# DETECTION AND DIFFERENTIATION OF HUMAN PROTOZOAN PARASITES IN STOOL SPECIMENS BY USING MULTIPLEX ALLELE SPECIFIC POLYMERASE CHAIN REACTION (MAS-PCR)

## In New Damietta City, Egypt

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**ABSTRACT**

Enteric protozoa continue to be the most commonly encountered parasitic diseases causing significant morbidity and mortality in developing regions of the world affecting millions of people. This study assessed the use of Multiplex Allele Specific Polymerase Chain Reaction (MAS-PCR) assay and microscopy for detection and identification of common pathogenic protozoan parasites in New Damietta city of North Delta region, Egypt. Between Jun 2013 until Sept 2013, fresh stool samples were obtained from 249 patients up to 65 years of age attending the internal clinic of the Damietta University Hospital and those visiting their general practitioner (GP) of outpatient clinics because of gastrointestinal symptoms. Stool samples collected was preserved at -200C for DNA extraction whilst the remaining was preserved in sodium acetate-acetic acid formalin and concentrated using the formol-ether technique for microscopic examination. DNA extracts were analyzed with the multiplex allele specific Polymerase Chain Reaction (MAS-PCR) for pathogenic protozoan parasites. The diagnostic results obtained using a multiplex allele specific PCR for the detection of *E. histolytica/dispar*, *G. lamblia* and *C. parvum*/*C. hominis* were compared with these obtained by routine microscopy of faecal samples from patients. 69 samples were positive by MAS-PCR assays , 9 cases of *G. intestinalis* infection, 34 cases of *D. fragilis* infection, 3 cases of *E. histolytica* infection, 17 cases *E.dispar* and 6 cases of *Cryptosporidium* infection in the clinical samples. By microscopy, only 32 samples were positive for one or more of the enteric protozoa, 5cases of *G. intestinalis* infection, 9cases of *D. fragilis* infection, 13 cases of *E. histolytica* infection, and 1 cases of *Cryptosporidium* infection in the clinical samples. However, there are no cases of *E.dispar* observed. Mixed infections were detected in 4 samples. The sensitivities varied from 58% for *D. fragilis* to 47% for *E. histolytica*, 35% for *Giardia*, and 30% for *Cryptosporidium*, while the specificities also varied from 97% for *E. histolytica* to 99% for *D. fragilis* and 100% for *E.dispar* . No cross-reactivity was detected in stool samples containing various other bacterial, viral, and protozoan species. This present study showed relatively high rates of protozoa infections in the study patients. The study has also demonstrated that the multiplex real time PCR assay was more sensitive compared to microscopy in the diagnosis of the intestinal protozoa parasites and thus, molecular methods must be considered the diagnostic methods of choice for enteric protozoan parasites.

**Keywords:** Human intestinal protozoa, Stool specimens**,** Microscopy, Multiplex Allele Specific Polymerase Chain Reaction (MAS-PCR), Diagnosis.

