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Molecular differentiation of *Entamoeba Spp.* isolated from Cameroonian human immunodeficiency virus (HIV) infected and uninfected patient

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***Entamoeba histolytica* is an utmost important cause of dysentery. *Entamoeba* spp. has been frequently reported in human immunodeficiency virus (HIV) positive individuals. Routine microscopic examination of stool sample is a most widely used technique but microscopy alone has low sensitivity and it is insufficient for differentiation among *Entamoeba* spp. Molecular techniques are newer methods which are currently used for the identification of *Entamoeba* spp. The present study was planned to differentiate the *Entamoeba* species by gene sequencing for the confirmation of microscopic findings in stool samples of HIV positive and negative patients of Cameroon. Out of 265 patients diagnosed microscopically for *Entamoeba,* 90 positive stool samples (28 from HIV patients) were collected and studied for the differentiation of *Entamoeba* species. DNA was extracted from infested stool samples and used to amplify a part of the genus *Entamoeba* small-subunit ribosomal RNA gene (SSU rDNA) as well as the serine rich *E. histolytica* protein gene and chitinase gene. The SSU rDNA were sequenced to identify the other species that could not be done by polymerase chain reaction (PCR), and for the differentiation of *E. histolytica* from *Entamoeba dispar* and *Entamoeba moshkovskii.* Sequence analysis identified seven different species of *Entamoeba* which were related to *Entamoeba*; *E. histolytica* (28.7%), *E. dispar* (25%), *E. moshkovskii* (10%), *Escherichia coli* (16.3%), *Entamoeba hartmanni* (6.2%), *Entamoeba polecki* (11.3%) and *Entamoeba struthionis* (7.5%), with the higher prevalence of *E. histolytica among* HIV infected patients than uninfected individuals. The phylogenetic analysis within the sequences of *E. histolytica* isolates suggested two distinguishable variants present among Cameroonian HIV patients. There is a possibility that specific genotypes may be more prevalent among HIV positive patients, and molecular diagnosis is important in establishing the correct diagnosis of amoebic dysentery.**

Key words: *Entanoeba* spp, HIV/AIDS, gene sequencing, Cameroon.

INTRODUCTION

Various *Entamoeba* species are often found in the stools of humans. Although, the majority of these *Entamoeba* spp. are considered to be harmless, care should be taken when *Entamoeba histolytica*, the causative agent of

amoebiasis, is involved. Infection with this gastrointestinal parasite may cause hemorrhagic dysentery, extra intestinal pathologies (example, liver abscesses) and death (Santos et al., 2010). Moreover, amoebiasis remains a significant cause of morbidity and mortality in the world. This infection is of major concern in public health, causing up to 100,000 deaths worldwide each year (WHO, 1997, 1997; Stauffer et al., 2006). In African countries, prevalence of *Entamoeba* spp. has been reported to vary from 1.4 to 12.4% (Gassama et al., 2001; Brink et al., 2002; Hailemariam et al., 2004; Samie et al., 2010).

Following the Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) pandemic, numerous studies demonstrated that intestinal parasites such as *Cryptosporidium* spp., *Microsporidia* spp., *Cystoisospora belli* and *Cyclospora cayetenensis* were frequently associated with episodes of severe, and often fatal diarrhea in both industrialized and developing countries (Stark et al., 2009; Nissapatorn et al., 2011, O'Connor et al., 2011). Currently, little is known about the occurrence of different *Entamoeba* spp. and their genotypes in co infection with HIV in Cameroon. However, some studies conducted in Mexico, South Africa and Taiwan on the *E. histolytica* and HIV co-infected patients demonstrated a high prevalence of infection with *E. histolytica* (Moran et al., 2005; Tsaï et al., 2006; Nkenfou et al., 2013). These studies were based on the detection of cysts or trophozoites in stool samples by using light microscopy or by detection of specific antibodies by serology in serum samples. However, differentiation between *E. histolytica* and other *Entamoeba spp.* (such as *Escherichia coli, Entamoeba hartmanni* and *Entamoeba polecki* like organisms) based on morphological features is difficult, and when *Entamoeba dispar or Entamoeba moshkovskii* is involved, it is impossible. Therefore, molecular methods, such as DNA-based tests, have aided in improving some of the sensitivity and specificity deficiencies associated with traditional methods for the detection of protozoan pathogens. A number of DNA-based assays like gene amplification with specific primers, multiplex polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and real-time PCR (RT-PCR) and gene sequencing have been developed for the identification of *Entamoeba* species infections (Fotedar et al., 2007; Samie et al., 2008; Bruijnesteijn van Coppenraet et al., 2009). To the best of this study knowledge, there is no study available from Africa in which PCR along with gene sequencing have been used for the identification of *Entamoeba* species and its subtypes isolated from HIV infected and uninfected patients. However, previously published studies are either based on serology (Jackson et al., 2000), microscopy or PCR (Zaki et al., 2003). The *Entamoeba* spp. that can be found in these patients remain unknown in Cameroon, and most of Sub-Saharan Africa. To fill this gap a molecular differentiation of *Entamoeba spp.* was performed among HIV positive and negative patients in two cities of Cameroon (Dschang and Ngaoundere).

METHODOLOGY

Ethics statement

This study was approved by the Cameroon National Ethic Committee (CNE) under the registration No. 131/CNE/SE/2012. The rules and regulations of good clinical laboratory practice were followed during the study. Participants consulting at the hospitals were kindly requested by the study team to participate in the study. All interested adult subjects provided written informed consent, and an interested parent or guardian of any child participant provided written proxy consent. All participants were offered professional counseling before and after HIV testing for those who had never done it before. All diagnostic results were kept strictly confidential. Anti-amoebic therapy treatments (metronidazole) were given to all participants who were found to be infected with *E. histolytica*.

