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

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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.[2,3] However, there is as yet no definitive evidence demonstrating that these two species are pathogenic to humans.[4]

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.[5] 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.[8] 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.[9]

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.[2] Moreover, this PCR-based approach is suitable for molecular epidemiological studies, which have been strongly encouraged by the World Health Organization.[8] 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 diarrhoeic 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 Hajiya 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* among *Entamoeba* spp. Molecular techniques are newer methods currently used for the identification of *Entamoeba* spp.[6]

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.[7] In previous studies, many *E. dispar* and *E. moshkovskii* infections were most probably confused with *E. histolytica* infections and were unnecessarily treated.
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.

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.[10] 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

positive samples for *Entamoeba* species were stored at -20^{0}C prior to DNA extraction.
The DNA extraction of all microscopy-positive LC DNA isolation kit (Roche Applied Sciences) according to the manufacturer's instructions. Briefly, genomic DNA was lysed in a buffer containing guanidine isothiocynate and bound to magnetic glass particles under chaotropic conditions. The unbound substances and

Standard strains
The standard strains of *E. histolytica* HM-1: IMSS, *E. dispar* SAW760 and *E. moshkovskii* Laredo strains were used as positive control in this study. The lyophilized DNA of these strains was donated by C. Graham Clark from the London School of Hygiene and Tropical Medicine, London, UK.

NM-PCR
Nested multiplex PCR targeting 16S-like ribosomal RNA gene was used to genetically characterize *E. histolytica*, *E. dispar* and *E. moshkovskii* according to Ngui *et al*.[11] Primary PCR for the detection of Entamoeba genus used forward primer E-1 (5'-TAAGATGCA GAGCGAAA-3') and reverse primer E-2 (5'-GTACAAAGGGCAGGGACGTA-3').[11]
Entamoeba species genomic DNA (positive control) was included in each PCR run.[11] The PCR was carried out in a 25µl volume with the final mix containing 10× PCR buffer, 1.25 mM dNTPs, 25 mM $MgCl_2$, 10 pmole of each primer, 0.3µl of Taq polymerase and 2.5 µl of DNA template.[11] The sample was heated to 96°C for 2 min, followed by 30 cycles of 92° C for 1 min (denaturing), 56°C for 1 min (annealing), 72°C for 1 min 30 s (extension) and a final extension at 72°C for 7 min.[11]

Subsequently, the primary PCR products were subjected to secondary PCR for Entamoeba species-specific characterization.[11] Amplification was achieved using primer sets EH-1 (5'-AAG CATTGTTTCTAGATCTGAG-3') and EH-2 (5'-AAGAGGTCTAACCGAAATTAG-3') to detect *E. histolytica* (439 bp); ED-1 (5'-TCTAATTTCGATTAGAACTCT-3') and ED-2 (5'-TCCCTACCTATTAGACATAGC-3') to detect *E. dispar* (174 bp); Mos-1 (5'-GAAACCAAG AGTTTCACAAC-3') and Mos-2 (5'-CAATATAAGGCTTGGATGAT-3') to detect *E. moshkovskii* (553 bp). The secondary amplification reagent concentrations were similar to the first PCR except that 2.5µl of primary PCR product was added instead of genomic DNA

DNA extraction

samples was carried out using the MagNa Pure 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^{0}c untill required for PCR amplification. template.[11]

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).[11]

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 uktraviolet 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 X^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 i*nfection based on microscopy and nested multiplex PCR assay of faecal samples according to locations

Location	**NE**	**Microscopy**		**PCR assay**	
		n	**%**	**n**	***%**
GH Kafanchan	88	7	8.0	3	42.9
YDGH Kaduna	88	5	5.7	1	20.0
GH Kachia	88	11	12.5	3	27.0
HGSGH Zaria	88	6	6.8	1	16.7
GH Saminaka	88	8	9.1	2	25.0
GH Birni Gwari	88	9	10.2	6	66.7
Total	**528**	**46**	**8.7**	**16**	**34.8**

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

Location	**NP PCR**	***E. histolytica***		***E. dispar***		***E. moshkovskii***	
		n	**%**	**n**	**%**	**n**	**%**
GH Kafanchan	3	2	66.7	2	66.7	1	33.3
YDGH Kaduna	1	0	0	1	100	0	0
GH Kachia	3	1	33.3	2	66.7	0	0
HGSGH Zaria	2	1	50	1	50	1	50
GH Saminaka	2	1	50	2	100	0	0
GH Birnin Gwari	5	1	20	3	60	1	20
Total	**16**	**6**	**37.5**	**11**	**68.8**	**3**	**18.8**

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

Type of infection	No. of samples positive by PCR	Percentage of stool positive (%)
E. histolytica (mono infection)	3	8.8
E. dispar (mono infection)	7	43.8
E. moshkovskii (mono infection)	2	12.5
E. dispar + *E. histolytica* (mixed)	3	18.8
E. dispar + *E. moshkovskii* (mixed)	1	6.3
Total	**16**	**100**

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,*[12] 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.[13] Also a study in India showed similar findings, where 49.5% patients were infected with *Entamoeba dispar* and only 7.4% with *Entamoeba histolytic,*[14] 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.[15]

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,[16] India,[17] and Australia.[2] Another study in India highlighted that *E. moshkovskii* infection was associated with dysentery.[17] 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.*

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.

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Conflict of Interest: None declared