## INTRODUCTION

Enteric protozoa continue to be the most commonly encountered parasitic diseases and to cause significant morbidity and mortality throughout developing regions of the world, affecting millions of people each year[36&60]. Numerous protozoa inhabit the gastro-intestinal tract of humans. The majority of these protozoa are non-pathogenic, or only result in mild disease. Some of these organisms can cause severe disease under certain circumstances. For example, *Giardia lamblia* can cause severe acute diarrhea which may lead to a chronic diarrhea and nutritional disorders; *Entamoeba histolytica* can become a highly virulent and invasive organism that causes a potentially lethal systemic disease[36].Apicomplexa and microsporidia species can cause severe and life-threatening diarrhea in AIDS patients and other immunocompromised individuals [61].Intestinal protozoa are transmitted by the fecal-oral route and exhibit life cycles consisting of a cyst stage and a trophozoite stage. The cysts consist of a resistant wall and are excreted in the feces. The cyst wall functions to protect the organism from desiccation in the external environment. Unhygienic conditions promote transmission of most protozoa [32&51]. Laboratory diagnosis of these protozoan parasites for many years has relied on the traditional microscopic examination of stool samples. This is regarded as the gold standard when performed by an experienced and a highly skilled microscopist However, the sensitivity and specificity of the microscopic technique has been found to be rather low[44&30]. It is laborious and requires long professional training and may present false positive and negative results. 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].To optimize parasite detection and identification, other diagnostic methods have been developed such as the Immunofluorescence (IF), Enzyme-linked immunosorbent assay (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, also have limitations[56]. Recently, more specific and sensitive alternative PCR methods have been introduced for all of these parasitic infections[45&58]. However, the incorporation in a routine diagnostic laboratory of these parasite-specific methods for diagnosis of each of the respective infections is time-consuming and increases the costs of a stool examination. Traditionally parasites have been identified by simple microscopy, serologic and PCR methods[25&48].The traditional PCR protocols require further processing of the amplicon, which is time-consuming and prone to false-positive results due to possible cross-contamination In an effort to improve on the PCR protocol, the multiplex allele specific PCR has been developed which is able to circumvent the problems associated with the traditional PCR and the other detection methods. This method allows specific detection of the amplicon, discriminating between *E. histolytica*, *E. dispar*, *G. lamblia*, and *C. parvum* in a single assay by binding to one or two fluorescence-labeled probes during PCR. A Multiplex Allele Specific Polymerase Chain Reaction (MAS-PCR) was developed for the simultaneous detection of intestinal protozoa infections in stool samples, it is capable of detecting the minimum amounts of organisms required to cause disease and the presence of multiple protozoan species in a single clinical sample. This improves the diagnosis of parasitic diarrhoeal infection, hence patient management[66&76].The multiplex PCR also included an internal control to determine efficiency of the PCR and detect inhibition in the sample. The assay was performed on species-specific DNA controls and a range of well-defined stool samples, and it achieved 100 percent specificity and sensitivity. The use of this assay in a diagnostic laboratory would provide sensitive and specific diagnosis of the main parasitic infections and could improve patient management and infection control[23].*Cryptosporidium* is an important diarrhea-causing parasitic protozoan found in both humans and animals[1&19]. Conventional methods for detecting *Cryptosporidium* oocysts in faecal specimens involve microscopic detection of oocysts using either a direct fluorescent antibody (DFA) assay with broadly reactive *Cryptosporidium* species antibodies or a modified acid-fast staining technique. However, neither of these methods can identify *Cryptosporidium* at the species level, and their diagnostic strength depends on the skills of the examiner[10&64]. An ELISA using monoclonal antibodies against *Cryptosporidium* antigens has been developed and successfully used; however, this method cannot identify *Cryptosporidium* at the species level, despite being practical as a screening method [26].Various PCR formats have been employed to distinguish species of *Cryptosporidium*. PCR-based detection has been shown to be sensitive and specific for the detection of *C. parvum* in clinical specimens and environmental samples[4,40,62,73,&75].PCR-RFLP and PCR followed by DNA sequencing analysis have been described as reliable approaches for the distinction of *C. hominis* from *C. parvum* (formerly known as *C. parvum* genotypes 1 and 2, respectively) [37,52&53]. Nevertheless, they are time-consuming and labour-intensive, making them inadequate for a rapid diagnostic response during outbreak investigations. A Multiplex Allele Specific Polymerase Chain Reaction (MAS-PCR) with specific primers and probes represents an alternative to conventional PCR for increasing the speed of sample analysis while decreasing the potential risks for contamination of the laboratory environment with amplicons[23]. The causative agent of amebic colitis and liver abscess is *E. histolytica* [54]. The non pathogenic parasites *E. dispar* and *E. moshkovskii* are more common and identical in appearance to *E. histolytica* [29]. *E. dispar* and *E. histolytica* are morphologically identical and phylogenetically closely related[71]. Both *E. histolytica* and *E. dispar* are able to colonize humans but only *E. histolytica* is able to cause invasive disease (colitis and extraintestinal manifestations)[47]. Tissue destruction is not seen with *E. dispar* in vivo. Earlier a panel of researchers concluded that colonization with *E. dispar* has never been documented to cause invasive disease in humans therefore the parasite does not necessitate treatment[69].
*Giardia* is a binucleated flagellated protozoan and these parasites can be found in mammals and other animals, including reptiles and birds[41].*G. lamblia*

is the most commonly isolated intestinal parasite throughout the world. Prevalence rates of 20-40% are reported in developing countries, especially in children[20]. There are two distinct genotypes of *G. lamblia* that infect humans, commonly referred to as assemblages A and B. Molecular analyses have shown the genetic variance between the two assemblages to be greater than that used to delineate other species of protozoa . *Dientamoeba fragilis* is a pathogenic protozoan parasite that infects the mucosa of the large intestine, causing gastrointestinal disease in humans .Diagnosis of *D. fragilis* relies on direct visualization of the trophozoites in stained fixed fecal smears by light microscopy. *D. fragilis* may be difficult to distinguish from nonpathogenic protozoa [24,14&57]. The objective of this work is to detection and identification of common pathogenic protozoan parasites in New Damietta city of North Delta region in faecal samples of patients and to compare the prevalence of *E. histolytica, G. lamblia* and *C. parvum* using microscopy and multiplex allele specific PCR diagnostic methods. Also, to compare the test performance characteristics of microscopy and MAS-PCR to an expanded gold standard in the diagnosis of protozoa parasites in faecal samples of patients with gastrointestinal symptoms.

