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RESEARCH ARTICLE

Evaluation of multiplex real-time PCR and ELISA in detection of intestinal protozoan parasites from children with diarrheal disease

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Abstract

Background: Infection with intestinal parasites regard as the most important causative agent for diarrhea, and *Giardia lamblia*, *Entamoeba histolytica* and *Cryptosporidium parvum* are three of the most common intestinal protozoan and the most important diarrhea-causing protozoa.

Objectives: To assess the prevalence of *E. histolytica, G.lamblia and Cryptosporidium spp.* in fecal samples of children with diarrhea and to compare the test performance characteristics of microscopy, ELISA and multiplex real time PCR to an expanded gold standard in the diagnosis of protozoa parasites in fecal samples of children**.**

Materials and Methods: This study included 100 patients who were examined by pediatric physician and attend to the parasitology laboratory in AL-Imamin AL-Kadhimin Medical City, suffering from gastrointestinal complaints with diarrhea. General fecal samples were taken from them during the period from May 2014 to February 2015. The age range was 1month to 18 years. All stool samples were laboratory diagnosed by microscopy, Enzyme-linked immunosorbent assay (ELISA) and multiplex real time PCR**.**

Results: We examined 100 child with diarrhea, Most common parasite was *G. lambila* (42%), overall best results were obtained by multiplex real-time PCR in detection of *G. lamblia, E. histolytica and C. parvum*. Statistically, the difference was significant between multiplex RT-PCR with microscopic and ELISA in detection of these protozoa.

Conclusion: Intestinal parasitic infection is common among diarrheal children and *G. lamblia* is the main parasite that causes infections in children, and there were a significance variants among diagnostic methods and detection of parasitic infections. The best results obtained by a multiplex RT-PCR for detection and differentiation between the most important causative agent of diarrhea.

INTRODUCTION

The World Health Organization (WHO) ranks diarrheal disease as the second most common cause of morbidity and mortality in children in the developing world (1), where poor sanitary and hygienic conditions exist. More than 5000 children are dying every day as a result of diarrheal disease. The etiological agents of diarrhea include viruses, bacteria, and parasites. The prevalence of intestinal parasitic infections is one of the most accurate

indicators of climate and socioeconomic conditions of a population and may be associated with personal hygiene, dietary habits and educational level plays an important role in the intergenerational transmission of poverty (2) .

Among children who survive diarrhea, the morbidity burden may affect their development depending on the intensity of diarrhea and type of pathogen. Malnutrition, anemia, cognitive delays, growth restrictions, irritability, increased susceptibility to other infections and acute complications are some of the consequent morbidities intestinal parasitic infections have detrimental effects on the appetite, survival, growth and physical fitness (3), school attendance and cognitive performance of school age children. In young children, diarrhea is particularly prevalent during the first 2 years of life and caused by parasitic diseases associated with impaired cognitive performance in later childhood (4).

G. lamblia, E. histolytica and *C. parvum* are three of the most common intestinal protozoan and the most important diarrhea-causing protozoa. *G. lamblia* is a flagellate parasite with a worldwide distribution and is considered one of the main nonviral causes of diarrhea in industrialized countries. However, clinical presentation ranges from asymptomatic carriage to acute and chronic gastrointestinal infections . this protozoan causes giardiasis typically characterized by diarrhea, steatorrhea, maldigestion abdominal cramps, bloating, malabsorption and weight loss (5).

E. histolytica, the causative agent of intestinal amoebiasis affects more than 50 million people worldwide. Amoebiasis is considered to be the most common parasitic infection particularly in the tropics and subtropics. It is the second leading cause of the death from parasitic diseases worldwide. *E. histolytica* is capable of invading the intestinal mucosa and may spread to other extra intestinal organs; mainly the liver, the kidneys, lungs, and brain. Thus, *E. histolytica* is unique among the intestinal amebae because it is able to invade tissue and clinical presentation may range from an asymptomatic infection to a disseminated fatal disease. Difficulty In the diagnosis of amoebiasis is due to the presence of similar amoeba that can be misdiagnosed such as *Entamoeba dispar* and other noninvasive amoebae (6).

Cryptosporidium species have a worldwide distribution and the ability to infect a wide range of vertebrate hosts. *C. parvum* and *Cryptosporidium hominis* are the species most commonly associated with human. The highest burden of disease occurring in children under 5 years of age. In immunosuppressed patients, the disease is often more severe, is usually associated with chronic diarrhea and wasting, and can be life threatening (5).

D. fragilis is a pathogenic parasite with a worldwide distribution that has been associated with acute and chronic gastrointestinal symptoms most frequently abdominal pain, diarrhea, and loose stools (7-9). Microscopic diagnosis of *D. fragilis* is hindered by its quick decomposition and thus relies on the analysis of fresh stool samples or stool samples fixated immediately and permanent staining. Such techniques are time-consuming and require experienced personnel to interpret the stained smears. The introduction of highly sensitive molecular diagnostic methods for intestinal parasites, including the recent developed real-time PCR for the diagnosis of *D. fragilis* (10).

