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Research Article

Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* Complex by Multiplex Polymerase Chain Reaction

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Abstract: This study emphasised the importance of distinguishing the two species of amoebiasis for accurate treatment and management. Molecular techniques for differentiation of *Entamoeba histolytica* and *Entamoeba dispar* is significant in the management and treatment of amoebiasis. Many studies have claimed that the two species are morphologically identical, but genetically different. This study, therefore, was carried out to test whether these two species are different using a molecular technique. In the study, 169 faecal specimens were collected from patients seeking medical services, and showing symptoms of amoebiasis in Naivasha District (County Referral) Hospital Kenya. The results showed that out of 169 stool samples, 36 (21.3%) had E. histolytica/ dispar complex by microscopic examination. Consequently, multiplex-PCR detected 6 (16.7%) samples positive for E. histolytica and 27 (75%) samples for E. dispar. Two (1.5%) of the E. histolytica positive samples and seven (5.3%) of the E. dispar positive samples were among negative samples not detected by microscopic examination. The results show that Multiplex PCR is more sensitive that microscopy and therefore can adequately be used to identify and distinguish between the two species. The study recommends that the ministry of health adopt the use of multiplex PCR for detecting amoebiasis and differentiating the species before any treatment is prescribed for patients.
Keywords: Multiplex-PCR, amoebiasis: *Entamoeba histolytica*: *Entamoeba dispar*.

INTRODUCTION

Amoebiasis is among the most common protozoan infections worldwide, third after malaria and schistosomiasis [1]. It infects approximately 50 million people worldwide causing 40,000 to 100,000 deaths per annum[2]. *Entamoeba histolytica* infects approximately 10% of the world population and its prevalence varies among countries and areas with different socioeconomic conditions and poor hygiene practices (Aseel and Sarmad, 2010; Gonin and Louise, 2003). Amoebiasis infection is common in Africa [3-5] and is responsible for approximately 100,000 fatalities in Central and South America, Africa, and India [6]. The global prevalence of *E. histolytica* (amoebiasis) reported may not be absolutely reliable since much of this information was generated in the era when microscopy was the only tool for diagnosis; given its technical limitation in separating species within the *Entamoeba* complex [7].

However, recently, specific and sensitive alternative molecular methods such as polymerase chain reaction (PCR) have been introduced for diagnosis and species separation[8]. Clinical manifestations of amoebiasis infection are due to the existence of two morphologically identical species of *Entamoeba*, but with different biochemical and genetic make-up: the non-pathogenic *Entamoeba dispar* and pathogenic *Entamoeba histolytica* [9]. Individuals infected with *E. histolytica* may present a wide range of clinical manifestation, from symptomatic colonisation to amoebic dysentery and invasive extra intestinal amoebiasis. However, a majority of infected individuals are asymptomatic[10, 11]. The WHO recommends administration of anti-amoeba drugs only after clear identification of *E. histolytica* and *E. dispar*. Whenever possible no patient should be treated based on microscopic findings alone [12].

Treatment of amoebiasis primarily relies on drugs derived from 5-nitro-imidazole such as metronidazole, which has been in use since 1959 with no new drug released [13]. The current modification of the drug, and which is common in Kenya, is *Dyrade-M*

(Metronidazole and Diloxanide). It is recommended that, in order to reduce resistance, proper diagnosis should be made for effective treatment and greater effort put into prevention [14, 13]. In this study, we aimed at distinguishing *E.histolytica* and *E.dispar* by multiplex polymerase reaction and determine the relative proportions of the two members of *Entamoeba* complexes in clinical faecal specimens. The results should facilitate the pathway for improving management and treatment of patients suffering from amoebiasis

METHODOLOGY

The study was carried between January 2012 and April 2012 in Naivasha District (County Referral) Hospital, located on the shores of the lake Naivasha (0° 43' 0" South, 36° 26' 0" East), Nakuru County. Naivasha is about 90 km from Nairobi and it covers an area of 1707 Km^2 inhabited by approximately 350,000 people (KNBS, 2009). Main human activities are farming, fishing, business and pastoralism. The study group consisted of patients who sought medical services in the outpatient department at the Hospital and were already presenting clinical symptoms of amoebiasis. Stool samples were collected from patients between 2-60 years who had not taken any anti-amoeba drugs within the preceding week.

