Differentiation of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* from diarrhoeic stools using Polymerase Chain Reaction in Kaduna, Nigeria

Dawah I.S*, Inabo H.I, Abdullahi I.O and Machido A.D

Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria

*Corresponding author: dawailiyah@yahoo.com

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ABSTRACT

**Background:** *Entamoeba* species have been reported to cause a high morbidity and mortality rate. **Aim:** The study was aimed at detecting and differentiating *E. histolytica*, *E. dispar* and *E. moshkovskii* using molecular technique (PCR). **Methods:** Microscopic examination of the faecal samples was carried out by the Formol-Ether concentration technique. DNA was extracted from microscopic positive stool samples and used to amplify a part of the genus *Entamoeba* small-subunit ribosomal RNA gene (SSU rDNA), using the Nested Multiplex Polymerase Chain Reaction (NM-PCR). **Results:** Of the 528 faecal samples, 46 (8.7%) were positive for Entamoeba by microscopy. The PCR results showed that out of the 46 microscopy positive samples, 16 (34.8%) successfully generated species-specific amplicons of *Entamoeba* species DNA. The infection with *E. dispar* (68.8%; 11/46) was the most common, followed by *E. histolytica* (37.5%; 6/46) and *E. moshkovskii* (18.8%; 3/46). Of these, 7 (43.8%) were shown to contain only *E. dispar*, 3 (18.8%) contained only *E. histolytica* and 2 (12.5%) contained only *E. moshkovskii*. Mixed infection with *E. histolytica* and *E. dispar* was found in 3 (18.8%) and *E. dispar* and *E. moshkovskii* in 1 (6.3%) sample. **Conclusion:** This study therefore highlighted the great importance of the use of molecular techniques to differentiate between *E. histolytica*, *E. dispar* and *E. moshkovskii* because it obviates unnecessary chemotherapy with possible costs, side effects and drug resistance. The use of PCR in the diagnosis of amoebiasis and epidemiological survey in Nigeria is thus recommended.

**Key words:** *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, DNA, Polymerase Chain Reaction, amoebiasis

INTRODUCTION

The protozoan parasite *Entamoeba histolytica* is estimated to infect 50 million people and causes 40,000 to 100,000 deaths annually, making it the third leading parasitic cause of death worldwide.[1] Other *Entamoeba* species, such as *E. dispar* and *E. moshkovskii*, have also been found in patients.
with gastrointestinal symptoms. However, there is as yet no definitive evidence demonstrating that these two species are pathogenic to humans.

E. histolytica, E. dispar and E. moshkovskii are morphologically identical but are biochemically and genetically different. Laboratory diagnosis of the aetiological agent of diarrhoea/dysentery is of utmost importance for the timely management of dysentery cases. Routine microscopic examination of stool sample is the most widely used technique, but microscopy alone has low sensitivity and it is insufficient for differentiation.

In fact, E. dispar is a harmless commensal protozoan and its presence in clinical specimens does not justify treatment. It has actually been established that misidentification of E. histolytica infection may occur if the diagnosis is based solely on stool microscopy. For final confirmatory identification, biochemical techniques, immunologic assays for detection of E. histolytica antigens or molecular methods are needed.

Amplification of amoeba DNA fragments by PCR has proved its usefulness for differential detection of E. histolytica, E. dispar and E. moshkovskii directly from stool samples. Moreover, this PCR-based approach is suitable for molecular epidemiological studies, which have been strongly encouraged by the World Health Organization. Therefore, the purpose of this study was to obtain more reliable and appropriate epidemiological data concerning E. histolytica, E. dispar and E. moshkovskii infections in diarrheic patients attending some hospitals in Kaduna State, Nigeria, using the nested multiplex PCR.