G. lamblia, E. histolytica, and *Cryptosporidium* spp. are not only three of the most important and common diarrhea-causing parasitic protozoa, but they often have similar clinical presentations. Microscopic diagnosis of these parasites is neither sensitive nor specific. Detection of trophozoites, cysts, or oocysts in fresh or preserved stool specimens using microscopy is the most common method of diagnosis particularly in resource limited countries. Though microscopy is fairly inexpensive, it can be labor intensive and time consuming, and diagnosis usually depends on the microscopist's level of expertise, the principal limitation of this method is its inability to differentiate closely related species and heterogeneity within species, as it is often difficult to differentiate cysts of the pathogenic from the non-pathogenic intestinal protozoa (11), particularly *E. histolytica*, has led to unnecessary or delayed treatment. In addition, studies have shown that excretion of trophozoites, cysts, or oocysts in the feces can be intermittent and sporadic from day to day and therefore could lead to missed infections due to the low numbers of the diagnostic stages in the stool sample (12).

To optimize parasite detection and identification, other diagnostic methods have been developed such as the Immunofluorescence (IF), (ELISA), culture and subsequent differentiation by isoenzyme analysis and the Polymerase Chain Reaction (PCR). These have been introduced as alternative methods that are more sensitive and specific. These applications however, Real-time PCR reduces labor time, reagent costs and the risk of cross-contamination, and offers the possibility of detecting multiple targets in a single multiplex reaction.

A multiplex real-time PCR has been described for the simultaneous detection of the most important diarrhea-causing parasites, *E. histolytica, G. lamblia, C. parvum / C. hominis* and *D. fragilis* and has demonstrated high sensitivity

and specificity with species-specific DNA controls and a range of well-defined stool samples. However, the role of this assay as a diagnostic tool in a routine clinical laboratory requires further evaluation with respect to large scale screening and improved patient diagnosis (13).

The purpose of the present work were to study the prevalence of *E. histolytica, G. lamblia , D. fragilis* and *Cryptosporidium spp.* in fecal samples of children with diarrhea and to compare the test performance characteristics of microscopy, ELISA and multiplex real time PCR to an expanded gold standard in the diagnosis of protozoa parasites in fecal samples of children, this would provide sensitive and specific diagnosis of the main parasitic diarrheal infections and could improve patient management and infection control.

Materials and Methods

Patients and samples : The patients of this study included 100 who were examined by pediatric physician and attend to the parasitology laboratory in AL-Imamin AL-Kadhimin Medical City, suffering from gastrointestinal complaints with diarrhea.

General fecal samples were taken from them during the period from May 2014 to February 2015. The age range was 1month to 18 years.

Stool samples Examinations

1-Macroscopical Examination

It was performed by observing grossly the consistency of stool samples, presence of blood, mucus and other substances.

2- Microscopical Examination

-Direct Method

From each stool samples, smears with normal saline and lugols iodine were examined. Two direct smears were examined from each fecal sample, by preparing two clean dry microscope slides, one with normal saline and the other with lugols iodine solutions. By using clean wood stick, the stool specimen was touched in different sites, especially where streaks of blood or pus were noticed, then mixed thoroughly with each drop of normal saline and lugols iodine solutions on the prepared slides, then each slide was covered with a cover slip. The smear was examined thoroughly under the low (x10) and high (x40) powers of the microscope **.**

3-Microscopic examination: Stool samples staining with modified Ziehl-Neelsen (acid-fast stain) (14).

ELISA test for human parasites Antigen

The *E. histolytica/dispar*, *G. lamblia* and *C. parvum* antigen detection ELISA kits **(**RIDASCREEN® *Entamoeba* test. Germany), **(**RIDASCREEN® *Giardia* test. Germany), and **(**RIDASCREEN® *C. parvum* test. Germany) respectively, are a qualitative determination of these parasites antigen in stool samples. These tests were performed according to the manufacturer's specifications**.**

DNA-extraction from Stool Samples

AccuPrep® Stool DNA Extraction Kit provided by BioNeer/ Korea was adopted by the manufacturer of DNA extraction kit**.**

Multiplex real-time PCR . (RIDA®GENE Parasitic Stool Panel. Germany) is a multiplex real-time PCR for the direct, qualitative detection and differentiation DNA extraction of *G. lamblia, C. parvum, E. histolytica* and *D. fragilis* in human stool samples. This test was performed according to the manufacturer's specifications **.**

The study was approved by the ethical committee of the College of Medicine/ Al-Nahrain University.