ETHICS CONSENT

Ethical clearance for the collection of clinical samples was granted and approved by Egerton University Research Ethical Committee (EU/DVRE/028). The permit was obtained from Ministry of Health in Kenya. A written consent was obtained and signed by both the medical superintendent of the hospital and the participating patients prior to sample collection. An informed written consent was directly obtained from adults patients before recruitment whereas for minors (below 18 years), consent was directly obtained from their parents and guardians on their behalf. Participation of patients was voluntary and those who declined to give faecal samples were excluded from the study.

Stool Specimen collection

A pre-field assessment was conducted prior to the study to inform the authorities, explain the objectives and procedures of the study to the hospital medical superintendent. Stool was collected in clean transparent 50 ml plastic bottles. Two grams of each sample was aliquoted into 1.5 ml screw-cap tube and stored at -20°C. The samples were then placed in cold chain boxes and transported to Walter Reed U.S Army Medical Research Unit Microbiology-Hub Kericho, Kenya for PCR analysis.

STOOL ANALYSIS

DNA extraction: DNA was extracted using ZR Faecal DNA MiniPrep™ according to the manufacturer's instructions [15]. The purity of DNA was assessed using NanoDrop 2000 spectrophotometer (Thermo Scientific).

MICROSCOPY

Direct wet smear and formol-ether concentration techniques were performed within two hours after faecal collection as previously described [16]. Briefly, on two separate microscope glass slides one drop of normal saline was placed and mixed with one gram of the stool sample. On one of the slides, a drop of lugos Iodine was added and mixed and coverslips placed on the two smears. To visualise samples with low amoeba density, formol-ether concentration was performed. Two grams of the sample were placed on a mortar and mixed with 10 ml of 10% formol saline using a pestle. The mixture was then sieved through wet gauze. Afterwards 7 ml of the filtered material was mixed with 3 ml of diether and centrifuged at 1500 rpm for 2 minutes and the supernatant discarded. A drop of the pellet was placed on a glass slide and mixed with a drop of lugos Iodine and the smear was covered with a cover slip. All the smears were examined under a light microscope at 10X and confirmed at 40X. The results were recorded either as positive, if cysts or trophozoites of either species were detected or not.

MULTIPLEX PCR

Multiplex PCR was carried out according to the protocol described by Nunez *et al* [17] with some modifications. Based on the tandem repeat sequences in the respected extra chromosomal circular DNAs of *E. histolytica* and *E. dispar*, a set of primers specific for *E. dispar* (EDPI-5′-ATGGTGAGGTTGTAGCAGAGA-3′ and EDP2- 5' CGATATTGACCTAGTACT-3') and *E. histolytica* (EHP1-5'-CGATTTTCCCAGTAGAAATTA-3' and EHP25'-CAAAATGGTCGTCTAGGC-3') were used. A PCR product of 132 bp from *E. histolytica* and a 96 bp from *E. dispar* was expected from the primer pairs EHP1/EHP2 and EDP1/EDP2 respectively. Multiplex PCR reaction was performed in a volume of 50 µl reaction using Dream Taq™ PCR Master Mix (2 X) (Fermentas Life Sciences, USA); 40 pmoles of each oligonucleotide primer and 60 ng of DNA template. Amplification was carried in a GenAmp PCR system 9700 (Applied Biosystems USA) under the following PCR conditions: initial denaturation, 94°C for 5 minutes followed by 35 cycles of 30 seconds at 94°C, annealing at 55°C for 30 seconds and extension at 72°C for 40 seconds with final extension at 72°C for 7 minutes. Amplified products were resolved in 2.0% agarose gel and visualised.

RESULTS

Comparison of Microscopy and multiplex-PCR

A total of 169 faecal samples were analysed by both microscopy and multiplex PCR. Thirty six (21.3%) samples examined by microscopy were positive for *E.*

histolytica/E. dispar complex whereas 33 (91.7%) were positive by multiplex PCR, 6 (16.7%) of which were for *E. histolytica* and 27 (75%) for *E. dispar*. One (2.8%) sample had mixed infection while another 133 were negative by microscopy. However, among the negative samples by microscopy, 2 (1.5%) had *E. histolytica* specific DNA products while 7 (5.3%) had *E. dispar* specific DNA (Table 1). The Chi-Square ($\chi 2$) was conducted to test the efficacy of the two techniques in detecting the two species. The total number of *E. histolytica and E. dispar* were 8 and 34 respectively. We found there was significant relationship between Microscopy test and multiplex PCR test at $\chi 2$ (1df, n=169) = 14.444, $p<0.05$. The sensitivity and specificity of microscopy was 73.3% and 98.2% respectively, while for multiplex PCR the sensitivity and specificity was 93.3% and 100% respectively. Multiplex-PCR detected and distinguished (24.9%) while microscopy detected 21.3%) there was a significant sensitivity variation (Wilks' Lambda = 0.848, F (2,167) = 14.959, p =0.000.Where the PCR was more sensitive than microscopy in differentiating the two species. (See Table 1).

