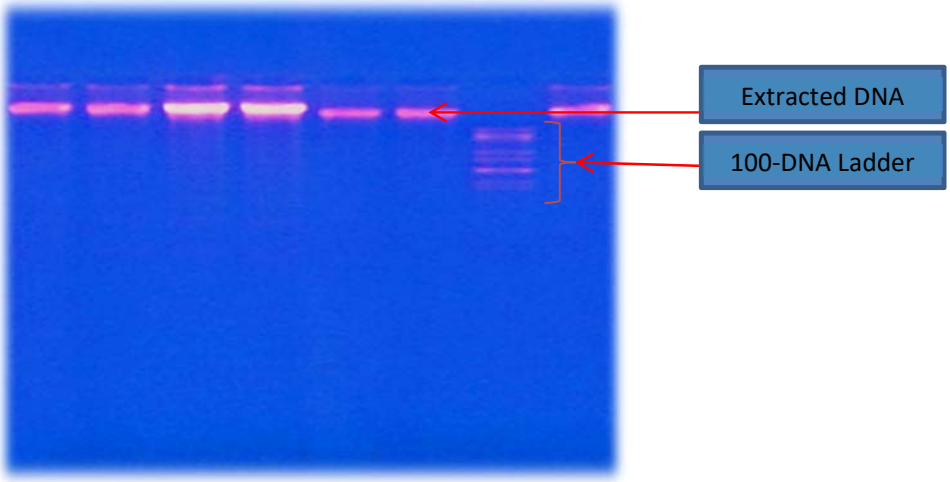

Figure (1): Agarose electrophoresis of total DNA parasites extraction from fecal samples.

Polymerase chain reaction was performed to amplify the tpi gene for the primary PCR, a PCR product of 605 bp was amplified by using primer set forward primerAL3543 and reverse primer AL3546 designed by Sulaiman *et al.* [15]. Polymerase chain reaction amplification mixture was performed in 20µl final volume with 2µl of template DNA in PCR PreMix (1U of Taq polymerase, 250µM each of deoxynucleoside triphosphate (dNTP),{ dATP, dCTP, dGTP, dTTP},10mM Tris-HCl, 30mM KCl, 1.5mM $MgCl_2$, stabilizer and tracking dye), 1µl of each primer, 16µl distilled water. The thermo cycling conditions briefly, the first stage was started by 5 min of an initial denaturation at 95 °C and following that, thirty-five cycles consisted denaturation at 94 ºC, 45 sec, annealing at 50 °C, 45 sec, extension at 72 °C, 60 sec and 10 minutes final extension at 72 °C.

The amplified products were analyzed by electrophoresis in 1.5% agarose gel stained with 0.5 mg/ml ethidium bromide.

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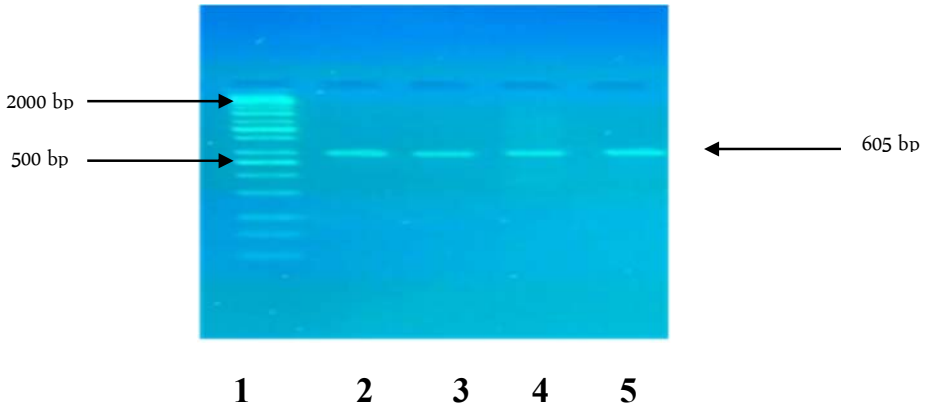

Figure (2): Agarose electrophoresis of PCR amplification for *tpi* gene (605bp). Line 1 represents DNA ladder (100bp), line 2,3,4,5 represent PCR product of *G. lamblia* from examined samples. Fragments were resolved on 1.5% agarose gel and visualized by ethidum bromide staining.

Culture for bacteria

Agars were prepared according to the manufacturer company; all stool specimens were inoculated on the MacConkey agar and Salmonella shigella agar, then incubated at 37°C for 24 hours [16]. In next day many bacteriological and biochemical tests performed to recovered types of bacteria such as indole, catalase, methyl red, production of gas was positive, urease, oxidase, voges-proskauertei, citrate utilization and H2S production according to [17].

Chromatographic immunoassay

Cer Test *Shigella* one-step card test is colored chromatographic immunoassay (Zaragoza-Spain) were used for detection of *Shigella* and one step *E coli* O157:H7 card test for detection of E. *coli*, after incubation period typical colonies were collected from each culture and then transport into collection tube and mixed with diluents solution to good sample dispersion. Cer test for each bacterium was removed just before used it. Four drops from sample collection tube were added in the circular window marked with the letter (s) after 10 minutes the results were read according to manufacturer's instructions so green-red mean positive result while green only mean negative result any other result mean invalid result.

