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PCR DETECTION OF *ENTAMOEBA HISTOLYTICA* IN MICROSCOPICALLY POSITIVE STOOL SAMPLES OF HOSPITAL PATIENTS IN SOROTI, EASTERN UGANDA

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ABSTRACT

Amoebiasis is an infection caused by water borne protozoan parasite *Entamoeba histolytica*. In Uganda where sanitation infrastructure and health education was not adequate, amoebiasis was thought to be still an important health problem. However there was little or no data on prevalence of this very important protozoan infection. In addition, microscopy remained the main method for the diagnosis of amoebiasis but could not differentiate between *Entamoeba dispar/moshkovskii* and *Entamoeba histolytica* infections. This made determination of true prevalence of *Entamoeba histolytica* infections difficult. It was against this background that this study was designed to carry out species specific diagnosis of *Entamoeba histolytica* and *Entamoeba dispar/moshkovskii* in Uganda where these species had been reported to be endemic. This study used microscopy and polymerase chain reaction amplification of Serine-rich *Entamoeba histolytica* (SREHP) gene. It was shown that 36.7% (n=22) of the samples initially diagnosed as positive by microscopy were positive by PCR. The true prevalence of E. *histolytica* and *E.dispar/ moshkovskii* was found to be 7.31% and 12.6% respectively. It was concluded that Entamoeba infection in Soroti, Eastern Uganda is more frequently due to *E. dispar /moshkovskii* (13.3%) the non-pathogenic forms than to *E. histolytica*, the pathogen (7.31%).

Key words: *Entamoeba histolytica*, Microscopy, Polymerase chain reaction, Prevalence.

INTRODUCTION

New efforts are being made to improve the understanding of the epidemiology of the helminths and intensifying the control efforts against these parasites. In contrast, relatively few studies are being carried out in this direction for the intestinal protozoa (1). Microscopy remains the main method used for the diagnosis of amoebiasis in most African countries, however, it cannot differentiate between *Entamoeba dispar* and *Entamoeba histolytica.*In Bangladesh, only 40% of patients diagnosed by microscopy were proven to have *E. histolytica* infection when specific methods were used. In addition, the accuracy of this method in detecting *Entamoeba histolytica* depends heavily on skills of the technician and has been shown to be less sensitive and less specific as compared to other methods such as immuno-florescence (IFA), antigen detection and PCR(2).

Molecular methods, such as PCR, have aided in alleviating some of the sensitivity and specificity

deficiencies associated with traditional methods for the detection of protozoan pathogens. A number of PCR-based assays like gene amplification with specific primers, multiplex PCR, restriction fragment length polymorphism and real-time PCR have been developed for the identification of *E.histolytica* infections (3). In Mexico, *E.histolytica* prevalence of 25.3% in the HIV/AIDS group and 18.5% in the HIV-negative group was described using PCR (4). Dhawan (5) estimated the prevalence rate of amoebiasis in the United States to be approximately 4% with *E dispar* infection, which is always asymptomatic, being 10 times more common than *E histolytica* infection. Despite the development of sensitive antigen-based and molecular techniques, there was no information on the true prevalence of *E. histolytica* in Uganda (6). Very few studies in Africa have used molecular methods to determine the prevalence of *E.histolytica*. As a consequence, large gaps remain on prevalence rates of *Entamoeba histolytica*. Estimates of prevalence rates of *E. histolytica* is an important decision making tool in allocation of limited public health resources, its treatment, prevention and control. Also feasibility of developing an amoebiasis vaccine depends on the estimation of the disease burden among the populations in the high risk areas (7, 8). It was against this background that this study was designed to carry out species specific diagnosis of *Entamoeba histolytica, Entamoeba dispar* and *Entamoeba moshkovskii* in Uganda where *Entamoeba histolytica* and *Entamoeba dispar* had been reported to be endemic (9).

MATERIALS AND METHODS

Isolation of amoebic DNA from isolates of Entamoeba histolytica

Sixty faecal samples initially diagnosed as positive by microscopy were stored frozen. For DNA isolation, 200 µl of faecal suspension (0.5 g/ml PBS) was added to 200 µl of 4% polyvinylpolypyrolidone (PVPP) (Sigma) suspension and heated for 10 min at 100 °C (10). DNA isolation was then done according to the method of Samie *et al*.(11). The genomic DNA was purified from stool samples using the QIAamp DNA Stool Mini Kit from Qiagen (Qiagen GmbH, Hilden, Germany) with some modifications. Briefly, 250 µl of liquid stool or diluted stool material was added to 50 µl of potassium hydroxide and 15 µl of 1 mol/L dithiothreitol and mixed thoroughly. After a 30-minute incubation period at 65°C, 8.2 µl of 25% HCl and 80 µl of 2 mol/L Tris-HCl (pH 8.3) were added to the mixture. After a brief vortexing, the protocol was continued with the Qiagen mini kit following the manufacturer's instructions.