**Material and Methods**

**Study site.** The Damietta University Hospital is a major hospital serving New Damietta City and other parts of Damietta government of North Delta, Egypt. The hospital's coverage population is approximately 100,000 people. The laboratory department and molecular biology unit of the hospital offers diagnostic as well as research services. **Study population.** All 175 symptomatic (70.30%) and 74 a symptomatic (29.70%) population up to 65 years old and permanently residing in New Damietta City who attend the internal Clinic of the hospital were included in this study and fulfilled the inclusion criteria i.e. Attending the internal clinic of the Damietta University Hospital visiting their general practitioner (GP) of outpatient clinics because of gastrointestinal symptoms and these who attended the clinic for their normal checkups. The age of the patients range from 0–65 years (median 33 years). The group examined contained significantly fewer children aged <15 years .Fecal specimens ($n$ = 249) submitted to the Department of parasitology and molecular biology unit, Faculty of Medicine (Damietta) at University Damietta Hospital. Specimens from outpatients were collected and submitted to the laboratory as a fresh specimen for investigation from Jun 2013 until Sept 2013, along with a portion mixed with sodium acetate-acetic acid-formalin (SAF)preservative[65].

**Sampling.** Labeled sterile containers with a collecting spoon were provided to all the cases and evaluation of clinical symptoms and sign was made at clinic

**Sample processing**. stool sample was collected from each case. The Fresh stool samples were transported to the laboratory immediately for analysis. Stool samples (0.2g) were kept in a labeled 2.0ml Eppendorf tubes and frozen at -20oC without preservative for molecular analysis. The remaining portions of the stools were preserved in Sodium-acetate acetic acid formalin solution (SAF) for microscopy and the formol-ether concentration method. The concentrate (sediments) was divided into two portions. One portion in a 15ml Falcon tube was stained with Lugol's iodine. Smear preparation of the other portion on clean dry 76mm x 26mm microscopy slide was stained with the modified Ziehl–Neelsen stain[23].

**Microscopy.** Unpreserved samples were investigated for trophozoites and cysts by microscopy of iodine-stained wet-mount preparations of a formalin–ether concentrate[8].Sodium acetate acetic acid formalin-preserved samples were first screened by iodine-stained direct smears. Parasite-like structures were confirmed by microscopy of Modified Ziehl–Neelsen staining for the detection of *Cryptosporidium* was performed and examined by oil immersion microscopy (×1,000magnification)[46&27].

**DNA extraction**. DNA of up to 30kb of *Giardia lamblia, Cryptosporidium parvum, Entamoeba histolytica, Entamoeba dispar,* Dientamoeba fragilis in the unpreserved stool samples were extracted and purified using the QIAamp DNA Stool mini kit (Qiagen, Hilden, Germany). The extraction was done according to the manufacturer's protocol and according to[58&69]. In brief, 100 µL of faecal suspension was added to 2 mL of lysis buffer and incubated at room temperature for 10 min, after which an internal control (Phocin herpes virus-1 (PhHV-1); *c.* 6000 copies/sample) and 50 µL of magnetic silica particles were added. The mixture was mixed and incubated for 10 min at room temperature. After centrifugation for 2 min at 1500

*g*, the supernatant was removed by aspiration and the pellet of silica–nucleic acid complexes was resuspended and washed in three washing buffers. Each washing step was conducted for 30 second on step 1 of the miniMAG instrument, with the exception of wash buffer 3 (15 s on step 1), after which the fluid was removed by aspiration. DNA was eluted in 100 µL of elution buffer for 5 min at 60°C on a thermo shaker (Eppendorf, Hamburg, Germany) at 1400 rpm. The extracted DNA was stored at −20°C. **MAS-PCR.** Multiplex Allele Specific Polymerase Chain Reaction PCR assay was performed on DNA eluates from all the stool samples using the Corbett Rotor Gene 6000 (Corbett life sciences, Australia) from the high-copy-number, ribosomal DNA-containing *Amoeba, Giardia, D. fragilis* and *Cryptosporidium* episomes with the following specific primers (Widmer *et al*.,2000).*E.histolytica*,Ehd239F(5′ATTGTCGTGGCATCCTAACTCA3′),Ehd88R(5′GCGGACGGCTCATTATAACA3′),andhisto(VIC-5′TCATTGAATGAATTGGCCATTT-3′-nonfluor; *G. intestinalis*, Giardia-80F (5′-GACGGCTCAGGACAACGGTT3′),Giardia127R(5′TTGCCAGCGGTGTCCG3′);*Cryptosporidium*sp.,CrF(5′CGCTTCTCTAGCCTTTCATGA3′),CrR(5′CTTCACGTGTGTTTGCCAAT3′),andCrypto(TexasRed5′CCAATCACAGAATCATCAGAATCGACTGGTATC-3′*D.fragilis* MAS-PCR was performed on all samplesDF3(5′GTTGAATACGTCCCTGCCCTTT3′)andDF4(5′TGATCCAATGATTTCACCGAGTCA-3′) with the following changes to the reaction conditions; 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Oligos were designed from the region of maximum mismatch in the 18S rRNA and ITS-2. The following primers were used to differentiate *E. histolytica* from *E. dispar*. *E. histolytica* specificprimerswere:Eh5AGAGAAGCATTGTTTCTAGATCTG-3(18S)
Eh 2 5-TTAATTATTAGACAAAGCCT-3(18S)Eh 3 5-TTATTGGTCTGGTCTGTC-3(ITS-2) *E. dispar* specific primers were:Ed 15-GAAGAAACATTGTTTCTAAATCCA-3(18S)Ed25CTACCTATTAGACATAGCCT-3(18S)3 5-TTTATTAACTCACTTATA-3(ITS-2).To directly demonstrate the authenticity of the *E. dispar* DNA , PCR amplification was carried out with species-specific primers. The sequence of 18S rDNA and ITS1 and 2 of *E. dispar* is known (Novati *et al* 1996; Som *et al* 2000).Amplification conditions were: denaturation at 94°C for1 min, annealing at 45°C for *E. histolytica* specific primersand 40°C for *E. dispar* specific primers, followed by extension at 72°C for 1 min. The amplification was carried outfor 30 cycles in a DNA Thermal cycler (MJ Research,USA) PCR was performed in 25-µL volumes containing PCR buffer, 5 mM $MgCl_2$, 2.5 µg of bovine serum albumin, 12.5 pmol of forward primer (021F) annealing to both *E. histolytica*, *E. dispar* , 6.25 pmol of *E. histolytica*-specific reverse primer (CP-HR) and 6.25 pmol of *E. E. dispar C.*specific reverse primer (CP-CR). Amplification comprised 15 min at 95°C, followed by 45 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, with a final extension for 5 min at 72°C. Amplification was detected following electrophoresis in agarose 2% w/v gels stained with ethidium bromide.

