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Evaluation of Multiplex Real-time PCR for Detection of three Diarrhea Causing Intestinal Protozoa

KEYWORDS: Entamoeba histolytica, Giardiasis, Cryptosporidiosis, Multiplex Real-time PCR .

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ABSTRACT *Background: Diarrheal diseases are one of the leading cause's childhood morbidity and mortality in many tropical and subtropical countries, in addition to have similar clinical presentation, although it remains a diagnosis of these parasites by microscopy, but microscopy lacks sensitivity and specificity. There is many study referred to used different immunological and molecular assay for diagnosis of parasitic infection.*

Objective: To evaluate a Multiplex Real-time PCR assay for the identification of Entamoeba histolytica, Giardia lamblia, and Cryptosporidium Spp in human fecal samples.

Materials and methods: One hundred eighty stool sample collected from children attending to the central teaching hospital for pediatric in Baghdad city (93 females and 87 males) ranging in age from 6 -12 years was collected during the period from June 2014 to October 2014.The diagnosis was established based on direct microscopic examination of stool and then re-diagnosed by using Multiplex Real-time PCR technique.

Result: In the present study the 180 stool samples tested the PCR for the detection of Entamoeba histolytica, Giardia lamblia, and Cryptosporidium Spp achieved a sensitivity and specificity of 100% compared to sensitivity of microscopy ranged from 74% for Entamoeba histolytica, 84% for Giardia lamblia and 70% for Cryptosporidium Spp, Entamoeba histolytica showed high frequency compare to Giardia lamblia and Cryptosporidium spp.

Conclusion: The Multiplex Real-time PCR described here is a sensitive and specific method for the detection of intestinal protozoa. It also offers the possibility of introducing DNA detection in many laboratories and should be considered the gold standard methods for the diagnosis of enteric protozoan disease.

Introduction:

Diarrhoea is a main health problem global, killing 3-4 million individuals annually. Those most affected by diarrhoea are children and immunocompromised persons living in developing countries. Although the mortality rate from diarrhoea in developed countries has fallen considerably, morbidity remains high [1].

The etiologies of diarrhea include viruses (e.g., Norwalk-like viruses, rotaviruses, and enteric adenoviruses), bacteria (e.g., *Campylobacter jejuni*, *Shigella*, *Salmonella*, enterotoxigenic *Escherichia coli*, and cytotoxigenic *Clostridium difficile*), and parasites [2]. Of which *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* are considered to be the most important [3].

Entamoeba histolytica is a potentially invasive pathogen and the causative agent of amebiasis, with approximately 50 million cases and 100,000 deaths annually [4].

Giardia lamblia is one of the most important causes of diarrhea. According to the center for disease control (CDC) about 2% of all adults and 6-8% of all children in developed countries and about a third of all people in developing countries are infected with giardiasis [5].

Cryptosporidium parvum has been recognized as the cause of large waterborne and food-borne outbreaks of gastroenteritis [6] Cryptosporidium parvum-associated diarrhea has become well known as a result of the severe manifestations in AIDS patients. *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* are three of the most important diarrhea-causing parasitic protozoa [7].

Traditionally, the diagnosis of these infections has relied upon direct microscopic detection of cysts, trophozoites, or oocysts in fresh or fixed stool specimens. However, these methods are laborious, time-consuming, and require expertise [8]. Moreover, for biological reasons such as the intermittent pattern of cyst or trophozoite excretion, sensitivities greater than 90% are rarely obtained [9].

The sensitivity of parasite identification has been reported to increase up to 85% when microscopic examination is performed on three fecal samples obtained on different days [10]. However, this leads to problems concerning patient compliance and delays in the final diagnosis. On the other hand, more specific and sensitive alternative methods such as enzyme-linked immunosorbent assay, direct fluorescent-antibody assay and PCR have been introduced for all three of these parasitic infections [7].

This study was designed to evaluate a multiplex PCR assay for the identification of *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium Spp.* in human fecal samples.