Table 1: Faecal multiplex-PCR and microscopy sample analysis

Microscopy Examination		**Multiplex-PCR**			
	E.h/E.d	E.h/ E.d	E.h	E.d	Negative
Positive	36	1	6	27	3
Negative	133	0	2	7	124
Total	169	1	8	34	127

[E.h-*Entamoeba histolytica*; E.d-*Entamoeba dispar*; E.h/E.d –*Entamoeba histolytica Entamoeba dispar* complex]

Multiplex –PCR Amplified products

Multiplex PCR was used to identify *Entamoeba* species and to compare its sensitivity with microscopy. Using species-specific primers 96 bp and 132 bp diagnostic PCR products were amplified for *E. dispar* and *E. histolytica* respectively (Figure 1). Lane 4 (132 bp) represents *E. histolytica* samples, lane 1, 5 and 6 (96 bp) represent *E. dispar* and the two distinct products (96 bp and 132 bp) on lane 9 represent a double infection with the two species. This is an indication that Multiplex PCR can adequately not only identify but also distinguish among the species (See Figure 1).

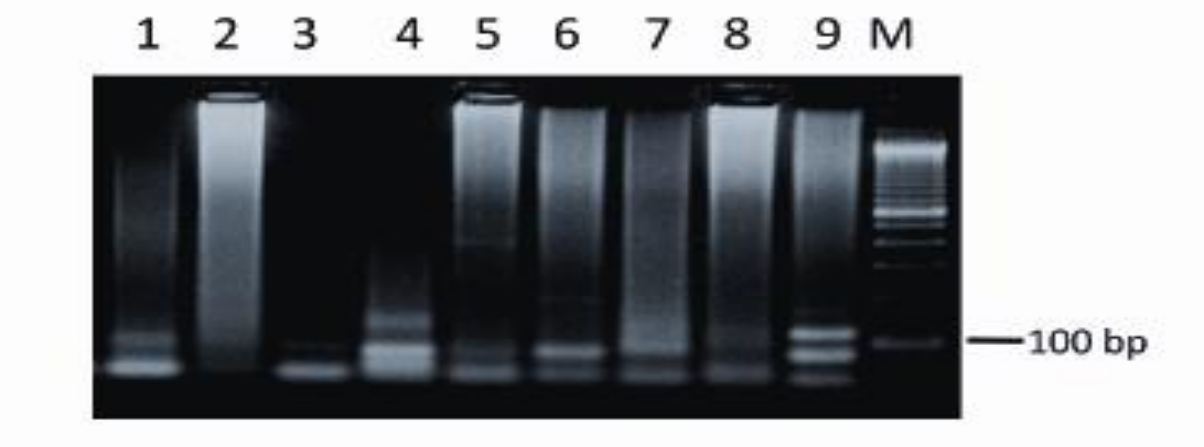

Figure-1: Agarose gel of Multiplex PCR products amplified by *E. histolytica* specific primers (EHP1 \ EHP2) and *E. dispar* specific primers (EDP1\EDP2). Molecular size ladder (M), *E. dispar* positive control (1), patients positive *E. dispar* positive samples (5, 6), *E. histolytica* positive control (4), mixed infection with *histolytica* and *E. dispar* (9, (96 bp and 132 bp respectively)), negative control (3), negative patient samples (2, 7, 8).