**PCR amplification and detection.** Amplification reactions were performed in 25-µL volumes containing PCR buffer (Hotstar mastermix; Qiagen, Venlo, The Netherlands), 5 mM $MgCl_2$, 2.5 µg of bovine serum albumin (Roche Diagnostics, Almere, The Netherlands), 3.125 pmol each of the *E. histolytica*- and *G. lamblia*-specific primers, 12.5 pmol of the *Cryptosporidium*-specific primer, 1.25 pmol of VIC-labelled MGB-*Taq*man probe (Applied Biosystems, Warrington, UK) for *E. histolytica*, 2.5 pmol of FAM-labelled double-labelled probe (Biolegio, Nijmegen, The Netherlands) for *G. lamblia*, 2.5 pmol of Texas-red-labelled double-labelled probe for *Cryptosporidium*, and 5 µL of template DNA. The PhHV-1-specific primers and probe set consisted of 3.75 pmol of each PhHV-1-specific primer and 2.5 pmol of Cy5-labelled double-labelled probe. Amplification comprised 15 min at 95°C, followed by 50 cycles of 15 s at 95°C and 30 s at 60°C. Amplification, detection and data analysis were performed using the I-cycler Real-Time PCR System and v.3.1.7050 software (Bio-Rad, Hercules, CA, USA).MT-PCR is a two-step assay using nested primer pairs in which the first step involves a highly multiplexed reaction to pre amplify multiple targets for between 15 and 20 cycles. These are then aliquoted into individual reaction tubes containing nested specific PCR primers as templates for the second-step reaction, which is performed using a liquid-handling robotics system provided by AusDiagnostics Pty. Ltd. (Sydney, Australia) For MT-PCR, the following items were placed on the deck of the

liquid-handling system: a strip tube containing step 1 multiplexed primers was placed in the thermal cycler; a gene disc containing lyophilized step 2 primers was placed in a loading block; and oil (for covering PCR mixtures), master mix, and water tubes (all supplied in a kit form) were placed in a reagent block. The samples were directly added to the strip tube in the thermal cycler. A software template for the reaction was then selected, and all operations for performing the step 1 multiplexed pre amplification, dilution, The gene disc was then hermetically sealed in a heat sealer, and step 2 amplification was carried out in a Rotor-Gene RG6000 thermal cycler. At the end of step 2, the presence or absence of each target was automatically called using a software routine (AusDiagnostics Pty. Ltd.) that compared the melting temperature of the product with expected values and checked the purity and quantity against predetermined threshold values, which were all manually verified. Master mix reagents and 72-well gene discs containing lyophilized primers were prepared and supplied by AusDia

**Control group .**Control DNA extracted from an infected clinical sample was used as a positive control for the PCR assays. This control group underwent DNA extraction and MAS-PCR as described above.

**Data analysis.** Statistical analyses were performed using SPSS v.11.0.1 (SPSS Inc., Chicago, IL, USA).

## Results

More than seventy percent (70.30%) of the cases who provide stool samples were symptomatic. The ages of study cases ranged from 5 months to 65 years. The mean ages of the symptomatic and asymptomatic were 33 years. Socioeconomic and clinical characteristics of study cases were shown in (Table 1).