Sample collection

A total of 265 patients (60 HIV positive patients) were recruited and diagnosed in the present study from July, 2012 to May, 2013 from two cities of Cameroon (Ngaoundere and Dschang) after obtaining their written informed consents. Out of 265 patients diagnosed for *Entamoeba* spp. infection, 90 stool samples (28 from HIV patients and 62 from HIV uninfected individuals) in which cysts or trophozoïtes of *Entamoeba* were detected by microscopic observation were further processed for confirmation by molecular method. Stool samples were kept in 2 ml Eppendorf tubes and stored at -20°C till further use.

Genomic DNA isolation from stool samples

For DNA extraction, stool samples of patients from Cameroon and *E. histolytica* strain grown on polyxenic medium at the Department of Medical Parasitology of Postgraduate Institute of Medical Education and Research, Chandigarh, India were used. Approximately, 200 mg of stool sample was taken to extract DNA using QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's protocol with few modifications: all the centrifugations steps were carried out at 800 g except the final step of purification in which centrifugation was done at 1300 g. *E. histolytica* strain was harvested from culture at mid log phase and centrifuged at 3000 rpm. The pellet was washed with PBS buffer pH 6.8 and resuspended in the same buffer. A 200 µl volume of this suspension was used to extract DNA from cysts and trophozoites of *Entamoeba* as described above. The extracted DNA from culture was used as positive control for the amplification reactions. The purity of the extracted DNA was estimated from the absorbance ratio 260/280 and its concentration in all the samples was estimated from the 280 nm readings.

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PCR amplification of the targeted genes

Three loci have been targeted for the amplification reactions: small subunit of ribosomal DNA (SSU rDNA), chitinase gene and serine rich *E. histolytica* protein (SREHP). The SSU rDNA gene has been previously used for the identification of the species (Clark and Diamond, 1991; Novati et al., 1996; Verweij et al., 2001). In fact, the chitinase and SREHP genes have polymorphic DNA loci which have been used to study the molecular epidemiology and the geographical diversity among human isolates of *E. histolytica* (Ghosh et al., 2000; Haghighi et al., 2002; Takano et al., 2007). Specific primers used for the three set of genes were as follows: the Sense Primer known as Entam1 5'GTT GAT CCT GCC ATT ATA TG 3' and the Antisense Primer known as Entam2 5'CAC TAT TGG AAT TAC 3'for the small subunit of ribosomal RNA (Ghosh et al., 2000), Sense Primer or SREHP1 5'GCT AGT CCT GAA AAG CTT GAA GAA GCT G and the Antisense Primer or SREHP2 5'GGA CTT GAT GCA GCA TCAAGG T 3'for the amplification of SREHP gene, the Sense Primer or EHF 5' GGA ACA CCA GGT AAA TGT ATA 3' and the Antisense Primer or EHR 5'TCT GTA TTG TGC CCA ATT 3' for the chitinase gene (Haghighi et al., 2002; Takano et al., 2007). PCR amplification of the SSUrDNA gene was performed in a total volume of 40 µl containing 2.5 µl of 10× PCR buffer, 3 µl of 25 mM $MgCl_2$, 250 µM of each dNTP, 25 pmol of each primer (Entam1 and Entam2), 2.5 U of Taq DNA Polymerase (Promega) and 3µl of genomic DNA sample. PCR mixture was submitted to denaturation at 94°C during 5 min, then to 35 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min followed by the final step of extension at 72°C for 10 min. For the amplification of the SREHP gene and chitinase, only the samples found positive for the genus *Entamoeba* (SSUrDNA) gene amplification were chosen. PCR amplification reactions with SREHP and chitinase primers were performed in a total volume of 40 µl containing 2.5 µl of 10× PCR buffer, 6 µl of 25 mM $MgCl_2$, 250 µM of each dNTP, 25 pmol of each primer (EHF and EHR), 2.5 U of Taq DNA Polymerase (Promega) and 3 µl of genomic DNA sample. PCR mixture was submitted to denaturation at 94°C during 5 min, then to 45 cycles at 94°C for 1 min, 60°C (SREHP) or 50°C (chitinase) for 1 min and 72°C for 1 min followed by the final step of extension at 72°C for 7min. To visualize the amplified genes, 5 µl of the PCR mixture were submitted to 1.5% agarose gel electrophoresis containing ethidium bromide. The migration was done under a voltage of about 78 to 80 mV and a current of 34 mA. This migration was followed by mixing the sample with the loading buffer containing bromophenol blue dye. After migration, the gel was visualized by Transluminscence (UVITEC Transluminator, Cambridge CB4 1QB-England) and photographed.

Gene sequencing and sequence analysis

The 550 bp PCR products containing the SSUrDNA locus were directly sequenced with appropriate primers in both directions. All of the PCR samples that were found to contain single bands on the agarose gels were treated with a Pre-Sequencing kit (USB Corporation, Cleveland, Ohio) before sequencing. Each 550 bp DNA fragment of the PCR samples that showed double or triple bands by agarose gel electrophoresis were excised and treated using a QIAquick gel extraction kit (Qiagen, Hilden-Germany). Individual PCR products were then sequenced using an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), according to the manufacturer's directions. The SSUrDNA PCRs products that generated multiple sequencing products which appeared as mixed profile in sequencing reaction, were purified with Qiaquick gel extraction Kit (Qiagen, Hilden, germany) and cloned using pCR2.1-TOPO vector as described in the protocol from the TOPO TA cloning Kit (Invitrogen, Carlsbad, CA,USA) (Santos et al., 2010). The sequences obtained were manually edited and aligned using ClustalW2. The phylogenetic tree based on the partial 16S like SSUrDNA sequences showing the distance among clinically important species of *Entamoeba* (*E. histolytica, E. dispar* and *E. moshkovskii*) were constructed (Saitou and Nei, 1987). Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2004; Tamura et al., 2013). The accession numbers of the nucleotide sequences used as reference in that construction were as follows: *E. dispar* (Z49256.1) *E. histolytica* (AB197936.1) and (X64142), *E. struthionis* (AJ566411.1), *E. coli* (ST1 or AF149915, and ST2 or AF149914), *E. polecki* (EF110881.1), *E. hartmanni* (AF149907.1), *E moshkovskii* (AF149906.1), *Entamoeba invadens* (AF149905) and *E. chattoni* (AF149912).