Subjects, Materials and Methods:

Patient Selection

A total of 180 patients suffering from diarrhea attending the outpatient clinic of central teaching hospital for children in Baghdad city (93 females and 87 males) ranging in age from 6 -12 years was collected during June 2014 till October 2014.

Stool Collection and examination

Stool Sample from each patient was collected in a clean, dry, tight fit cover and examined within half hour in microbiology laboratory at hospital by direct wet mount methods with normal saline and lugols iodine for the detection trophozoite and cyst stage of *Entamoeba histolytica* and

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Giardia lamblia in addition to direct Exam by acid fact stain for *Cryptosporidium Spp.*

Extraction of DNA from Stool

Stool samples were screened for *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium Spp.* DNA by multiplex real time PCR for the direct, qualitative detection and differentiation of three parasites in human stool samples. 0.2 gm of stool sample was used for extraction of DNA by the Zymo research method, the stool pellet was Exposure to three time freeze-thaw, DNA was extracted by using The ZR-96 fecal DNA Kit (Zymo Research Corporation,USA)

Multiplex Real Time PCR

The RIDA GENE Parasite stool panel II from (R-Biopharm Company,Germany)this Kit designed to detection and differentiation of specifically *E. histolytica*, *Giardia lamblia* ,and *Cryptosporidium parvum* ,were used for detection of these parasites in stool samples according to the manufacturer's instructions.

Statistical analysis:

Mc Nemar's test was used for statistical analysis on case-control data. Sensitivity and specificity for microscopic examination and the Real time Multiplex PCR were calculated according to Knapp and Miller [11].

Results:

A total of 180 stool specimens were examined for three protozoa collected from children ranging in age from 6 -12 years, 93 females and 87 males.

According to type of method detection 53 were positive for *Entamoeba histolytica*, 38 were positive for *Giardia lamblia* and 4 positive for *Cryptosporidium spp* by direct microscopic examination, while the number of positive cases was increased by used multiplex real-time PCR. The results of all tests are shown in the table (1)

Table (1): Distribution of parasite infections according to microscopic examination and Multiplex real time PCR

Organisms	Microscopic Examination	Multiplex real time PCR
E.histolytica	53	70
G.lambelia	38	45
Cryptosporidium Spp.	4	7
Negative for three Parasite (diarrhea only)	85	58
Total	180	180

The specificity of the multiplex real-time PCR was evaluated by using a range of controls: DNAs from *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium spp*. The specificity was very high (100%) for each parasite in multiplex real-time PCR compare with direct microscopic examination as show in in table (2) (3).

Table (2): Sensitivity and Specifity of diagnostic methods

Method	E. histolytica		G.lambelia		Cryptosporidium	
	Sensitivity %	specificity %	Sensitivity %	specificity %	Sensitivity %	specificity %
Microscopic Examination	74	100	84	100	70	100
Multiplex real time PCR	100	100	100	100	100	100

Table (3): Summary of Sensitivity and Specifity for each method

Methods	No. of sample	No. of Positive sample			Sensitivity %	specificity %
		E. histolytica	G.lambelia	Cryptosporidium Spp.		
Microscopic Examination	180	53	38	4	100	100
Multiplex real time PCR	180	70	45	7	70 - 84	100

Regarding total number of cases, table (4) which revealed that positive and negative results by used direct microscopic examination and multiplex real time PCR.

Table (4): Result of Multiplex real time PCR compared with Microscopic Examination in total stool sample

Organisms	Microscopic Examination		Multiplex real time PCR	
	Positive	Negative	Positive	Negative
E. histolytica	53	127	70	110
G. lambelia	38	142	45	135
Cryptosporidium Spp.	4	176	7	173

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Discussion

The intestinal protozoa most commonly causing gastroenteritis are *Giardia lamblia* and *Cryptosporidium spp*, together with *Entamoeba histolytica*, and the clinical presentation of these protozoal infections is often similar [12].

This study design to investigate prevalence of intestinal protozoa in children by used direct microscopic examination and compared to multiplex real-time PCR. Intestinal parasitic infection is among the most common infections worldwide. It is estimated that approximately 3.5 billion people are affected and that 450 million are ill as a result of these infection, the majority being children [13].