**Table 1: Socioeconomic and clinical characteristics of study cases.**

| **Socioeconomic and health care behavior No. ( %)** | | **Housing & Waste disposal No. ( %)** | | **Water supply & water contact No. ( %)** | | **Clinical symptoms No. ( %)** | | **Stool consistency No. ( %)** | |
|---|---|---|---|---|---|---|---|---|---|
| Low | 105 (42.3) | Modern building | 219 (87.9 ) | Piped | 235 (94.3) | Anemia | 19 (7.6) | Firm | 90 (36.1) |
| Moderat e | 90 (36.1) | Primitiv building | 21 (8.4) | River Nile | 45 (18.7) | Diarrhea Abdomin al pain | 78 (31.3) | Loose | 66 (26.5) |
| High | 24 (9.6) | Sewage disposal | 8 (3.2) | Shallow wells | 19 (7.6) | Nausea & Vomiting | 62 (24.8) | Mucoid | 78 (31.3) |
| consulta tion for treatme- nt | 30 (12.4) | Well with chamber disposal | 0 (0.0) | Deep wells | 0 (0.0) | Headach e, fever, fatigue, pallor & Weight loss | 90 (36.1) | Blood stained | 15 (6.2) |

A total of 69/249 samples(table 3&4) were positive by MAS-PCR *assays* detected 9 cases of *G. intestinalis* infection, 34 cases of *D. fragilis* infection, 3 cases of *E. histolytica* infection, 17 cases *E.dispar* and 6 cases of *Cryptosporidium* sp. infection in the clinical samples. MAS-PCR showed 100% sensitivity and specificity. With microscopy, only 32/249 samples(table 2&4)were positive for one or more of the enteric protozoa. Microscopy detected only 5cases of *G. intestinalis* infection, 9cases of *D. fragilis* infection, 13 cases of *E. histolytica* infection and 5 cases of *Cryptosporidium* sp. Mixed infections of pathogenic and non-pathogenic protozoa, *Entamoeba coli,Entamoeba hartmani, Endolimax nana,* were detected in 4 samples. However, there is no casas of *E.dispar* observed. It should be noted that microscopy cannot differentiate the nonpathogenic, morphologically identical *E. dispar* from the pathogenic *E. histolytica*. Out of the 13

microscopy-positive *E. histolytica* samples, compared to the PCR methods, only 3were true *E. histolytica* positives . When microscopy was compared to molecular method, the sensitivities varied from 58% for *D. fragilis* to 47% for *E. histolytica*, 35% for *Giardia*, and 30% for *Cryptosporidium*, while the specificities also varied from 97% for *E. histolytica* to 99% for *D. fragilis* and 100% for *E.dispar*. None of the control samples run by MAS-PCR produced a product. No cross-reactivity was seen with the other organisms. A total of 249 fecal samples results included in the study were summarized in Table 4.

**Table 2: Infection rate of protozoa in study cases by microscopy**

| **Parasites** | **Total N=249** n (%) | **Symptomatic N =175** n (%) | **Asymptomati N =74** n (%) | ***P value*** |
|---|---|---|---|---|
| *Entamoeba histolytica/ dispar* | 13(5.2) | 3(1.8) | 10(13.6) | <0.097 |
| *Giardia lamblia* | 5(2.0) | 3(1.8) | 2(2.7) | <0.001 |
| *Cryptosporidium species* | 5(2.0) | 4(2.3) | 1 (1.4) | <0.999 |
| *Dientamoeba.fragilis* | *9* (3.7) | *5*(2.9) | *4*(5.4) | <0.996 |

**Table 3: Infection rate of protozoa in study cases by MAS-PCR**

| **Parasites** | **Total N=249** n (%) | **Symptomatic N =175** n (%) | **Asymptomati N =74** n (%) | ***P value*** |
|---|---|---|---|---|
| *Entamoeba histolytica/ dispar* | 20(8.0) | 3(1.8) | 17(22.9) | <0.0001 |
| *Giardia lamblia* | 9(3.7) | 7(4.0) | 2(2.7) | <0.001 |
| *Cryptosporidium species* | 6(2.4) | 3(1.8) | 3 (4.1) | <0.969 |
| *Dientamoeba.fragilis* | 34 (13.7) | 23(13.1) | 11(14.9) | <0.997 |

**Table 4: summarized results included in the study**

| Parasites | PCR Total N=69 n (%) | Microscopy TotalN=32 n (%) |
|---|---|---|
| *Entamoeba histolytica* | 3(1.2) | 13( 5.2) |
| *Giardia intestinalis* | 9(3.6) | 5(2.0) |
| *Cryptosporidium sp.* | 6(2.4) | 5 (2.0) |
| *Entamoeba dispar* | 17(6.9) | 0 (0.0) |
| *Dientamoeba fragilis* | 34 (13.6) | 9 (3.6) |
| *Mixed Infection* | 0 (0.0) | 4(1.8) |

Primers were designed from regions of maximum sequence divergence between *E. histolytica* and *E. dispar*. Primer sequences are given in 18S rDNA and ITS-2 is shown in figure 1. The primer pair (1 + 3) in which one primer was derived from 18S rDNA and the second from ITS-2 amplified the expected 1.29 kb fragment when the *E. histolytica*-specific primer was used with *E. histolytica* DNA but not with *E. dispar* DNA. Similarly, the *E. dispar*-specific primer pair amplified the 1.29 kb fragment only from *E. dispar* DNA (figure 1A). Since the *E. dispar* DNA used in this study showed absolutely no amplification with the *E. histolytica* primer pair (figure 1A, lane 4), the possibility of any contamination was ruled out. The cloned *Eco*RI fragments of *E. dispar* rDNA were also tested for amplification with *E. histolytica*-specific and *E. dispar*-specific primer pairs derived from 18S rDNA (primer pairs 1 + 2). Both fragments amplified the expected 600 bp band only with the *E. dispar*-specific primer pairs (figure 1B).