**METHODOLOGY**

**Study area and population**

The present study was carried out in six government hospitals. Two hospitals each from the three Senatorial Districts in Kaduna State, Nigeria, namely: Kafanchan and Kachia General Hospitals in the South, Yusuf Dantsoho General Hospital Kaduna and Birnin Gwari General Hospital in the Central, Saminaka General Hospital and Hajjiya Gambo Sawaba Memorial Hospital, Zaria in the North. This study was conducted between August, 2013 and August, 2014. A total of 528 diarrheic faecal samples were examined for the presence of Entamoeba.

**Consent, sample collection and laboratory procedures**

The study was approved by the Ethical Committee of the Ministry of Health, Kaduna State. Description of the objectives and methodology of the study was explained to the patients or parents/guardians prior to sample collection. Five hundred and twenty eight (528) stool samples were aseptically collected from the patients at the selected hospitals in sterile capped bottles.

**Microscopy**

The stool samples were analyzed using the Formol-Ether concentration method as described by Cheesbrough. Briefly, about 1g of the faecal matter was mixed in about 4ml of 10% formol water in a screw-cap tube and shaken for about 20 seconds. The emulsified faeces was sieved and the filtrate transferred to a conical centrifuge tube with an equal volume of ether. The tube was centrifuged for 1 minute at 3000rpm. The faecal debris was discarded and the sediment transferred to a clean glass slide. After the addition of a small amount of iodine, the glass slide was covered with a cover slip. The entire preparation was microscopically examined under x10 objective to identify the cysts. Microscopically

It is important to emphasize that earlier reports have been relying upon results of microscopic examination of stool specimens that cannot differentiate the pathogenic E. histolytica from the morphologically identical species E. dispar and E. moshkovskii, which occur worldwide. In previous studies, many E. dispar and E. moshkovskii infections were most probably confused with E. histolytica infections and were unnecessarily treated.

E. histolytica, Entamoeba dispar and Entamoeba moshkovskii infection via a combination of microscopy and Nested Multiplex Polymerase Chain Reaction (NM-PCR) targeting 16S ribosomal RNA of Entamoeba species.

All patients presenting to the selected hospitals with acute and persistent diarrhoea or dysentery within the period of the study were enlisted having consented to participate and fulfilled the inclusion criteria which included acute or persistent diarrhoea and dysenteric syndrome. Patients without diarrhoea were excluded.

Molecular techniques are newer methods currently used for the identification of Entamoeba spp.
positive samples for Entamoeba species were stored at -20°C prior to DNA extraction. The DNA extraction of all microscopy-positive samples was carried out using the MagNa Pure LC DNA isolation kit (Roche Applied Sciences) according to the manufacturer’s instructions. Briefly, genomic DNA was lysed in a buffer containing guanidine isothiocyanate and bound to magnetic glass particles under chaotropic conditions. The unbound substances and impurities were removed by washing the magnetic particles. The washed DNA was eluted from the magnetic particles under conditions of low salt concentration and elevated temperatures. The extracted genomic DNA was then stored at -20°C until required for PCR amplification.

DNA extraction

The secondary PCR had a similar cycling condition except that the annealing temperature (48°C instead of 56°C) and extension duration (1 min instead of 1 min 30 sec) were modified. In both amplifications, samples were incubated in the MyCycler thermal cycler (Bio-Rad, Hercules, USA).

Agarose gel electrophoresis

Electrophoresis was used to separate 3µL of the amplification products through 1.8% Agarose gel in 0.5 x Tris-borate-EDTA at 120V for 45 minutes. This was then visualized by ethidium bromide staining under ultraviolet light for bands of DNA of appropriate sizes. Control reactions were included with each batch of samples analyzed by nested multiplex PCR.

Statistical analysis

The data entry and analysis was carried out using the SPSS software (Statistical Package for the Social Sciences) program for Windows version 17 (SPSS, Chicago, IL, USA). Qualitative data were estimated and presented as frequencies and percentage. The prevalence and 95% confidence intervals (CIs) were calculated for each parasite. Associations between proportions were explored using chi-square \( \chi^2 \) (test) and a \( P \)-value of <0.05 was considered significant at 95% confidence interval.

