GENETIC ANALYSIS OF A REGION OF 16S-LIKE RIBOSOMAL RNA GENE OF *ENTAMOEBA* SPECIES FROM DIARRHOEIC STOOL SAMPLES IN KADUNA STATE, NIGERIA

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ABSTRACT

Background: Genetic mutations and other polymorphisms in genes, gene systems, or whole genomes are vital in the pathogenesis and epidemiology of Amoebiasis.

Aim: The study aimed at investigating intra-species genetic variation in *E. histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* from stool samples of diarrhoeic patients in Kaduna State, Nigeria.

Methods: The DNA extracted from microscopic positive stool samples was used in the amplification of a part of the genus *Entamoeba* small-subunit ribosomal RNA gene (SSU rDNA) with Nested Multiplex Polymerase Chain Reaction (NM-PCR) and followed by DNA sequencing.

Results: This study revealed that 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*. The study also revealed considerable number of nucleotide polymorphisms in the form of deletion, substitution and punctual insertion mutation at different positions of the 16S-like ribosomal nucleotide sequences of the three Entamoeba species. A total of 14 genotypes of *Entamoeba* species, comprising six *E. histolytica* genotypes, six *E. dispar* genotypes and two *E. moshkovskii* genotypes were identified. The phylogenetic analysis within the sequences of *Entamoeba* species isolates suggested three different variants present among the diarrhoeic patients.

Conclusion: The existence of high level of diversity reported in this study suggests that a rapid generation of new *Entamoeba* variants is occurring in *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* infecting humans. Further studies in the complete ribosomal RNA gene of these species would possibly reveal more genetic information on Entamoeba.

Key words: *Entamoeba histolytica*, Nested Multiplex PCR, DNA sequencing, diarrhoea

INTRODUCTION

The World Health Organization (2010), reported that *Entamoeba histolytica* an intestinal protozoan parasite, accounts for invasive amoebiasis in about 40–50 million people, leading to about 40 000–100 000 global deaths annually. Since amoebiasis was first described more than a century ago by Lösch (1875), there is still uncertainty as to why symptoms of the disease appear only in 10% of those infected, while majority remain asymptomatic (Parija, 2006).

Some factors have been reported to contribute to the outcome of amoebic infection in a susceptible host. These include the virulence of the *E. histolytica* strains and the variability in host immunity against amoebic invasion. While the variability of human immunity against amoebic infection is not well understood, the existence of genetic variation in *E. histolytica* has been studied in depth recently by Rivera *et al*. (2006); Ali *et al*. (2007); Parija and Khaimar (2008) and Sylvain *et al*. (2015).

Studies have revealed the genetic variation in protein-coding sequences of *E. histolytica*, such as those for the serine-rich *E. histolytica* protein by Rivera *et al*. (2006) and chitinase by Haghighi *et al*. (2003), as well as non-protein-coding regions such as the ribosomal RNA (rRNA) genes by Sehgal *et al*. (1993) and loci 1–2 and 5–6 by Ali *et al*. (2007; Parija and Khaimar (2008). The existence of genetic variation in non-protein-coding loci 1–2 and 5–6 has been reported by Pinheiro *et al*. (2005) as well as protein-coding chitinase gene of *E. dispar* has also been documented by Ramos *et al*. (2005).

In studying genetic variation, the rRNAs, especially the 16S rRNA, have been widely used due to their conservative nature and wide distribution (Khamar and Parija, 2007). The existence of genetic variation among *E. histolytica* isolates collected from different geographical areas such as South Africa, India, Bangladesh, the Philippines, Mexico, Venezuela, and Georgia among others has been reported by Zaki and Clark (2001); Simonshvili *et al*. (2005) and Revera *et al*. (2006).