PCR amplification of Ser-rich Entamoeba histolytica Protein gene

The amoebic *Ser-rich* protein gene repeats were amplified using PCR and primers specific for *E. histolytica*. An *E. histolytica* Ser-rich protein-specific sense primer was GCTAGTCCTGAAAAGCTTGAAGAAGCTG (Primer1), while an *E. histolytica Ser-rich protein*-specific antisense primer was GGACTTGATGCAGCATCAAGGT (12). The procedure was as follows: all solutions were gently vortexed and briefly centrifuged after thawing. Using a thin-walled PCR tube on ice, the reaction mixtures were added as follows: 10 X PCR buffer 2.5 µl, 10 mM dNTPs 5.0 µl, 25 mM $MgCl_2$ 5.0 µl, 50 pmoLPrimer1 1.0 µl, 50 pmoL Primer2 1.0 µl, Taq polymerase 0.3 µl, distilled water 1.5 µl, DNA sample 3.0 µl. Samples were again gently vortexed and briefly centrifuged to collect all drops from walls of tubes. Samples were then placed in a thermocycler and set as follows: denaturation at 94ºC for 3 minutes, annealing at 55ºC for 1 minute, extension at 72ºC for 2 minutes and whole process repeated for 35 cycles. The PCR products were identified on 12% polyacrylamide gel electrophoresis at 80 volts for four hours. Standard strain E. *histolytica* HM-1: IMSS to be used as positive control, could not be obtained. However, a negative control was used. The negative control was prepared as follows: 10 X PCR buffer 2.5 µl, 10 mM dNTPs 5.0 µl, 25 mM $MgCl_2$ 5.0 µl, 50 pmoLPrimer1 1.0 µl, 50 pmoL Primer2 1.0 µl, Taq polymerase 0.3 µl, distilled water 4.5 µl.

RESULTS

It was shown that only 22 of the 60 samples (33.3%) initially diagnosed as positive by microscopy were positive by PCR (Figure 1).Two strains of *E. histolytica* were common, being detected in five separate patients. The patients whose samples were identified as: 4, 9, 11, 12, 16 seemed to be infected by a similar strain. While patients whose sample identity were: 2, 7, 18, 20 and 22 also seemed to be infected with another strain. However, each of the six strains (8, 10, 13, 14, 15, 21) were detected in only one patient.