Nucleotide sequence accession numbers

The nucleotide sequence data reported in the present work have been submitted to the GenBank/EMBL/DDBJ database under accession numbers AB845670 to AB845674; AB851494 to AB851500; KF515235 to KF515253 and KF870200 to KF870233.

Statistical analysis

Data were registered in Microsoft excel 2010 and analyzed with Statistical Package for the Social Science (SPSS) version 11.0 statistical software. Chi square (χ^2) test allowed us to compare the prevalence of *Entamoeba* infection according to HIV status. Associations were tested at 95% confidence.

RESULTS

A total of 265 patients were recruited and their stools were examined microscopically for *Entamoeba* spp. Of these, 90 samples were diagnosed as *Entamoeba* positive (28 samples from HIV patients and 62 from non-HIV patients) were collected and studied for the differentiation of *Entamoeba* species. Out of 90 stool samples positive for all the *Entamoeba* species, 45 (50%) samples were positive for *E. histolytica* as initially diagnosed by microscopy; 80 (88.9%) (Table 1) were positive for PCR of the genus specific *Entamoeba* with the SSUrDNA primers set and 23 (28.7%) were positive for *E. histolytica* with the chitinase and SREHP primers set (Table 1). Ten samples (11.1%) initially diagnosed microscopically positive for *Entameoba* spp. were negative by PCR (Table 1). After performing sequencing and Basic Local Alignment Search Tool (BLAST) similarity of the different sequences, the result (Table 2) showed that 7 different species of *Entamoeba* that is, *E. histolytica* (28.7%), *E. dispar* (25%), *E. moshkovskii* (10%), *E. coli* (16.3%), *E. hartmanni* (6.2%), *E. polecki* (11.3%) and *E. struthionis* (7.5%) were found in 80 PCR confirmed stool samples. *E. histolytica*, *E. coli*, *E. hartmanni* and *E. struthionis* were found to be more prevalent in HIV infected patients (33.3, 20.8, 8.3 and 8.3% respectively) than in negative cases (25, 14.3, 5.4 and 7.1% respectively). *E. dispar*, *E. moshkovskii* and *E. polecki* (25, 11.7 and 11.3%) were more prevalent within the HIV uninfected individuals. However, multispecies

Table 1. Overall table showing the species of *Entamoeba* isolated from stool samples of Cameroonian HIV infected and uninfected patients by PCR and gene sequencing after microscopy diagnosis. EH= *E. histolytica* cyst; EC = *E. coli* cyst; NIA= Non Identified Amoeba cyst; += positive; - = negative. NB; three cases of double species infection occurred and are mentioned in the table.

Samples code	HIV status	Microscopy	PCR amplification			Sequencing of SSUrDNA gene
			SSUrDNA	SREHP	Chitinase	
TA	-	NIA	+	-	-	*E. coli*
DS	-	NIA	+	-	-	*E. polecki*
NS	-	EH	+	-	-	*E. dispar*
TE	-	EH	+	+	+	*E. histolytica/E.moshkovskii*
DM	-	NIA	+	-	-	*E. coli*
TM	-	NIA	+	-	-	*E. coli*
SD	-	EH	+	-	-	*E. dispar*
EM	-	EH	+	-	-	*E. dispar*
CN	-	NIA	+	-	-	*E. coli*
KC	+	EC	-	*Not done*	*Not done*	*Not done*
DR	-	EC	+	-	-	*E. coli*
LM	-	EH	+	+	+	*E. histolytica*
LF	-	EC	+	-	-	*E. hartmanni*
AF	-	NIA	+	-	-	*E. coli*
AI	-	EH	+	+	+	*E. histolytica/E. dispar*
AJ	-	EH	+	-	-	*E. dispar*
LA	-	EH	+	-	-	*E. moshkovskii*
ME	-	EH	+	-	-	*E. dispar*
1010	-	NIA	-	*Not done*	*Not done*	*Not done*
1011	-	EH	+	+	+	*E. histolytica*
1012	+	EC	+	-	-	*E. coli*
1013	-	EH	+	+	+	*E. histolytica*
1014	-	EH	+	+	+	*E. histolytica*
1015	-	EC	+	-	-	*E. struthionis*
1016	+	EH	+	-	-	*E. dispar*
1017	+	NIA	+	-	-	*E. hartmanni*
1018	+	EH	+	-	-	*E. moshkovskii*
1019	-	EH	+	-	-	*E. dispar*
103	-	EC	+	-	-	*E. polecki*
109	-	EC	+	-	-	*E. coli*
1234	+	NIA	+	-	-	*E. polecki*
1145	+	EC	-	*Not done*	*Not done*	*Not done*
1211	+	EH	+	+	+	*E. histolytica*
230	+	EH	+	-	-	*E. dispar*
235	+	EH	+	+	+	*E. histolytica*
434	+	EC	+	-	-	*E. struthionis*
S1	-	EH	+	-	-	*E. moshkovskii*
S2	-	EC	-	*Not done*	*Not done*	*Not done*
S3	-	EC	-	*Not done*	*Not done*	*Not done*
113	-	EC	+	-	-	*E. coli*
121	+	EH	+	+	+	*E. histolytica*
1262	+	EH	+	+	+	*E. histolytica*
1277	-	EC	+	-	-	*E. polecki*
1273	-	EC	+	-	-	*E. polecki*
114	+	EH	+	+	+	*E. histolytica*
139	+	EH	+	+	+	*E. histolytica*
67	-	EH	-	*Not done*	*Not done*	*Not done*

Table 1. Contd.