Parija and Khaimar (2008), revealed that mutations and other polymorphisms in genes, gene systems, or whole genomes may play important roles in the pathogenesis and epidemiology of *Entamoeba*. DNA sequencing is considered the gold standard for identifying such mutations). However, to the best of our knowledge, there is no documented evidence of the existence of genetic variation among *Entamoeba* species in Nigeria. In the present study therefore, PCR and DNA sequencing were used to investigate intra-species genetic variation in *E. histolytica, Entamoeba dispar* and *Entamoeba moshkovskii* from diarrhoeic stools in Kaduna State, Nigeria.

MATERIALS AND METHODS

Study area

The present study was carried out in six hospitals in Kaduna State, Nigeria, namely: Kafanchan General Hospital, Kachia General Hospital, Yusuf Dantsoho General Hospital Kaduna, Birnin Gwari General Hospital, Saminaka General Hospital and Hajiya Gambo Sawaba Memorial Hospital, Zaria. Of the six selected hospitals, two hospitals were located in each of the three Senatorial Districts of the State namely; Northern, Central and Southern Senatorial Districts. The hospitals were chosen because they are government hospitals and the most patronized health institutions in the state.

Inclusion and exclusion criteria

Patients who presented with acute and persistent diarrhoea or dysentery syndrome within the period of study were recruited for the study. Patients without diarrhoea or with diarrhoea but on antiparasitic agents were excluded.

Consent and sample collection

The study was approved by the Ethics Committee of the Ministry of Health, Kaduna State, Nigeria. Informed consents were obtained from the patients or parents/guardians. Five hundred and twenty-eight stool samples were aseptically collected from the patients in sterile capped bottles.

Laboratory procedures

Microscopy

The stool samples were analyzed using the Direct Smear and Formol-Ether concentration methods as described by Cheesbrough (2005). Briefly, the Direct Smears were made by placing a drop of normal saline at the center of a microscope slide and small amount of the stool sample was picked and emulsified on the normal saline using a sterile wire loop. A cover slip was gently placed on the sample and using a tissue paper to press gently on the cover slip to make a thin preparation.

While in the Formol-Ether concentration method, 1g of the faecal sample was emulsified in 10ml of normal saline, filtered through a two-layered gauze into a centrifuge tube and spun at 2,500rpm for 2 minutes. The clear supernatant was then discarded and 7ml of formol saline was added to the sediment followed by 3ml of diethyl ether. The preparation was then shaken vigorously and centrifuged for 2 minutes at 2,500rpm. After discarding the faecal debris and ether, the sediment was transferred to a clean glass slide and a drop of iodine was added. The two preparations were covered with cover slips and examined microscopically under x40 objective to detect trophozoites and identify the cysts.

DNA extraction

The DNA extraction of all microscopy-positive samples was done with MagNa Pure DNA isolation kit (Roche Applied Sciences) according to the manufacturer's instruction. Briefly, genomic DNA was lysed in a buffer containing guanidine isothiocynate and bound to magnetic glass under chaotropic conditions. 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 -20^0c until required for PCR amplification.

Primers used

The primer sequences for the nested multiplex PCR (NM-PCR) (Table 1) were as designed by Dawah *el al*. (2016).

Table I: Primer sequences for NM-PCR

	Genus specific primers (First PCR)	Length of PCR products (bp)
Entamoeba genus	Forward-E-15'-TAAGATGCACGAGACGAAA-3' Reverse-E-2 5'-GTACAAAGGGCAGGGACGGTA-3'	222
	Species specific primers (second nested multiplex PCR)	
E.histolytica species	Forward-EH-1 5'- AAGCATTGTTTCTAGATCTGAG-3' Reverse-EH-2 5'-AAGAGGTCTAACCCGAAATTAG-3'	439
E.moshkovskii species	Forward-EM-1 5'-GAACCAAGAGTTTCACAAC-3' Reverse-EM-2 5'CAATATAAGGCTTGGATGAT-3'	553
E. dispar species	Forward-ED-1 5'-TCTAATTTCGATTAGAACTCT-3' Reverse-ED-2 5'-TCCCTACCTATTAGACATAGC-3'	174

Standard strains

E. histolytica HM-1**:** IMSS, *E. dispar* SAW760 and *E. moshkovskii* Laredo were the standard strains used as positive control in this study.