RESULTS

A total of 528 stool samples were examined microscopically for Entamoeba spp. Of these, 46 (8.7%) samples were diagnosed as Entamoeba positive and subjected to PCR for differentiation of Entamoeba species. Males were more infected (10.8%) than females (6.6%) but the difference was not statistically significant (\( P > 0.05 \) (result not shown). Out of the 46 microscopy-positive
samples, 16 (34.8%) samples successfully amplified *Entamoeba* species DNA by nested multiplex PCR. Birnin Gwari had the highest prevalence (66.7%), followed by Kafanchan (42.9%), Kachia (27.0%), Saminaka (25.0%), Kaduna (20.0%), while Zaria (16.7%) had the least (table 1).

The PCR differentiation of *Entamoeba* species in table 2 revealed that *Entamoeba dispar* infection appeared to be the most dominant [11 (68.8%)], followed by *Entamoeba histolytica* [6 (37.5%)] and *Entamoeba moshkovskii* [3 (18.8%)]. Out of the 16 PCR positive samples, 3 (18.8%) contained only *Entamoeba histolytica*, 7 (43.8%) contained only *Entamoeba dispar* and 2 (12.5%) contained only *Entamoeba moshkovskii*. Mixed infection with *Entamoeba dispar* and *Entamoeba histolytica* was found in 3 (18.8%) samples, while only 1 (6.3%) sample had *Entamoeba dispar* and *Entamoeba moshkovskii* as shown in table 3.

Table 1: Prevalence of *Entamoeba* infection based on microscopy and nested multiplex PCR assay of faecal samples according to locations

<table>
<thead>
<tr>
<th>Location</th>
<th>NE</th>
<th>Microscopy</th>
<th>PCR assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>GH Kafanchan</td>
<td>88</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>YDGH Kaduna</td>
<td>88</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>GH Kachia</td>
<td>88</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>HGSGH Zaria</td>
<td>88</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>GH Saminaka</td>
<td>88</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>GH Birni Gwari</td>
<td>88</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>528</td>
<td>46</td>
<td>16</td>
</tr>
</tbody>
</table>

Key: NE= Number Examined, n = Number positive, *= based on number positive by microscopy, YD= Yusuf Dantsoho, HGS= Hajiya Gambo Sawaba, GH= General Hospital, PCR: Polymerase Chain Reaction

Table 2: Differentiation of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* as determined by nested multiplex PCR in microscopically positive samples according to locations

<table>
<thead>
<tr>
<th>Location</th>
<th>NP PCR</th>
<th>E. histolytica</th>
<th>E. dispar</th>
<th>E. moshkovskii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>GH Kafanchan</td>
<td>3</td>
<td>66.7</td>
<td>2</td>
<td>66.7</td>
</tr>
<tr>
<td>YDGH Kaduna</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>GH Kachia</td>
<td>3</td>
<td>33.3</td>
<td>2</td>
<td>66.7</td>
</tr>
<tr>
<td>HGSGH Zaria</td>
<td>2</td>
<td>50</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>GH Saminaka</td>
<td>2</td>
<td>50</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>GH Birni Gwari</td>
<td>5</td>
<td>20</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>16</td>
<td>37.5</td>
<td>11</td>
<td>68.8</td>
</tr>
</tbody>
</table>

Key: GH = General Hospital, YD = Yusuf Dantsoho, HGS = Hajiya Gambo Sawaba, NP PCR = Number Positive by Polymerase Chain Reaction
Table 3: Pattern of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* infection as determined by nested multiplex PCR in microscopically positive samples

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>No. of samples positive by PCR</th>
<th>Percentage of stool positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. histolytica</em> (mono infection)</td>
<td>3</td>
<td>8.8</td>
</tr>
<tr>
<td><em>E. dispar</em> (mono infection)</td>
<td>7</td>
<td>43.8</td>
</tr>
<tr>
<td><em>E. moshkovskii</em> (mono infection)</td>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td><em>E. dispar</em> + <em>E. histolytica</em> (mixed)</td>
<td>3</td>
<td>18.8</td>
</tr>
<tr>
<td><em>E. dispar</em> + <em>E. moshkovskii</em> (mixed)</td>
<td>1</td>
<td>6.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>16</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