Statistical analysis

Prevalence of infection was compared between different variables by Chi-squared test. Significance was attributed to probability values $P \leq 0.05$. Computer SPSS and Microsoft excel programs were used for determination of probability values.

Results

Comparative analysis of (microscopy, ELISA and multiplex real time PCR) for detection of intestinal parasites in study diarrheic children

Microscopy detected only 24 cases of *G. lamblia* infection, 27 cases of *E. histolytica/dispar* infection, and 2 cases of each *Cryptosporidium* spp. and *D. fragilis* infections in the clinical samples (Table1). While ELISA test detected 32 cases of *G. lamblia* infection, 26 cases of *E. histolytica/dispar* infection, and 3 cases of *C. parvum* infection in the clinical samples at the same times there is no ELISA kit for detection *D. fragilis* .
However, multiplex RT-PCR test detected 42 cases of *G. lamblia* infection, 7 cases of *E. histolytica* infection, 11cases of *C. parvum* and 5cases of *D. fragilis* infection in the clinical samples. Among 100 diarrheal children the overall best results were obtained by multiplex real-time PCR in detection of *G. lamblia*, *E. histolytica and C. parvum*. Statistically, the difference was significant between RT-PCR with microscopic and ELISA in detection of these protozoa.

Out of the 27 microscopy-positive *E. histolytica/dispar* samples, compared to the multiplex RT- PCR methods, only 7 were true *E. histolytica* positives, it was determined that 20 were morphologically identical to *E. histolytica* like *E. dispar* . It should be noted that microscopy and ELISA in this study cannot differentiate the nonpathogenic, morphologically identical *E. dispar* from the pathogenic *E. histolytica*.

Table 1: Comparative analysis of various techniques for detection of intestinal parasites in study diarrheic children.

protozoa	methods	Positive	%	(X^2)		p-value
Giardia lamblia	Micro.	24	24	Micro.& ELISA	1.59	0.207NS
	ELISA	32	32	Micro. &PCR	2.14	0.143NS
	PCR	42	42	ELISA & PCR	7.3	0.006*
Entamoeba histolytica / dispar	Micro.	27	27	Micro.& ELISA	0.26	0.872NS
	ELISA	26	26	Micro. &PCR	13.1	<0.001**
	PCR	7	7	ELISA & PCR	14.2	<0.001**
Cryptosporedium parvum	Micro.	2	2	Micro.& ELISA	0.19	0.658NS
	ELISA	3	3	Micro. &PCR	6.5	0.010*
	PCR	11	11	ELISA & PCR	4.9	0.026*
Dientamoeba fragilis	Micro.	2	2	Micro. &PCR	1.33	0.248NS
	ELISA	-	-			
	PCR	5	5			

Significant *(p value ≤ 0.05) , **(P≤ 0.001) ; NS :Not significant

Comparative the sensitivity and specificity of various techniques in detection of protozoa from diarrheic children

The comparison between multiplex RT PCR and ELISA test for diagnosis of protozoa is shown in Table 2. The sensitivities varied from 27.27% for *C. parvum* to 76.19% for *G. lamblia* and 100% for *E. histolytica*, while the specificities 79.57% for *E. histolytica*, and 100% for each *G. lamblia* and *C. parvum*. While comparison between multiplex RT PCR and microscopy, the sensitivities varied from 18.18% for *C. parvum* to 40.00% for *D. fragilis*, 57.14% for *G. lamblia* and 100% for *E. histolytica*, while the specificities also varied from 78.49% for *E. histolytica* to 100% for *D. fragilis*, *G. lamblia* and *C. parvum*.

Table 2: Sensitivity and specificity of multiplex RT-PCR versus ELISA and direct microscopy for diagnosis of protozoa in 100 stool samples from diarrheic children

G. lamblia	**Positive**	**Sensitivity**	**Specificity**
PCR	42	100%	100%
ELISA	32	76.19%	100%
MICROSCOPY	24	57.14%	100%
E. histolytica	**Positive**	**Sensitivity**	**Specificity**
PCR	7	100%	100%
ELISA	26	100%	79.57%

MICROSCOPY	27	100%	78.49%
C. parvum	**Positive**	**Sensitivity**	**Specificity**
PCR	11	100%	100%
ELISA	3	27.27%	100%
MICROSCOPY	2	18.18%	100%
D. fragilis	**Positive**	**Sensitivity**	**Specificity**
PCR	5	100%	100%
ELISA	---		
MICROSCOPY	2	40.00%	100%

Discussion

The diagnosis of the etiological agents of diarrhea can be performed in the laboratory only, because clinical signs do not enable to differentiate between the different microorganisms (15**)**.