Nested Multiplex PCR

The NM-PCR was carried out as developed by Khaimar and Parija (2007) . Briefly, the reaction volume of 25µl comprised 2.5µl 10x PCR buffer, 1.5µl of 25mM $MgCl_2$, 1.4µl deoxynucleoside triphosphate (5mM each dNTP), 0.3µl (5 IU) of Taq polymerase, 0.3µM of each primer (IDT) and 2.5µl of template DNA was added in genus specific and species specific PCR. The PCR tubes were placed in a thermal cycler (Master cycler gradient).

The genus specific PCR mixture was subjected to an initial denaturation at 96^0C for 2 minutes, followed by 30 cycles – consisting of 92^0C for 60 seconds (Denaturation), 56^0C for 60 seconds (Annealing) and 72^0C for 90 seconds (Extension). Finally, one cycle of extension at 72^0C for 7 minutes was performed.

In the species specific nested multiplex PCR (which had multiple primer sets in the same tube), only the annealing temperature was changed to 48^0C, leaving the other parameters of the amplification cycles unchanged (Khaimar and Parija, 2007).

Agarose Gel Electrophoresis

Three micro litres of the amplification products was separated by electrophoresis through 1.8% Agarose gel in 0.5 x Tris-borate-EDTA at 120V for 45 minutes and was visualized by ethidium bromide staining under UV light for bands of DNA of appropriate sizes. Control reactions were included with each batch of samples analyzed by nested multiplex PCR.

DNA Sequencing

PCR products of 16S-like rRNA genes of *E. histolytica*, *E. dispar* and *E. moshkovskii* were sequenced on ABI3730XL sequencer (Macrogen, Seoul, South Korea) using species specific primers as described by the manufacturer. The sequences were compared to those available in the GenBank database with the BLAST program run on the National Center for Biotechnology Information Server (http://www.ncbi.nlm.nih.gov/BLAST) to validate these DNAs as those of *E. histolytica*, *E. dispar* or *E. moshkovskii* origin. The nucleotide sequences were edited with reference to chromatographs using Chromas (Version 1.6.2) and aligned using CLC Mainwork Bench (version 7.8.2) software. The phylogenetic tree was constructed using the Neighbor-Joining Method and bootstrap analysis of 100 replicates (Ngui *et al*., 2012).

RESULTS

The results revealed that 16 microscopically positive samples successfully amplified *Entamoeba* species DNA by Nested Multiple PCR as shown in figure 1 below.