The results of present study which revealed that high differences between two methods, this may be related with microscopic examination of stool samples has been considered to be the 'gold standard' for diagnosis of *E. histolytica*, *G. lamblia*, and *C. parvum* infections for many years ago [7]. However, microscopy has several important 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) *E. histolytica* cannot be differentiated from the non-pathogenic *Entamoeba dispar* simply on the basis of the morphology of cysts and small trophozoites; and (iv) in settings with relatively large numbers of negative results [1].

This results an agreement with study done by Stark *et al.,* (2011), who evaluation of a multiplex tandem PCR (MT-PCR) assay for the detection and identification of four pathogenic protozoan parasites, such as *Cryptosporidium spp., Dientamoeba fragilis*, *Entamoeba histolytica*, and *Giardia intestinalis*, from 472 fecal samples submitted to the Department of Microbiology at St. Vincent's Hospital [14].

Some researchers which demonstrated more specific and sensitive alternative methods such as enzyme-linked immunosorbent assay, direct fluorescent-antibody assay and polymerase chain reaction (PCR) have been introduced for all three of these parasitic infections but more expensive compared with direct microscopic examination [7].

So basically the present study selected molecular technique to immunological methods. Monoclonal antibodies against *cryptosporidium* antigens are successfully used for fluorescence microscopy and in antigen ELISAs [15, 16]. However, non-specificity of antibody based methods owing to cross-reactivity with other microorganisms and low sensitivity is reported to be problematic [17, 18, 19]. Alternatively, PCR has shown to be sensitive and specific for the detection of *C. parvum* in fecal samples [19].

A multiplex real-time PCR has been described previously for the simultaneous detection of

E. histolytica, G. lamblia and Cryptosporidium spp. DNA in faecal samples [13]. In the present study, the results obtained using this multiplex real-time PCR assay were compared retrospectively with the results obtained by routine microscopy in clinical laboratory practice for patients with diarrhea. And analysis of 180 faecal DNA samples demonstrated that prevalence of *E. histolytica* was 70 cases (38%), followed by *G. lamblia* was 45 cases (25%) and finally *Cryptosporidium spp* was 7 cases (3.88%) as compared to 53. 38 and 4 cases respectively by microscopy. The study revealed that multiplex real-time PCR approach is more sensitive and specific .The result of this study was in agreement with results of by Stark *et al.,* (2011). Who exhibited 100% sensitivity and specificity, while traditional microscopy of stained fixed fecal smears exhibited sensitivities and specificities of 56% and 100% for *Cryptosporidium spp.*, 38% and 99% for *D. fragilis*, 47% and 97% for *E. histolytica*, and 50% and 100% for *G. intestinalis* [14].

Also an agreement with the finding of Verweij *et al.,* (2003) who reported that the detection of parasite-specific DNA appears to be more sensitive than microscopy, for amoebic infection with *E. histolytica* and *E. dispar* specific (real-time) PCR [20]. And with finding of Rashidul *et al*., (2007) who observed these same results [21]. Other study as has been shown more sensitive for *G. lamblia* infections with real-time PCR [7, 22]. For *C. parvum* infections [18, 21].

Entamoeba histolytica was the most common pathogenic enteric parasite found in patient's diagnosed using microscopy and multiplex real-time PCR. This finding disagreement with other studies [23, 24]. Also disagreement with other which used real-time PCR (n = 950 cases), the rate of detection of *G. lamblia* increased to 8.6%, and the number of infected cases was more than double in children of school age. In addition, very high rates of *G. lamblia* infection were found in adopted foreign children, who had presumably been exposed in their country of origin [25].

In conclusion, the present study revealed that significant numbers of *Entamoeba histolytica*,*G. lamblia* and *Cryptosporidium* infections remain undetected by microscopy in patients with gastrointestinal symptoms or diarrhea. The Multiplex Real-time PCR described here is a sensitive and specific method for the detection of intestinal protozoa. It also offers the possibility of introducing DNA detection in many laboratories and should be considered the gold standard methods for the diagnosis of enteric protozoan disease

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