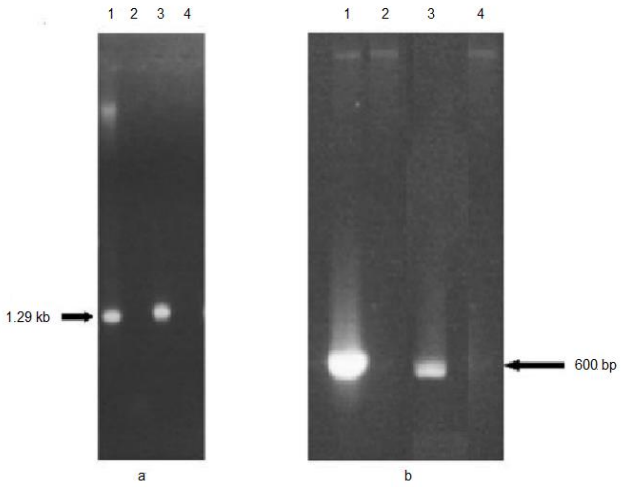


**Figure1.** PCR amplification using species-specific primer pairs. The location of primers (Eh1, 2, 3 and Ed1, 2, 3) in the 18S rDNA and ITS-2 is shown in the top panel. **(A)** PCR amplification of total genomic DNA of *E. histolytica* (lanes1 and 2) and *E. dispar* (lanes 3 and 4) with *E. histolytica*-specific primer pairs – Eh1 and Eh3 (lanes 1 and 4) and *E. dispar*-specific primer pairs – Ed1 and Ed3 (lanes 2 and 3). **(B)** PCR amplification of cloned *Eco*RI fragments of *E. dispar* rDNA – Ed (lanes 1 and 2) and Ed (lanes 3 and 4) with *E. dispar*-specific primer pairs – Ed1and Ed2 (lanes 1 and 3) and *E. histolytica*-specific primer pairs – Eh1 and Eh2 (lanes 2 and 4). Amplified products were separated by electrophoresis through 1% agarose gels for 6 h at 0□8 V/cm. Sizes of amplified fragments are indicated.

## DISCUSSION

*E. histolytica*, *G. intestinalis*, *Cryptosporidium*, and *D. fragilis* are the four most important and commonly occurring diarrhea-causing parasitic protozoa[63]. Therefore, it is essential that correct diagnosis be made, as all four protozoa can be successfully treated with a range of antiprotozoal drugs[31].Infection with these parasites is rare, but its high morbidity and, in particular, mortality make accurate diagnosis crucial[6].The Multiplex Allele Specific Polymerase Chain Reaction (MAS-PCR) assay for the detection of *Cryptosporidium*, *Dientamoeba*, *E. histolytica/dispar*, and *G. intestinalis* presented here provides an additional diagnostic tool for the rapid, sensitive, and specific detection of these enteric protozoa[23&66].The intestinal parasite with the highest prevalence in Delta region is *D. fragilis* followed by *Entamoeba histolytica* , *,Giardia lamblia*[16&50].About 40–50 million people develop clinical amoebiasis each year, resulting on up to 100 000 deaths[72]. In the present study a total of 13(5.2%) *Entamoeba histolytica/dispar* prevalence was observed by microscopy but *Entamoeba histolytica* and *Entamoeba dispar*-specific DNA amplification using the multiplex allele specific polymerase chain reaction identified only 3(1.2%) *E. histolytica* cases and revealed a considerably higher prevalence of *Entamoeba dispar 17*(9.6%). This observation compares well with results obtained in a similar study conducted in northern Ghana by[68&70],that showed a high prevalence (9.8%) of *E. histolytica*/*dispar* complex by microscopy and 8.8% of *Entamoeba dispar* but only one case of *Entamoeba histolytica* by PCR. These results agree with Samuel Ekuban[15]in North District of the Ashanti Region. In consonance with other studies[33]. This study showed a high prevalence of *Giardia lamblia* in asymptomatic cases. This suggests that *Giardia lamblia* infection either presents sub-clinically or the protozoa have limited pathogenicity. Analysis of 722 faecal DNA samples by Hove *et al.*, [24&31] in the Netherland revealed that a prevalence rate of 9.3% of *G. lamblia* by PCR, as compared to 5.7% by microscopy. From a total of 480 patients and apparently healthy Egyptian selected, the prevalence rate of *G. lamblia* infection detected by concentration-sedimentation method was 11.0%[17&26] The primers used in the current study were aimed at detecting *Cryptosporidium parvum* (type II). This is because *C. parvum* is known to infect almost all mammals, including humans, and is a major pathogen of calves. Humans are infected with *C. parvum* in a zoonotic cycle[28]. In this study 5.6% by PCR of symptomatic and asymptomatic recruited cases were found to be infected with *Cryptosporidium parvum*. The overall prevalence of 4.4% and 4.9% by PCR and microscopy respectively. This study has demonstrated that *C. parvum* infection is predominantly common among children and is detected more frequently from symptomatic children than asymptomatic indicating that children with diarrhoea and/or vomiting are more likely to be infected with the protozoa[28&45].This is consistent with a previous study in Egypt by Abdel-Messih *et al.*, [2]. However these findings contradict the findings of[21] in Keny and [3] in Accra who indicated that *Cryptosporidium* infections were highest among children and adult. Classically, diagnosis of *Giardia*, *Cryptosporidium* and *E. histolytica* infections is achieved by microscopical examination of faecal samples[22].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[66-70].Although molecular methods such as PCR have proven to be highly sensitive and specific for the detection of *E. histolytica*/*E. dispar*, *G. lamblia* and *C. parvum*/*C. hominis* infections[40], their use in routine diagnostic laboratories is still very limited[7&34].The introduction of molecular methods has been hindered by time-consuming methods for the isolation of DNA from faecal specimens and the presence of inhibitory substances in such samples[18,42&43]. Furthermore, amplification of DNA was previously laborious and expensive, and cross-contamination among samples was a notorious problem. However, newly developed methods have greatly reduced these obstacles[20&67].A multiplex PCR reduces labour time, reagent costs and the risk of cross-contamination, and offers the possibility of detecting multiple targets in a single multiplex reaction. A multiplex PCR has been described for the simultaneous detection of the three most important diarrhoea-causing parasites, i.e., *E.*