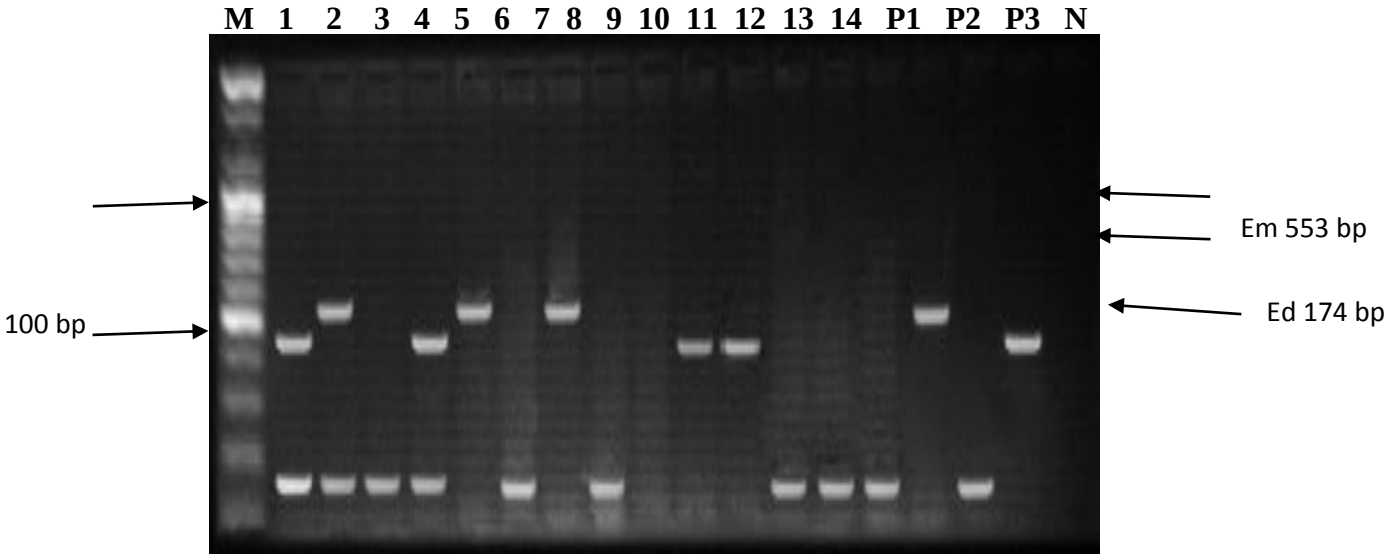

Figure 1: Nested Multiplex PCR on stool samples. EH = *E. histolytica*, ED = *E. dispar* and EM = *E. moshkovskii*, bp = base pair, M = DNA marker (Ladder), P1, P2, P3 = positive controls and N= negative control.

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 2.

Table 2: 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

Results of multiple sequence alignment of *Entamoeba histolytica* PCR products and the standard strain (*Entamoeba histolytica* HM-1: IMSS) showed an insertion of C at position 11 in stool sample 5, substitution of C with T at position 68 in stool samples 1, 4 and 5 respectively, deletion of A at position 147 in stool sample 4 and substitution of G with C at position 220 in sample 5, substitution of C with G at positions 211 and 238 in sample 6, while samples 2 and 3 showed no mutation as shown in figure 2 below

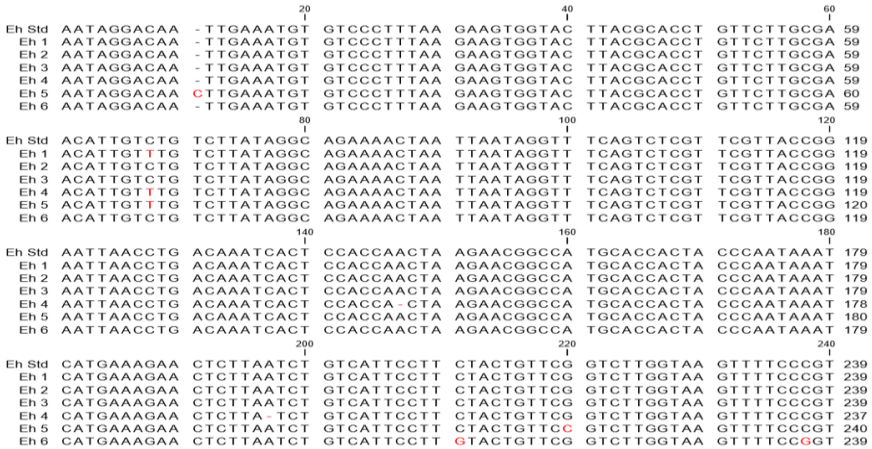

Figure 2: Multiple sequence alignment of *E. histolytica* specific 439 bp PCR products from stool samples 1 to 6 and the standard strain *E. histolytica* HM-1: IMSS (Eh-Std). The sequence variations are highlighted in red.

Results of multiple sequence alignment of *Entamoeba moshkovskii* PCR products and the standard strain (*Entamoeba moshkovskii* Laredo) in Figure 3 showed a deletion of T at position 36 in sample 1, substitution of T with C at position 34 and T with A at position 81 in stool sample 2, substitution of A with T at position 222 and A with T at position 358 in stool sample 3.