Statistical analysis

The Chi-square- x2 test and Fisher exact test were used to influence different factors in study parameters. The lower level of accepted statistical significant difference is bellow or equal to ($p \leq 0.05$). Odd ratio and confidence intervals were used to assess the risk effect of studied factor between groups. Sensitivity and specificity were calculated [18] by using the followings

Sensitivity= Number of true positive / Total number of individuals in population.

Specifity = Number of true negative / Total number of individuals in population.

3. Result and discussion

Out of total 100 enrolled cases, parasitic and bacterial infection were found in 60% (60/100) of cases and 40% (40/100) of cases may be associated with viral infection or other types of microorganisms. Highest infection rate was found in males than females, also age group (6-11) years followed by (2-5) years show high frequency than others, our study showed non-significant differences as shown in table (1)

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Table (1): Distribution of study group infected with diarrhea according to gender and age

Variable factors		
Gender type	**Positive (%)**	**No. of tested cases**
Male	38(66.66%)	57
Female	22(51.16%)	43
X^2	2.454	NS
P- value	0.117	P<0.05
Age groups		
<2 years	4(40%)	10
2-5 years	20(66.66%)	30
6-11 years	25(64.10%)	39
12-16 years	11(52.38%)	21
X^2	3.003	NS
p- value	0.3910	P<0.05

X^2: Chi square, **P:** Probability, **NS**: Not significant.

In the present study, the rate of *G.lamblia* infection obtained using conventional PCR assay were compared retrospectively with the results obtained by routine microscopy and ELISA technique in patients with diarrhea. Statistically, the most differences were significant between PCR with microscopic and with ELISA in detection of *G. lamblia* as shown in table 2. So basically 42% of studied group infected with G. *lamblia* this may be related with fact, *G. lamblia* and *Entamoeba histolytica* protozoans remain the most common enteric parasitic pathogens in the patients group. The high rate may be due to the existence of resistant cysts of the parasite in the study region [19].

Table (2): Comparative analysis of various techniques for *G. lamblia* detection in study group

Protozoan	Methods	Positive %	x^2Statistic		P-value
G. lamblia	Micro. ELISA PCR	24 24 32 32 42 42	Micro. & ELISA ELISA &PCR Micro.& PCR	1.587 2.145 7.327	0.207 NS 0.143 NS 0.006*

NS: Not significant,* =Significant (p ≤0.05),

The comparison between microscopy, ELISA test and PCR for diagnosis of *G. lamblia*. The sensitivities were 57.14% for microscopy and 76.19% for ELISA test, whereas PCR assay had sensitivity of 100% (42/42) and specificity was100% as shown in table (3). The sensitivity and specifty of *Giardia lamblia* detection was very low (24%) by microscopy due to required experience and skills of the microscopist. Followed by enzyme-linked immunosorbent assay (32%) while more sensitive and specific nucleic acid based methods are

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PCR (42%) these results agree with other researcher indicated molecular techniques which are better than others [20][21][22][23]. Diagnosis largely depends on direct stool sample examination, however, rapid copro-antigen detection kits and molecular techniques are being increasingly used [24] [25]. Also due to molecular tools for the detection and characterization of these parasites are increasingly being used however, particularly for research purposes due to increased specificity and sensitivity and the ability to identify species [26][27].

Table (3):Sensitivity and specificity of light microscopy, ELISA and PCR for diagnosis of *G. lamblia* in stool samples from patients group

G. lamblia	**Positive**	**Sensitivity**	**Specificity**
Light microscopy	24	57.14%	100%
ELISA	32	76.19%	100%
PCR	42	100%	100%

The results were obtained by PCR in detection of *G. lamblia*, 42 giardiasis patients detection by this method, we showed that infection was more common in males 29(50.88%) than females13(30.23%) and statistically significant as shown in table (4). This result agrees with that of those of Hussein and Mohammed in 2014 in Bagdad city and with result of study done by Hassen (2009) who found that infection rates of giardiasis were 55% and 44.9% in males and females respectively in Al-Nassiria city, also with study of Al-Saeed and Issa (2006) in Dohuk city. This is probably due to the higher activity of male children and more contact with environment outdoors, compared to females. Other studies done by Al-Joudi and Ghazal (2005) and Jaeffer (2011), who found that there were no significant differences between infection in males and females. The present study disagrees with result from Raza and Sami (2009) who found that the rate of infection for females was higher (19%) than in males (16%) [28-33].

According to age group, result showed that infection rate with giardiasis was highly in age group 2-5years 17(56.67%) followed by 6-11 years 16(41.03%) but there is no significance between age groups among giardiasis patients as shown in table (4). This result agrees with Ismail *et al. (*2016) who noticed the pre-school age group was the most vulnerable for giardiasis also with study done by Al-Warid (2012) who found that the maximum infection rate was in age group less than 10 years (51.61%). This result may be due to the poor hygienic inhabits of children beside the other socioeconomic conditions and immune status. Giardiasis is a worldwide infection that is detected in all age groups although it is encountered more frequently in children. The higher prevalence in children indicates some degree of acquired resistance to infection in adults [36] [37].