*histolytica*, *G. lamblia* and *C. parvum*/*C. hominis*, and has demonstrated high sensitivity and specificity with species-specific DNA controls and a range of well-defined stool samples[66].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 [9&35].This study highlights the lack of sensitivity that conventional staining techniques that are commonly used in most diagnostic laboratories provide for the diagnosis of these infections. The sensitivity of microscopy is as less as 60% and confounded with misleading results due to misidentification of macrophages as trophozoites,(polymorphonuclearleukocytes) PMNs as cysts (particularly when lobed nuclei of PMNs break apart), and other *Entamoeba* species .It was also showed that the assay is the most sensitive method for differential detection of *E. histolytica* and *E. dispar* because it is able to detect as little as 0.2 pg for *E. histolytica* and 2 pg each for both *E. dispar* DNA, whereas a single round PCR assay can detect 9.5 pg of *E. dispar* and 19 pg of *E. histolytica* [68&70].The main purpose of detection and differentiation of *E. histolytica* species in stool samples is the detection of the causative agent of amoebic dysentery. We showed that this multiplex PCR assay was capable of detecting nearly all of (17/20) the suspected *E. histolytica* cases and showed that some of them were actually positive for *E. dispar*, 17 cases of and only three cases of *E. histolytica.* The MAS- PCR was shown to possess a higher level of sensitivity (100%) for the detection of *E.dispar* in feces. This shows that our MAS-PCR is highly sensitive, capable of detecting target DNA at a copy number that the conventional microscopy unable to detect, agreement with[11,36&60].On the basis of MAS- PCR assay, the number of *E. histolytica* positive cases found in stool samples is about 3 times higher than *E. dispar*. This result clearly indicates that the method used in diagnosis of amoebiasis could significantly affect estimates of the actual number of *Entamoeba* infections in North Delta supports that *E. dispar* infection is, in general, much more common than *E. histolytica* coinceded with similar study in Netherlands by Hove[31].Microscopy detected only 32/249 positive samples compared to 69/249 for the MAS-PCR assay. Compared to both assays, the sensitivity of microscopy ranged from 38% for *D. fragilis* up to 56% for *Cryptosporidium*. Previous studies by [55], have produced similar findings. When comparing microscopy, conventional PCR, and PCR for the detection of *D. fragilis*, it was found that, compared to MAS-PCR, microscopy had a sensitivity of only 34%; this is similar to the 38% found in this study[56].Detection of the other parasite-specific DNAs has also been shown to be more sensitive than microscopy, as it has for *Giardia* infections, for *Cryptosporidium* infections, and for amoebic infection with *E. histolytica*- and *E. dispar*-specific[23&49]. The present study revealed that significant numbers of *E. dispar* and *Cryptosporidium* infections remain undetected by microscopy in patients with gastrointestinal symptoms who consult their GP. Furthermore, the number of additional parasites detected with microscopy was shown to be limited in this population. Therefore, the introduction of MAS-PCR for the routine detection of diarrhoea-causing protozoa would improve the diagnostic efficiency of laboratories dealing with faecal samples from this patient group[5,23&66].The data indicate that the use of microscopy alone for general, routine parasitological diagnosis has limited diagnostic value. It appears that the rationale for developing and implementing molecular screening platforms, combined with microscopy-based and specialized analyses where appropriate[8,36&59].In both the clinical samples and control samples tested the MAS-PCR for the detection of *Cryptosporidium*, *Dientamoeba*, *E. histolytica*, and *G. intestinalis* achieved a sensitivity and specificity of 100% Compared to previously published MAS-PCR assays targeting [36&38].The same organisms, in all samples tested in which microscopy revealed the presence of *Cryptosporidium*, *Dientamoeba*, *E. histolytica*, and *G. intestinalis*, specific amplification was detected. However, MAS-PCR detected an additional 69positive samples (6 *Cryptosporidium*, 34 *D. fragilis*, 17 *E. histolytica*,3 *E.dispar* and 9 *Giardia*). The assay also was found not to cross-react with various other viral, bacterial, and protozoal fecal pathogens. The four samples previously suspected as mixed infection cases of *Entamoeba coli* with *E. histolytica* and positive by our MAS-PCR assay were confirmed that they were *E. coli* infections. Therefore, further development of molecular diagnosis for detection of other nonpathogenic *Entamoeba* species commonly found in humans, such as *E. coli* and *E. hartmanni*, will lead to specific identification and provide the true prevalence of these amoebae in epidemiological studies[12,13&58].Because of the excellent specificity