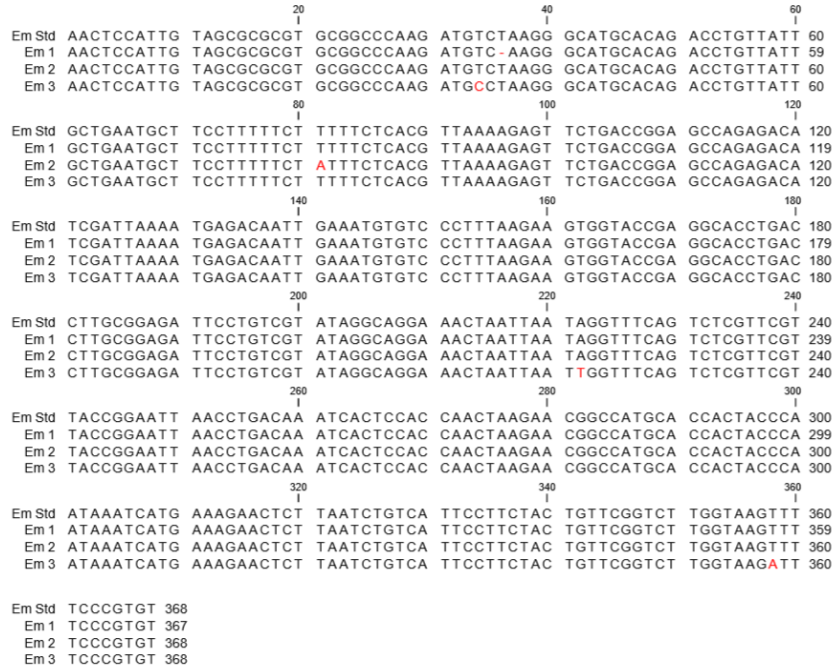

Figure 3: Multiple sequence alignment of the *E. moshkovskii* specific 553 bp PCR products from diarrhoeic stools (Samples 1 to 3) and the standard strain *E. moshkovskii* Laredo (Em-Std). The sequence variations are highlighted in red.

Results of multiple sequence alignment of representatives *Entamoeba dispar* PCR products and the standard strain (*Entamoeba dispar* SAW760) showed substitution of C with G at positions 40 in stool sample 2, deletion of C at position 53 in stool sample 3, substitution of G with C at position 65 in stool samples 1, 2 and 3 respectively, substitution of C with G at position 114 in stool sample 2, A with G at position 131 in Sample 4, A with T at position 121 and T with A at position 129 in sample 6 as shown in figure 4 below.

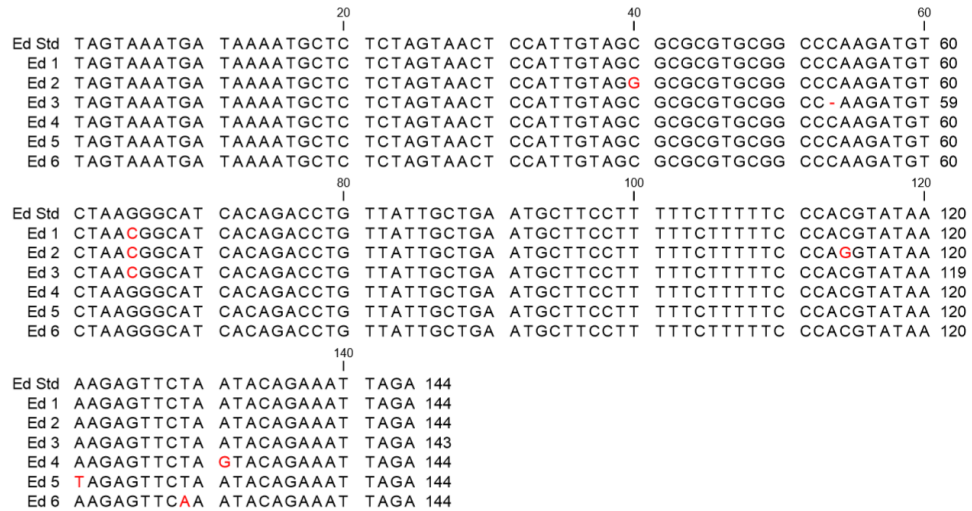

Figure 4: Multiple sequence alignment of *E. dispar* specific 174 bp PCR products from diarrhoeic stools (Samples 1 to 6) and the standard strain *E. dispar* SAW760 (Ed-Std). The sequence variations are highlighted in red.