94	-	EH	+	+	+	*E. histolytica*
833	-	EC	+	-	-	*E. polecki*
172	-	NIA	-	*Not done*	*Not done*	*Not done*
1083	-	EC	+	-	-	*E. polecki*
MO	-	EH	+	-	-	*E. dispar*
1040	-	EC	+	-	-	*E. coli*
4121	-	EH	+	-	-	*E. dispar*
0 3702	-	EH	+	+	+	*E. histolytica*
43121	+	EC	-	*Not done*	*Not done*	*Not done*
1874	-	EC	+	-	-	*E. dispar*
1062	+	EH	-	*Not done*	*Not done*	*Not done*
11664	-	EH	+	-	-	*E. dispar*
11804	+	EH	+	-	-	*E. dispar*
7335	+	NIA	+	-	-	*E. struthionis*
1073	+	NIA	+	-	-	*E. struthionis*
4142	-	NIA	+	-	-	*E. hartmanni*
11673	-	EC	+	-	-	*E. coli*
FC	-	EH	+	-	-	*E. dispar*
KH	+	EC	+	-	-	*E. coli*
AO	+	EH	+	+	+	*E. histolytica*
MJ	+	NIA	+	-	-	*E. polecki*
NM	-	NIA	+	-	-	*E. struthionis*
DF	-	NIA	+	+	+	*E. histolytica*
NR	-	EH	+	-	-	*E. moshkovskii*
TB	-	EH	+	+	+	*E. histolytica*
NT	-	NIA	+	-	-	*E. moshkovskii*
TH	-	NIA	-	*Not done*	*Not done*	*Not done*
SV	-	NIA	+	-	-	*E. struthionis*
TI	-	EH	+	+	+	*E. histolytica*
SR	-	EH	+	-	-	*E. dispar*
DO	-	EH	+	+	+	*E. histolytica*
VJ	-	NIA	+	-	-	*E. hartmanni*
NC	+	EC	+	-	-	*E. Coli*
MB	-	EH	+	+	+	*E. histolytica*
ER	-	NIA	+	-	-	*E. hartmanni*
SO	+	EH	+	+	+	*E. histolytica*
YU	+	EH	+	-	-	*E. dispar*
ML	-	EH	+	-	-	*E. moshkovskii*
TL	-	NIA	+	-	-	*E. moshkovskii*
TF	-	EH	+	+	+	*E. histolytica/E. dispar*
BB	-	EH				*E. dispar*
DJ	-	EH	+	-	-	*E. dispar*
DB	-	NIA	+	-	-	*E. polecki*

infection was rare in this study population. HIV individuals were infected with only one species while in HIV uninfected group, 3 individuals (3.2%) were infected with two species (*E. histolytical*, *E. moshkovskii* and *E. histolytica*/*E. dispar*).

The phylogenetic tree was constructed using the Neighbor-Joining Method (Figure 1), which shows the distances between sequences of three clinically important *Entamoeba species* (*E. histolytica*, *E. dispar* and *E. moshkovskii*) isolated from both HIV infected and uninfected individuals. This phylogenetic tree presents four clades (group of clusters different each to other) of

Table 2. Prevalence of *Entamoeba spp* differentiated in stool samples of Cameroonian HIV infected and uninfected patients tested by PCR and gene sequencing (p<0.002).

Species	HIV/AIDS patients (%)	HIV negative (%)	Overall population infected by each species (%)
E. histolytica	8 (33.3)	15 (26.8)	23 (28.7)
E. dispar	3 (12.5)	15+2* (30.3)	18+2* (25)
E. moshkovskii	1 (4.2)	6+1* (12.5)	7+1* (10)
E. coli	5 (20.8)	8 (14.3)	13 (16.3)
E. hartmani	2 (8.3)	3 (5.4)	5 (6.2)
E. polecki	3 (12.5)	6 (10.7)	9 (11.3)
E. struthIonIs	2 (8.3)	4 (7.1)	6 (7.5)
totaux	24 (100)	56+3* (100)	80+3* (100)

NB: *= double species infection.