and sensitivity of MAS-PCR in this study, we propose its application as an alternative tool in routine diagnosis and in epidemiological studies of intestinal parasites. This method will provide more accurate epidemiological data and a greater understanding of infections with these parasites in humans[38,39,76&77].In summary, Traditionally, microscopy has been the method of choice; however, for diagnosis of enteric protozoans, molecular methods are now considered the gold standard for diagnosis, given the excellent sensitivities and specificities achieved by molecular methods. Although PCR-based assays have been successfully used for all organisms, this assay to provide detection of the four different targets in one commercially available kit. This is study developed and evaluated a multiplex PCR (MAS-PCR) assay for the simultaneous detection and identification of *Cryptosporidium*, *D. fragilis*, *E. histolytica*, and *Giardia* in human fecal samples. In the future, 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.

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**دراسة لتشخيص وتصنيف الطفيليات المعوية بأستخدام البلمرة التسلسلية مقارنة بالتقنيات العادية المستخدمة فى التشخيص بمدينة دمياط الجديدة – مصر**

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**الهدف :لا تزال الطفيليات المعوية وحيدة الخلية هى الأكثرشيوعا بين الأمراض الطفيلية والتى تتسبب فى العديد من الأمراض والوفيات بين الملايين من الناس فى دول العالم وخاصة فى الدول النامية ويهدف البحث الى أكتشاف وتشخيص هذه الطفيليات المعوية وتصنيف الممرض منها وغير الممرض باستخدام تفاعل البلمرة المتسلسل والفحص المجهري لعينات البراز في المرضي المصابين وغير المصابين بمدينة دمياط الجديدة**

**الطريقة :تم تجميع عينات البراز من 249 شخص (175 مريض و74 شخص طبيعي) تتراوح اعمارهم بين 10 – 65 عام من المترددين علي العيادات الخارجية بمستشفي دمياط الجامعي والمقيمين بالأقسام الداخلية وتم عمل تشخيص لعينات البراز بالميكروسكوب المجهري بعد تركيزها وصبغها وأيضا تم التشخيص عن طريق تحديد الصفة الجينية لكل طفيل بواسطة تفاعل البلمرة التسلسلية**

**النتائج :وقد أظهرت نتائج الفحص بالميكروسكوب المجهري عن تشخيص وإكتشاف 32 حالة إيجابية للعدوي بالطفيليات المعوية 13 (5.2 % ) طفيل الإنتاميبا هيستوليتيكا 9 ( 3.6% ) طفيل الداي إنتاميبا فاراجايل 5 (2%) طفيل الجارديا لامبيا 5 (2%) طفيل الكريبتوسبريديوم في حين أظهر الفحص بإستخدام البلمرة التسلسلية لنفس العينات عن إكتشاف 69 حالة مصابة و تمييز الإنتاميبا الممرضة من غير الممرضة 34 (13.6% ) طفيل الداي إنتاميبا فاراجايل 17 ( 6.9% ) طفيل الإنتاميبا دسبار 9 ( 3.6% ) طفيل الجارديا لامبيا 6 ( 2.4% ) طفيل الكريبتوسبريديوم 3 ( 1.2% ) طفيل الإنتاميبا هيستوليتيكا**

**الأستنتاج :أظهرت النتائج إرتفاع معدل الإصابة بالطفيليات المعوية وحيدة الخلية بين الحالات التي شملتهم الدراسة ولا سيما بين المرضى الذي يعانون من أعراض النزلات المعوية كما وضح من خلال النتائج الحساسية الفائقة لتفاعل البلمرة المتسلسل في تشخيص الطفيليات المعوية وتمييز الممرض منها عن غير الممرض مقارنة بالتشخيص الميكرسكوبي المجهري العادى**