The phylogenetic analysis showed three clades (group of clusters different from each other) of sequences of *Entamoeba* species comprising one (1) clade of *E. histolytica*; one (1) clade of *E. dispar* and one (1) clade of *E. moshkovskii*. All sequences of the three *Entamoeba* species (Figure 5) isolated from the patients were closely related and had a common evolutionary origin. While a strain of *Escherichia coli* deposited at the GenBank served as an out-group.

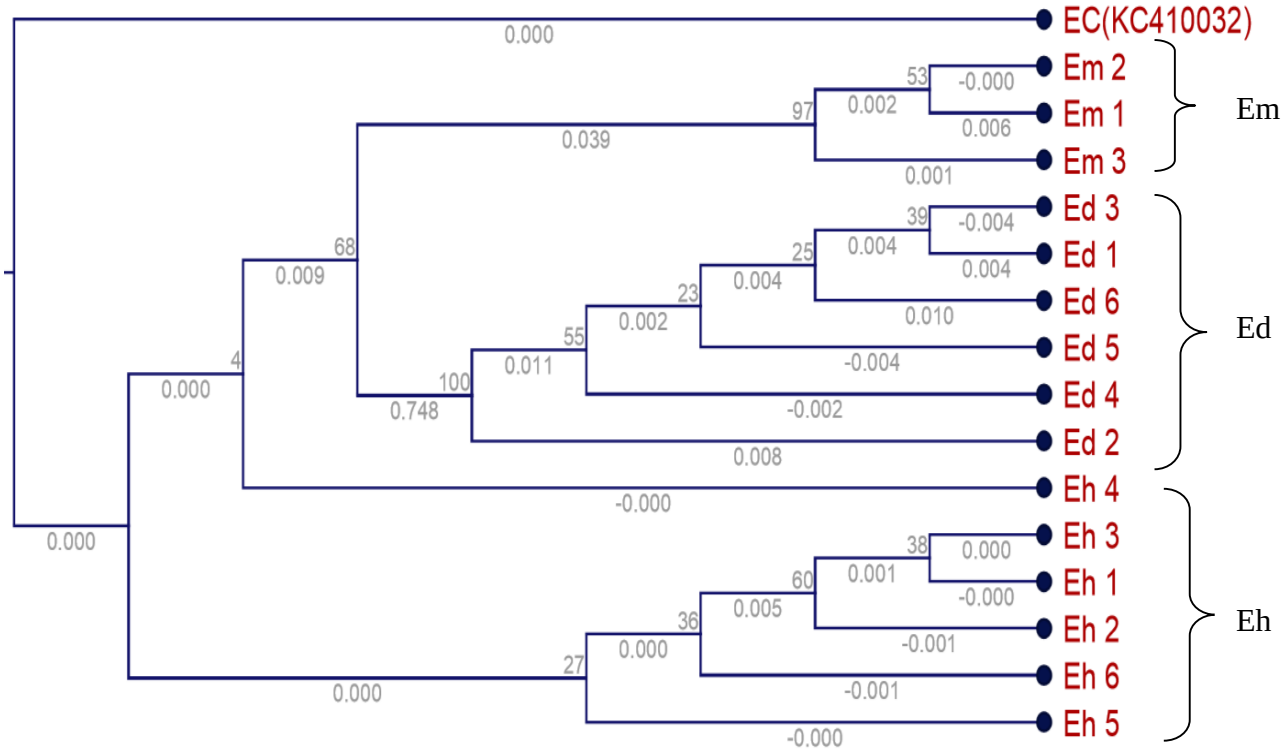

Figure 5: Phylogenetic tree based on partial 16S ribosomal RNA gene sequences, showing the relationships among the identified *Entamoeba* species: *E. histolytica* (Eh), *E. dispar* (Ed) and *E. moshkovskii* (Em) with an *Entamoeba coli (*EC) isolate deposited at the NCBI Genbank from Ghana and the standard strains (Std). The phylogenetic tree was constructed using the Neighbor-Joining method and a bootstrap analysis of 100 replicates. Numbers above branches are bootstrap values while numbers below are branch distances.

DISCUSSION

This study showed that out of the 16 PCR positive samples, 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 *et al*. (2012) 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. The PCR products of ribosomal RNA gene sequenced showed that all the *E. dispar* amplicons had 98% similarity to the *E. dispar* sequences in GenBank (e.g. accession no. KP722600.1),

whereas all the *E. histolytica* sequences showed high similarity (99%) to the *E. histolytica* sequences in GenBank (e.g. accession no. KP233840.1) and all of the *Entamoeba moshkovskii* amplicons showed 99% similarity to the *Entamoeba moshkovskii* sequences in GenBank (e.g. accession no. KP722605.1).These results confirmed the DNAs amplified as being of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* origin and an indication that they are all of the same origin.

Genetic analysis in this study showed the existence of intra-species genetic variation (mutation) in *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii*. Nucleotide polymophism in the form of substitution, deletion and insertion at different positions of the sequences were observed. These results agreed with the findings of Jaco *et al.* (2001); Tanyuksel *et al*. (2008); Parija and Khaimar (2007) who observed considerable number of nucleotide polymorphism in the of deletion, substitution and punctual insertion mutations at different positions of the DNA sequences of the three *Entamoeba* species.