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Table (4): Distribution of giardiasis cases according to gender and age groups

		Positive %	Total	p- value	OR	CI
Gender	Male	29(50.88%)	57	0.038 *	2.390	1.039 to 5.495
	female	13(30.23%)	43		1.0	
Age groups	<2 years	3(30.00%)	10	0.186 NS	1.071	0.205 to 5.584
	2-5 years	17(56.67%)	30		3.269	0.993 to 10.754
	6-11 years	16(41.03%)	39		1.739	0.555 to 5.447
	12-18 years	6(28.57%)	21		1.0	

NS: Not significant,* =Significant (p ≤0.05), **OR**: Odds Ratio; **CI**: Confidence interval.

Bacterial culture and one-step card chromatographic immunoassay were used to investigate presence of *E. coli* and *Shigella* in study samples and the result demonstrated that 12 out of 100 cases show positive result for *E. coli* and 6 out of 100 show positive result for *Shigella*. Co-infection between three microorganisms which revealed that 5 patients with *Giardia lamblia* positive test had co-infection with *E. coli* and 4 patients with *Giardia lamblia* positive test had co-infection with *Shigella* as shown in (Table 5).

Table 5: Distribution of studied sample according to co-infection

Co-infection	Frequency(%)	Examined no.(%)
G. lamblia+ *E. coli*	5(11.90%)	37(88.09%)
G. lamblia + *Shigella*	4(10%)	38(90%)
Total	9(21.42%)	33(78.57%)
P-value		

According to table (6) and (7), the diagnosis of 42 *G. lamblia* infections in stool samples by conventional PCR showed that there were 5/42 (11.90%) cases which had co-infection, these co-infections related to *E. coli* while 3/42(7.14%) cases had co-infection with *Shigella*. Statistically, there were no significant differences among co-infection cases. Co-infection can also increase treatment costs, probably as a result of clinical complications due to interactions among co-infecting pathogens [38]. *Giardia lamblia* has been linked to co-infections with other microorganisms. The importance of polymicrobial infections has gained tremendous impact in recent years and synergistic infections have been identified. In synergistic polymicrobial infections, one microbe creates a favorable environment in order for another one to more easily colonize a specific niche of their common host [38]. The result of present study comparable with study done by kim *et al*., (2016) who found that 3.4% (28/821) positive for *G. lamblia*; and 3.5% (29/821) for *E. coli* in school children in suburban areas near Yangon Myanmar.

Table (6): Distribution of co-infection between *E coli* and *G. lamblia* stratified by age

Age group	*G. lamblia* +ve No (%)	*G. lamblia* –ve No (%)	P-value
<2 years *E. coli* +ve *E. coli* –ve Total	1(33.33%) 2(66.66%) 3(100%)	0 7(100%) 7(100%)	0.107 NS
2-5 years *E. coli* +ve *E. coli* –ve Total	2(11.76%) 15(88.23%) 17(100%)	3(23.07%) 10(76.9% 13(100%)	0.678 NS
6-11 years *E. coli* +ve *E. coli* –ve Total	2(12.5%) 14(87.5%) 16(100%)	4(17.39%) 19(82.60%) 23(100%)	0.677 NS
12-16 years *E. coli* +ve *E. coli* -ve Total	0 6(100%) 6(100%)	0 15(100%) 15(100%)	0.000 NS
Total *E. coli* +ve *E. coli* –ve Total	5(11.90%) 37(88.09%) 42(100%)	8(13.79%) 50(86.20%) 58(100%)	0.098 NS

NS: Not significant,* =Significant (p ≤0.05)

Table (7): Distribution of co-infection between *Shigella* and *G. lamblia* stratified by age

Age group	*G. lamblia* +ve No (%)	*G. lamblia* –ve No (%)	P-value
<2 years *Shigella*, ve *Shigella* –ve Total	1(33.33%) 2(66.66%) 3(100%)	0 7(100%) 7(100%)	0.107 NS
2-5 years *Shigella* +ve *E Shigella* –ve Total	2(11.76%) 15(88.23%) 17(100%)	2(15.38%) 11(84.61% 13(100%)	0.772 NS
6-11 years *Shigella* +ve *Shigella* –ve Total	1(6.25%) 15(93.75%) 16(100%)	0 23(100%) 23(100%)	0.677 NS
12-16 years *Shigella* +ve *Shigella* -ve Total	0 6(100%) 6(100%)	0 15(100%) 15(100%)	0.000 NS
Total *Shigella* +ve *Shigella* -ve Total	4(9.52%) 38(90.47%) 42(100%)	2(3.44%) 56(96.55%) 58(100%)	0.206 NS

NS: Not significant,* =Significant (p ≤0.05)

4. Conclusion

Polymerase chain reaction highly sensitive and specific than other methods for *Giardia lamblia* detection, direct examination exhibited many false positive and negative results with parasitic infection.

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