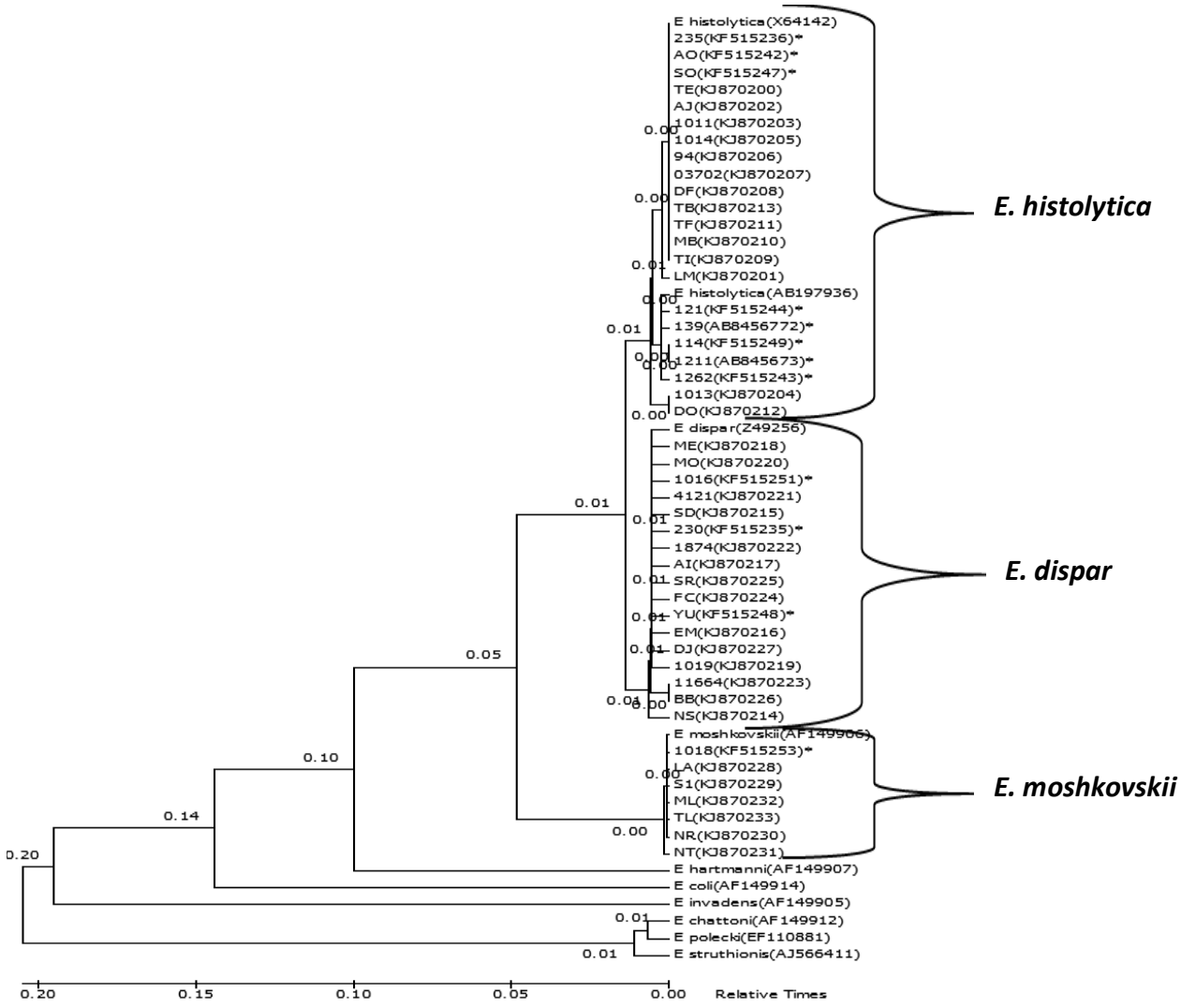

Figure 1. Phylogenetic tree based on partial SSUrDNA sequences, showing the relationships among clinically important identified species of *Entamoeba* (*E. histolytica*, *E. dispar* and *E. moshkovskii*). Phylogenetic analysis used two different approaches, distance-based analysis and maximum-likelihood (ML), produced trees with identical topologies of which only ML tree is presented. GenBank accession numbers are given in parentheses after the taxon name. Sequences without taxons were obtained during this study. Numbers above branches are relative time values from 1,000 replicates. N.B:* =sequences isolated from HIV positive patients.

sequences of clinically important *Entamoeba species* (2 clades of *E. histolytica*; 1 clade of *E. dispar* and 1 clade of *E. moshkovskii*). *E. histolytica* sequences isolated from all the patients is represented in two clades closely related respectively to the reference sequences of *E. histolytica* (AB197936) and (X64142). *E. histolytica*, *E. dispar* and *E. moshkovskii* were aligned with reference sequences of corresponding *Entamoeba* species retrieved from the gene bank (X64142, Z49256, and AF149906). Comparison of these sequences revealed that out of 8 sequences of *E. histolytica* isolated from HIV patients, 5 have 99.1% identity with the reference sequence X64142 whereas 3 isolates have 100% similarity with reference to the same sequence (Figure 2). *E. dispar* sequences isolated from HIV patients have 100% similarity with reference sequence Z49256. Among HIV negative patients, the majority of *E. histolytica* sequences have 100% similarity with reference sequence X64142 except three sequences (KJ870201; KJ870204 and KJ870212) that have 98.9% similarity with the same reference sequence. Only one sequence of *E. dispa*r (KJ870214) has 99.2% similarity with reference sequence Z49256 (Figure 3), and majority of the other sequences of *E. dispar* have 100% similarity with the same reference sequence. Same observation is made with *E. moshkovskii* sequences (Figure 4) among the sequences that have 99.2% similarity with reference sequence AF149906, and one sequence (KJ870231) has 100% similarity with the same reference sequence.

DISCUSSION

Intestinal opportunistic parasites such as *Cryptosporidium* spp., Microsporidia spp., *Cystoisoisospora belli* and *Cyclospora cayetanensis* are utmost importance cause of diarrhea among HIV positive individuals (Stark et al., 2009). *Entamoeba spp*. has been reported to colonize with increased frequency among HIV positive individuals (Hung et al., 2005; Watanabe et al., 2011). Recent data have shown an increase in the occurrence of *E. histolytica* among HIV patients in countries such as Japan, Mexico, Taiwan and South Africa (Moran et al., 2005; Hung et al., 2008; Samie et al., 2008; Watanabe et al., 2011). With the hall mark of HIV infection being the depletion of CD4+ T cells count (below 200 cells/µl) and the progressive decline of the mucosal immunologic defense mechanisms, HIV/AIDS patients become more prone to life-threatening gastrointestinal infections such as diarrhea due to opportunistic pathogens (Stark et al., 2009).

E. histolytica is an important cause of dysentery, and can also manifest as extra-intestinal invasive form. Majority of the infections are asymptomatic and in about 10% of the cases it is symptomatic (WHO, 1997). Laboratory diagnosis of the etiological agent of diarrhea/ dysentery is of utmost important for the timely management of dysentery cases. Routine microscopic examination of stool sample is the most widely used technique for identifying the parasitic cause of diarrhea. However, microscopy alone is insufficient for differentiation between *E. histolytica*, *E. dispar* and *E. moshkoweskii*. It also suffers from low sensitivity (<10%) and specificity (Huston et al., 1999; Fotedar et al., 2007). There are other diagnostic methods such as zymodeme analysis which is cumbersome to perform (Sargeaunt et al., 1978). Molecular techniques such as PCR (Tanyuksel et al., 2003; Solaymani et al., 2006), RFLP (Hooshyar et al., 2003), real time PCR (Hamzah et al., 2010) and genotyping (Ali et al., 2005; Kumari et al., 2013) are newer methods which are currently being used for the identification of *Entamoeba* speciesc.