This study revealed 14 genotypes of *Entamoeba* species, comprising six *E. histolytica* genotypes, six *E. dispar* genotypes and two *E. moshkovskii* genotypes. Ali *et al*. (2007) in Bangladesh, involved clinical specimens using six tRNA-linked STR loci, and revealed 85 genotypes in 111 unrelated samples. Haghighi *et al*. (2003) documented a total of 53 different genotypes among 63 isolates of *E. histolytica*, mostly from Japan and Thailand, using sequencing of four loci (two tRNA-linked STR loci, chitinase, and SREHP). Parija and Khaimar (2008) also identified four new *E. histolytica* genotypes and three new *E. moshkovskii* genotypes in India using PCR-SSCP analysis.

The phylogenetic tree presented three clades (group of clusters different each to another) of sequences of *Entamoeba* species comprising one (1) clade of *E. histolytica*; one(1) clade of *E. dispar* and one (1) clade of *E. moshkovskii*. All sequences of the three *Entamoeba* species isolated from the patients were closely related to the standard strains (*E. histolytica* HM-1: IMSS, *E. dispar* SAW760 and *E. moshkovskii* Laredo) respectively. This suggests that all the isolates have a common evolutionary origin. This result is consistent with the results of Sylvain *et al*. (2015) who reported three clades of sequences of clinically important *Entamoeba* species (one clade of *E. histolytica*; one clade of *E. dispar* and one clade of *E. moshkovskii*) isolated from HIV patients in Cameroon.

CONCLUSION

The existence of high level of genetic diversity reported in this study suggests that a rapid generation of new *Entamoeba* variants is occurring in *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* infecting humans. The close evolutionary relationship of *E. dispar* and *E. moshkovskii* genotypes with a recognized human pathogen like *E. histolytica* should prompt further studies. In addition, further studies will be useful to extend genetic variation analysis to the complete ribosomal RNA gene of these species, which would possibly elucidate more about genetic variation in *Entamoeba*.

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Conflicts of Interest

There is no competing interest in anyway.

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