Key: PCR = Polymerase Chain Reaction

**DISCUSSION**

The results of this study revealed that of the 46 microscopy-positive samples, 16 (34.8%) samples successfully amplified *Entamoeba* species DNA by nested multiplex PCR. Our molecular differentiation of *Entamoeba* species showed that *Entamoeba dispar* (68.8%) was observed to be the commonest species detected in this study, followed by *Entamoeba histolytica* (37.5%) and *Entamoeba moshkovskii* (18.8%). The high prevalence of *E. dispar* in the present study agreed with the worldwide distribution of *Entamoeba* species, which indicated that *Entamoeba dispar* is perhaps 10 times more common than *Entamoeba histolytica*, however, the local prevalence may vary significantly, thus necessitating the assessment of prevalence in different in geographical regions. Similar observation also reported that 70.8% of patients were infected with *E. dispar*, compared to 4.5% of *E. histolytica* and 61.8% of *E. moshkovskii* in Australia.[2]

A study in Brazil showed that the prevalence of *Entamoeba dispar* (90%) was more frequent compared to *Entamoeba histolytica* (10%) among infected individuals.[3] Also a study in India showed similar findings, where 49.5% patients were infected with *Entamoeba dispar* and only 7.4% with *Entamoeba histolytica*,[4] while another study in Netherlands reported 91.2% microscopically positive samples were identified as *Entamoeba dispar* and 6.7% were *Entamoeba histolytica* by both PCR and ELISA assay.[5]

This study also showed that 7 (43.8%) contained only *Entamoeba dispar*, 3 (18.8%) contained only *Entamoeba histolytica* and 2 (12.5%) contained only *Entamoeba moshkovskii*. Mixed infection with *Entamoeba dispar* and *Entamoeba histolytica* was found in 3 (18.8%) samples, while only 1 (6.3%) sample had *Entamoeba dispar* and *Entamoeba moshkovskii*. This result is consistent with that of Ngui and co-workers who reported 33 (65.5%) samples contained only *E. histolytica*, 10 (19.2%) contained only *E. dispar* and 3 (5.8%) contained only *E. moshkovskii*. Mixed infection with *E. histolytica* and *E. dispar* was found in 6 (11.5%) samples.[11]

The detection of *E. moshkovskii* (18.8%) in this study, to the best of our knowledge, was the first to be reported in Kaduna State, Nigeria. Many cases of humans infected with *E. moshkovskii* have been reported sporadically from different parts of the world including Thailand,[6] India,[7] and Australia.[8] Another study in India highlighted that *E. moshkovskii* infection was associated with dysentery.[7] In our study, it was noted that all individuals infected with *E. moshkovskii* were children and were symptomatic. Therefore, further investigation which includes the clinical impact of *E. moshkovskii* is imperative for a better understanding of a true pathogenic potential of *E. moshkovskii*.

**CONCLUSION**

The nested multiplex polymerase chain reaction (NM-PCR) method was effective in differentiating *E. histolytica* from *E. dispar* and *E. moshkovskii*. The NM-PCR method is an optional tool in the diagnosis and epidemiological studies of amoebiasis. The correct detection and differentiation of *E.
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**histolytica from E. dispar and E. moshkovskii** will avoid unnecessary treatment of *E. dispar* or *E. moshkovskii*-infected patients with anti-amoebic drugs. We propose the use of PCR in both the routine diagnosis of amoebiasis.

**ACKNOWLEDGEMENT**

We thank Dr C. Graham Clark from the London School of Hygiene and Tropical Medicine for providing us with the lyophilized DNA of standard strains of *E. histolytica* HM-1:1MSS, *E. dispar* SAW760 and *E. moshkovskii* Laredo. We are also grateful to the staff of the DNA Laboratory in Nigeria for their technical assistance.

**REFERENCES**


**Conflict of Interest:** None declared


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