The results of PCR amplification showed that only 51.1% (23/45) of the stool samples initially diagnosed as positive for *E. histolytica* by microscopy were found to be positive by PCR. The present study also highlights the limitation of microscopy in correctly diagnosing the *Entamoeba* spp. as compared to the molecular identification as reported by previously published studies (Krogstad et al., 1978; Tannich et al., 1989; Acuna-Soto et al., 1993; Diamond and Clark, 1993). Among Cameroonian patients, *Entamoeba* spp. other than *E. histolytica* was found to be present in higher number. These results are consistent with earlier observations that *Entamoeba* infection in Africa is more frequently due to other species of *Entamoeba* as compared to *E. histolytica* (Ekou et al., 2013). Similar observations have been made in Brazil, Nicaragua and Italy (Fotedar et al., 2007). Australia exhibits the highest frequency of *E. dispar* (73.3%) and *E. moshkovskii* (60.7%) infections, detected by molecular techniques in microscopic positive for *Entamoeba* cysts in general population (Fotedar et al., 2007). Thus, in immune compromised individuals also other species of *Entamoeba* may be mistaken for *E. histolytica* if only microscopy is used for diagnosis. Though, molecular techniques are much more sensitive and specific than microscopy but these are expensive to perform in routine clinical setting in developing countries.

The SSUrDNA was sequenced to identify the other species that could not be done by PCR because only the primers specific for *E. histolytica* were used in the amplification reaction. The reason of choosing SSUrDNA gene for sequencing and further analysis is attributed to the fact that it has polymorphic DNA loci and successfully used in previously published literature for phylogenetic study of *Entamoeba* spp. (Clark and Diamond, 1997; Silberman et al., 1999; Clark et al., 2006). After sequencing, 24 samples (52.2%) initially diagnosed as *E. histolytica* by microcopy and negative by PCR for *E. histolytica* were found to be positive for *E. moshkovskii* (15.2%) and *E. dispar* (37%). Some samples initially diagnosed microscopically as *E. coli* were found to be *E. struthionis*, *E. hartmanni*, or *E. polecki*. Earlier, *E. struthionis* was isolated from farmed ostriches in Spain

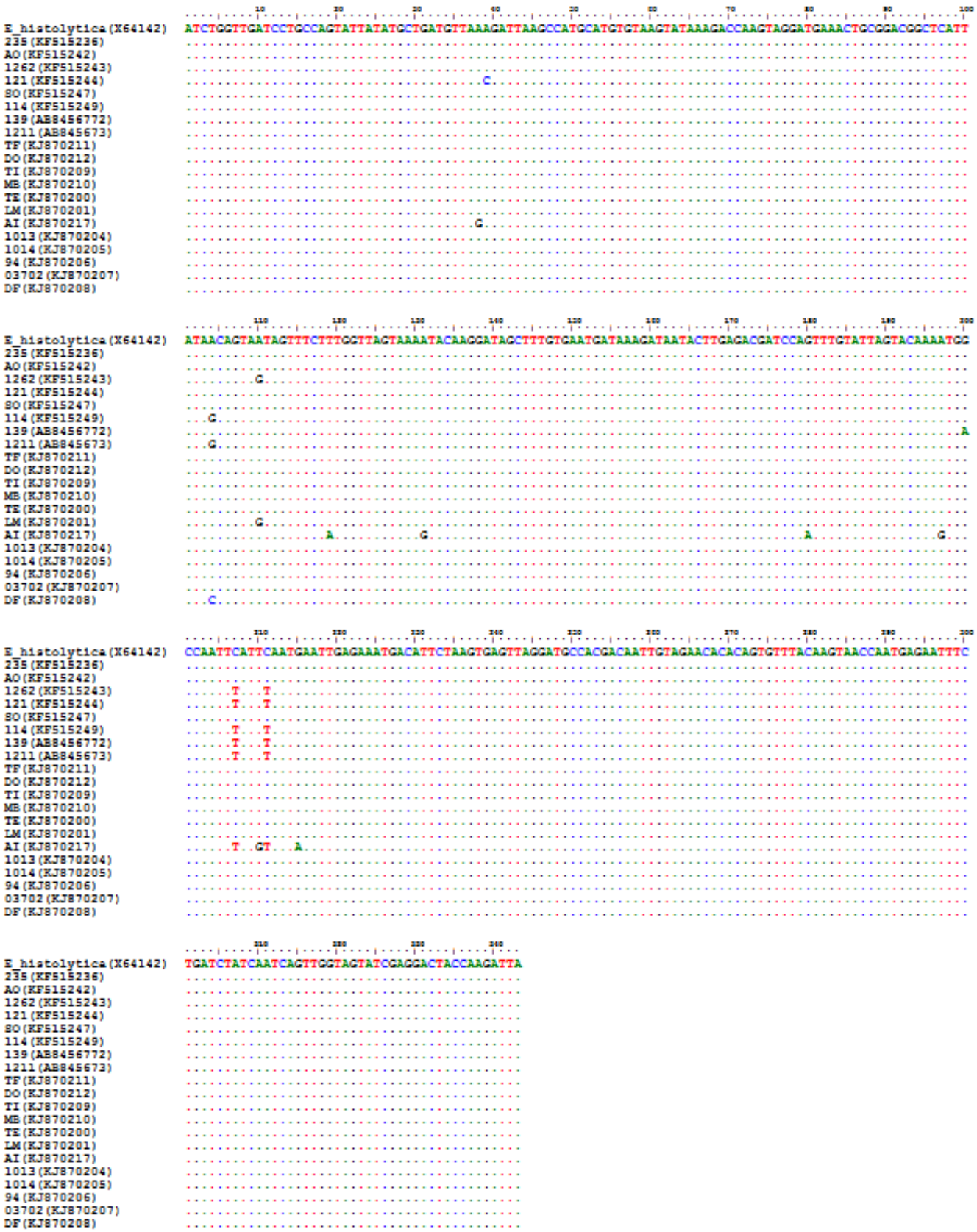

Figure 2. Multiples sequences alignment of *E. histolytica*, 16S like SSUrDNA gene sequences from Cameroonian HIV positive and negative patients with reference sequence of *E. histolytica* retrieved from the genbank.