DISCUSSION

The study included patients presenting symptoms of intestinal amoebiasis, had sought medical attention, and was suspected to be harboring *Entamoeba*. The common methods, the direct and the formol-ether for stool analysis[18-19] cannot distinguish between *E. histolytica* and *E. dispar*, so these parasites are indicated as *E. histolytica*/*E. dispar* complex. Distinguishing between the members of the complex in stool samples is essential for accurate diagnosis of intestinal amoebiasis and for establishing the prevalence of the pathogenic *E. histolytica* in the community. Currently, more specialized methods exist to distinguish them [20-21] but are not available in most of the developing countries[22]. In the present study, microscopic examination identified (21.3 %) patients infected with *E. histolytica/E. dispar* complex. In contrast, multiplex-PCR detected and distinguished (24.9%) patients infected with either/or both species. These findings corroborate other studies which showed that multiplex PCR is superior over microscopy in

species separation and the two species are genetically different [17,23,24].

An important factor also demonstrated was that mono-infection rate of *E. dispar* was higher (20.1%) than *E. histolytica* (2.4%) and co-infection (2.4%). The occurrence of co-infection observed in this study is in agreement with other studies done in South Africa and Australia [9,25]. The higher incidences of *E. dispar* compared to *E. histolytica* were consistent with other studies which reported such observations[23,24].

This huge difference of detection (mono-infection rate of *E. dispar* (20.1%) and *E. histolytica* (2.4%) explains the bigger margin of error that microscopy could be subjecting medical experts and thus leading to massive (of 17.7%) treatment of unwarranted cases. Under normal circumstances, only 2.4% of the patients should be treated if multiplex PCR was used, but unfortunately, 20.1% cases are treated under the reliance of microscopy. In addition, the multiplex PCR failed to detect 3 (8.3%) of the samples which were detected as positive by microscopy even after reanalysis. However, these samples turned positive after they were spiked with the positive controls SAW 760 strain and HMI-IMSS strain for *E. dispar* and *E. histolytica* respectively.

These negative results could be explained by a possible presence of other members of *Entamoeba* species complex such as *E. moshkovskii, E. polecki, E. coli*, and *E. hartmanni*, which are not easily distinguishable under microscopy. The Multiplex PCR could not detect them (other species) because it was specific to *E. dispar* and *E. histolytica*, and the researcher was not interested in the other species. These species were previously identified and reported in Ghana, Pondicherry in India and Bangladesh[26-27]. However, molecular identification of these species has not been done in Kenya and is hereby recommended for further findings.

The current study further observed that 6.8% of the stool samples, which were negative by microscopy, became positive by multiplex PCR and 1.5% was found to have the DNA of *E. histolytica* and 5.3% had that of *E. dispar*. These findings demonstrated that multiplex PCR is a more sensitive and reliable technique which allows for distinguishing simultaneous *E. histolytica* from *E. dispar* in a single PCR step as well as the presence of mixed infections which are in agreement with other studies [28-30].

Consequently, multiplex PCR use may be highly recommended as a technique in combination with microscopy for accurate diagnosis of amoebiasis. This will significantly improve diagnosis and managements of patients with amoebiasis. Only those patients who are infected with the pathogenic species (*E. histolytica*) should be treated. Those patients diagnosed with non-pathogenic species (*E. dispar*) should not be treated, thereby reducing the cost of treatment to the Public Health Care System in Kenya where currently treatment is based on microscopy results only. While the cost of molecular diagnostic techniques may appear to be high in relative terms in this part of the world, the need for its inclusion into routine diagnostic procedures is justified when the cost is viewed vis-à-vis that of continued medication among people who actually do not need it due to diagnostic limitations of microscopy. These findings underscore the need for proper diagnosis prior to administration of *Entamoeba* treatment[12].

CONCLUSION

Entamoeba species (*E. histolytica* from *E. dispar*) are common in Naivasha sub County area of Kenya. Multiplex PCR is a good and a simple procedure and easily adapted to routine use and can serve as a tool for detection and confirmation of microscopy results, for easier management of patients infected with amoebiasis. The technique is more sensitive and specific in detection and distinguishing between *E. histolytica* from *E. dispar* compared to microscopy. It is also a useful tool for detecting presence of mixed infection simultaneously in a single PCR round. This being the first report highlighting the difference between *E. histolytica* and *E. dispar* from human faecal samples in Kenya. The government of Kenya through the ministry of health should incorporate these findings into policy on *Entamoeba* diagnosis. In view of the limited samples, this study should be carried out in a broader scale possibly covering other neighbouring counties and incorporating other techniques such as serological and immunological for a more accurate scenario on prevalence of other species in this complex. However, multiplex PCR technique does not substitute microscopic stool examination, which widely screens for virtually most intestinal parasites but may rather be a used in combination.

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