In the present study three diagnostic methods were includes: Microscopic, ELISA and Multiplex Real Time PCR. Among 100 children with diarrhea, Microscopy detected only 24 cases of *G. lamblia* infection, 27 cases of *E. histolytica* infection, and 2 cases of each *Cryptosporidium* spp. and *D. fragilis* infections in the clinical samples (Table1). While ELISA test detected 32 cases of *G. lamblia* infection, 26 cases of *E. histolytica/dispar* infection, and 3 cases of *C. parvum* infection in the clinical samples at the same times there is no ELISA kit for detection *D. fragilis* .

This may be related to the fact that the microscopic sensitivity of morphodiagnostic technique is approximately 46% on a single step due to the intermittent excretion of cysts over time of intestinal protozoa, and at least three fecal samples have to be obtained over a 3-5 day period to achieve 94% accuracy in positive protozoa diagnosis (16,17) However, microscopic method for diagnosis protozoa, it is a time-consuming, requires expertise, unpractical and may prove inadequate in diagnosis of a small number of parasites (18).

These conventional techniques can be replaced by ELISA for its simplicity and the limited laboratory tools requirements and also the use of immunological methods has increased recently, the ELISA technique chosen due to the fact that, it is rapid and reliable and particularly suited to the analysis of large numbers of samples (19).

The results of our study were agreement with other researchers found that ELISA was more sensitive and more accurate than microscopic stool examination (20). It is also faster for rapid investigation of a large number of stool samples in laboratories. Similar results have been found in Egypt (21), and Germany (22).

According to Brown *et al*. (23), performing a range of techniques on a single sample may enhance the detection of parasites since different techniques vary in their sensitivity for different parasite species. Therefore, in this study more than one parasitological methods were used to diagnose intestinal protozoa infection to increase the diagnostic yield.

Based on the multiplex RT-PCR test detected 42 cases of *G. lamblia* infection, 7 cases of *E. histolytica* infection, 11cases of *C. parvum* and 5cases of *D. fragilis* infection in the clinical samples. Among 100 diarrheal children the overall best results were obtained by multiplex real-time PCR in detection of *G. lamblia*, *E. histolytica*, *C. parvum and D. fragilis*. Statistically, the difference was significant between RT-PCR with microscopic and ELISA in detection most of these protozoa.

The primary advantage of using PCR is the possibility of differentiation between *E. histolytica* and *E. dispar* is extremely important for accurate diagnosis of intestinal amoebiasis and for knowing the true prevalence of pathogenic *E. histolytica* in the community, where the presence of other *Entamoeba* species is common. PCR is more accurate to detect the epidemiology of *E. histolytica* and *E. dispar* infection, contrary to the microscopic and Sandwich ELISA test, because it allowed to distinguish the two *Entamoeba* species antibodies for *E. histolytica* that recognize antigen on the surface of the trophozoites only, which are generally identified in diarrhea, and not in the

cystic stage of the parasite (24). WHO recommended that *E. histolytica* should be specifically identified and if present, treated; and other amoeba identified, treatment are unnecessary.

Therefore multiplex real-time PCR is very useful for the detection of intestinal
protozoa infections, either for patient diagnosis, epidemiological studies or monitoring of the prevalence and intensity of intestinal parasitic infections during intervention programs. In this study, the multiplex real-time PCR on the selected protozoa showed a high prevalence of intestinal protozoa, *G.lamblia* was found in 42/100 stool samples with infection rate (42%) from total parasitic infections, this high rate may be due to that *Giardia* is a common cause of diarrhoeal illness and gastrointestinal disturbance in both high- and low-income countries. Giardiasis is an important unresolved health problem in developing countries, as it is related to poor sanitation and management of supplied water the problem that is exacerbated by the absence of a simple reliable diagnostic test (25).

Interestingly, this is the first study to develop and evaluate a multiplex RT- PCR assay for the simultaneous detection and identification of *C. parvum*, *D. fragilis*, *E. histolytica*, and *G. lamblia* in human fecal samples. Additionally, an internal control for the detection of inhibition of the amplification by fecal contaminants was included in the assay. Traditionally, microscopy has been the method of choice; however, for diagnosis of intestinal protozoans, molecular methods are now considered the gold standard for diagnosis (26), given the excellent sensitivities and specificities achieved by molecular methods.

In the future, other multiplex assays combining other parasitic targets could be developed, The implementation of such multiplex assays will have a tremendous impact on routine diagnostic laboratories, as these parasite targets could be combined with both viral and bacterial causes of diarrhea. This would represent a major advance in the differential laboratory diagnosis of diarrheal diseases in general.

In conclusion. Intestinal parasitic infection is common among diarrheal children and *G. lamblia* is the main parasite that causes infections in children, and there were a significance variants among diagnostic methods and detection of parasitic infections. The best results obtained by a multiplex RT-PCR for detection and differentiation between the most important causative agent of diarrhea.

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