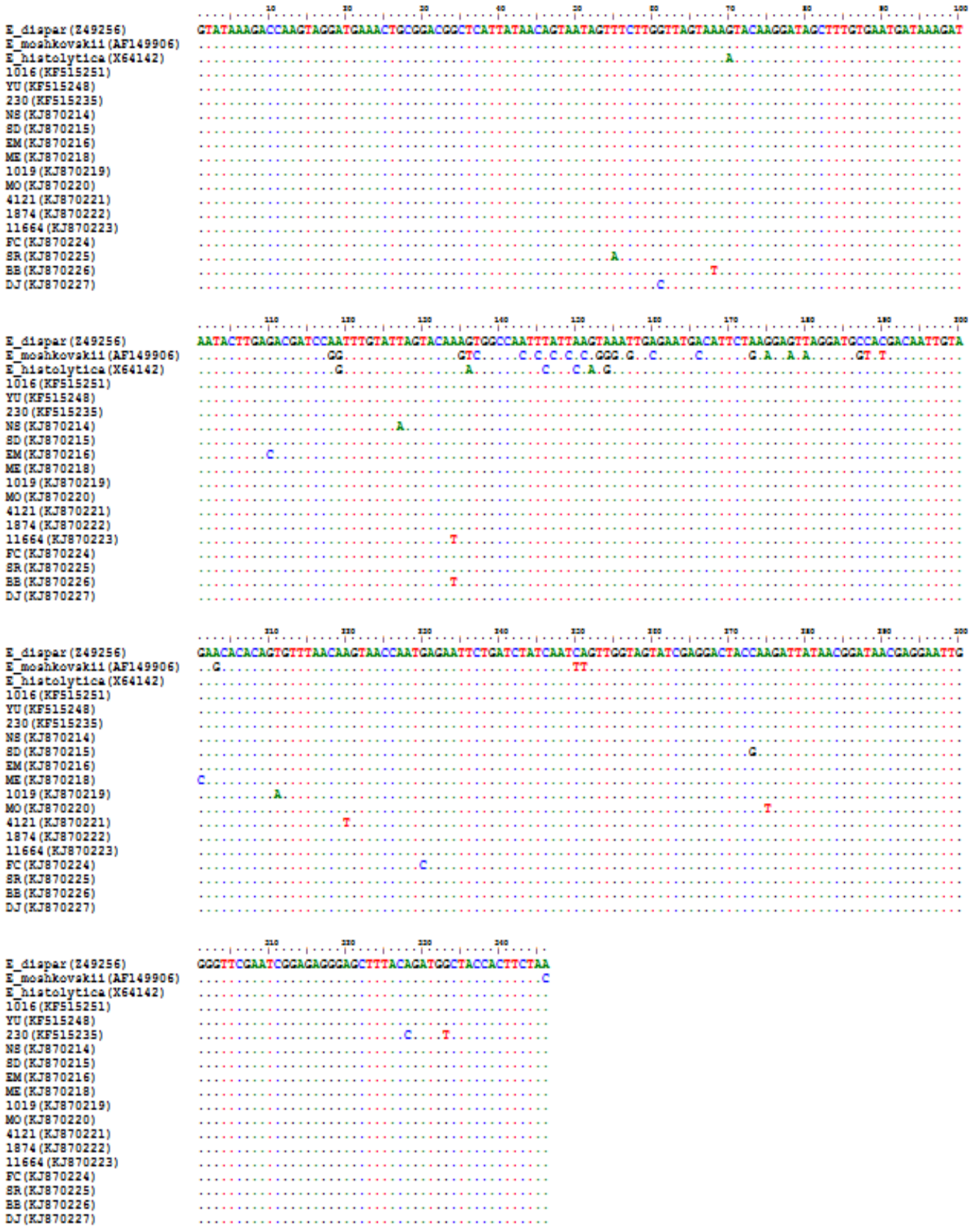

Figure 3. Multiples sequences alignment of *E. dispar* 16S like SSUrDNA gene sequences from Cameroonian HIV positive and negative patients with reference sequences of *E. histolytica*, *E. dispar* and *E. moshkovskii* retrieved from the genbank.

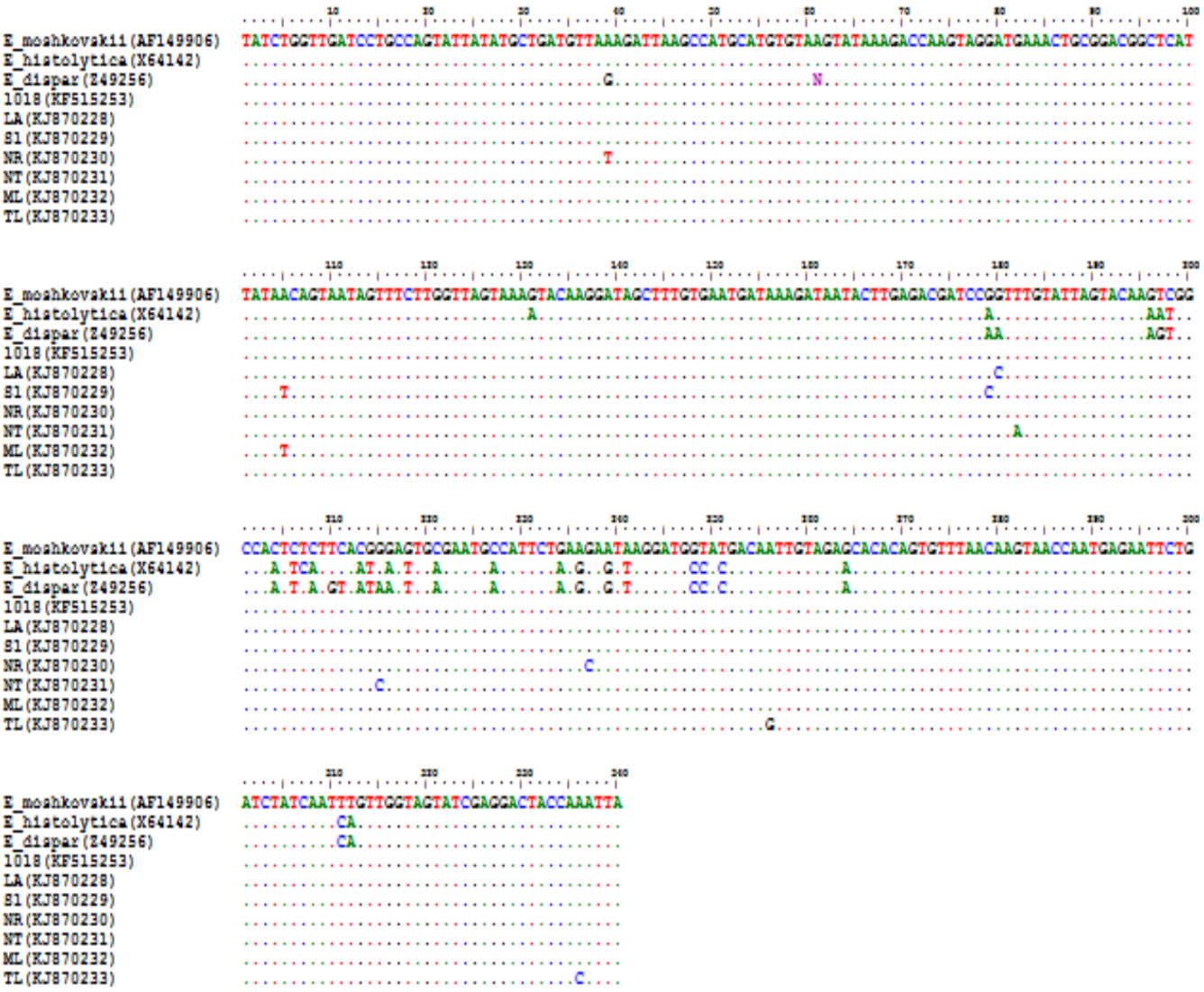

Figure 4. Multiples sequences alignment of *E. moshkovskii* 16S like SSUrDNA gene sequences from Cameroonian HIV positive and negative patients with reference sequences of *E. histolytica*, *E. dispar* and *E. moshkovskii* retrieved from the genbank.

and is known to be closely related to *E. polecki* (Ponce et al., 2004). It has been documented that it is not restricted to pigs and birds but can also infect humans (Clark et al., 2006). The cities where the samples used in this study were obtained, are well known for domesticating pigs and poultry where hygiene conditions are not very good. Thus, poor hygienic conditions may have led to the cross infection from pigs and birds to humans. Though, *E. struthionis* has been identified for the first time in human stool sample from Cameroon but its significance in humans is still unknown.

The phylogenetic analysis is in concordance with the previously published study as cluster of medically important *Entamoeba* spp. (*E. histolytica*, *E. dispar* and *E. moshkovskii*) is quite different from the other species of the *Entamoeba* (Clark et al., 2006). It also showed a difference among the closely related cluster of medically important *Entamoeba* species which were not identified correctly by microscopy. Analysis of the SSUrDNA suggested that nucleotide sequences of *E. histolytica* isolated from three HIV patient's samples (SO, AO and 235) belong to the clade closely related to the reference strain of *E. histolytica* (X64142) in which sequences isolated from HIV negative individuals are more predominant. Whereas, sequences isolated from HIV positive patients are more predominant in the clade comprised by reference strain of *E. histolytica* (AB197936). Thus, there is a possibility that two different variants of *E. histolytica* are more prevalent among HIV patients of Cameroon (Figure 1). Though, different variants have not been studied in *E. histolytica* but similar observation was made by Verweij et al. (2001) with

Entamoeba chattoni species in the phylogenetic tree presenting the distances between human isolates of uni and tetra nucleated cyst producing amoeba (Verweij et al., 2001; Ponce et al., 2004).

In the present study, PCR and gene sequencing to differentiate between various species of *Entamoeba* that infect HIV positive and negative patients in Cameroon were used. Therefore, further genotyping using *E. histolytica* specific primers as well as the correlation of the severity of *E. histolytica* infection and level of CD4+ T cells in AIDS patients are needed to highlight the relationship between HIV/AIDS and amoebiasis.

Conclusion

Cameroonian HIV patient stool samples tested present 7 species of *Entamoeba*; *E. histolytica* (28.7%), *E. dispar* (25%), *E. moshkovskii* (10%), *E. coli* (16.3%), *E. hartmanni* (6.2%), *E. polecki* (11.3%) and *E. struthionis* (7.5%). The phylogenetic analysis within the *E. histolytica* sequences isolated from Cameroonian HIV patients presented two distinguishable variants. Thus, there is a possibility that specific genotypes may be prevalent among HIV positive patients.

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Conflicts of interest

There is no competing interest between the authors.

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