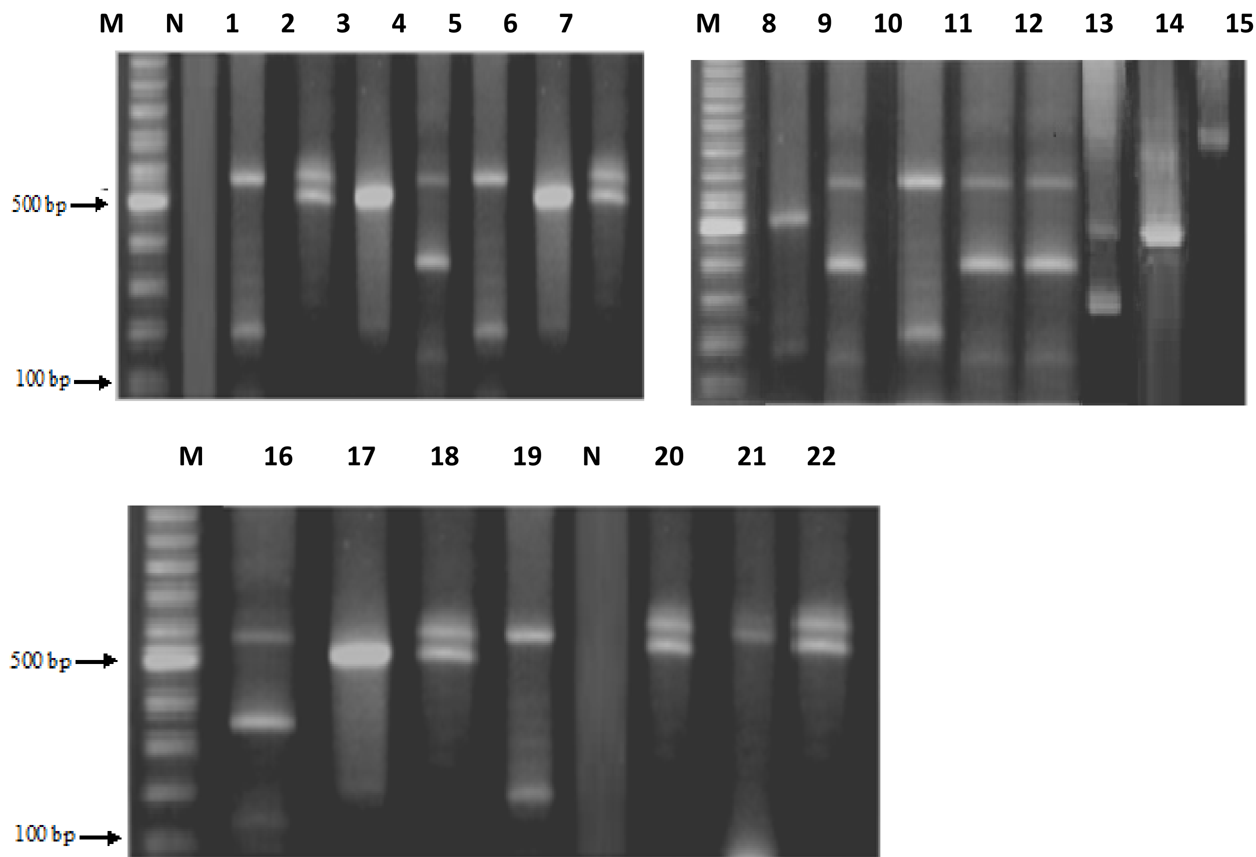

Figure 1: Gel photographs of SREHP PCR products amplified from DNA of *Entamoeba histolytica* positive stool samples from Soroti. 1-22 are the positive samples; M is a 100 bp marker while N is negative control.

DISCUSSION

Entamoeba histolytica infections are common in Sub-Saharan Africa but the true prevalence of infection and disease caused by *Entamoeba histolytica* was unknown for Uganda and most of Sub-Saharan Africa. This was attributed to the fact that until relatively recently the laboratory differentiation of *E. histolytica* from the morphologically identical non-pathogenic amoebic species *E. dispar/moshkovskii* was not possible. However, differential identification of *E. histolytica* and *E. dispar* is essential for both appropriate patient treatment and epidemiological purposes. Despite the development of sensitive antigen-based and molecular techniques; there was no other information on the diversity of *E. histolytica* strains in Uganda. As a consequence, large gaps remained in our knowledge of species prevalence rates. To address this, species specific diagnosis of *E. histolytica* was performed among isolates from Soroti.

In this study, the amoebic *Ser-rich protein* gene repeats were amplified using direct PCR using primers that are specific for *E. histolytica*. The results showed that only 33.3% of samples initially diagnosed as positive by microscopy were positive by PCR (Figure 1). This gave overall true prevalence 7.31% of *E. histolytica*.

These results are consistent with earlier observations that *Entamoeba infection* in Africa is more frequently due to *E. dispar* than to *E. histolytica*. Similar observations have been made in Brazil, Nicaragua, and Italy (13). Australia exhibits the highest frequency of *E. dispar* (73.3%) and *E. moshkovskii* (60.7%) infection, which were detected in a population referred to as a clinical laboratory service where 2.9% of samples were microscopically positive for *Entamoeba* cysts (13). In contrast, in countries along the Pacific coast, the frequency and prevalence data

for *E. histolytica* infection seem to be higher or similar to those obtained for *E. dispar.* The data available for the frequency of *E. histolytica* infection in the Middle East show that cases in the Gaza Strip in Palestine detected in hospitalized patients exhibited a 69.5% frequency, as compared to Saudi Arabia with a frequency of 2.7% in a similar population (14, 15).

The PCR resulted into mostly single but also multiple bands. This PCR product length polymorphism was thought to result from size variations within the SREHP gene. There is evidence to suggest that the *E. histolytica* SREHP genome is tetrapoid (16, 17). This could have accounted for the multiple bands that were observed and may reflect polymorphism among homologous loci on allelic chromosomes. Another factor that could account for multiple bands was the existence of the repeat loci at multiple locations in the *E. histolytica* genome each with a characteristic PCR product. To further support this argument, it has previously been shown that SREHP appears to be a single copy gene only when analyzed using Southern blots. However, when the repeat region is amplified from a clonal line of an *E. histolytica* isolate, often two or three bands are observed, not always of equal intensity but usually quite close in size (18). This suggested that multiple alleles can be present and that the ploidy is likely to be four. The major way in which the alleles differ was said to be in the number of 8 and 12 amino acid repeats that are present, but there are sequence differences between the DNA repeats also. It could also be argued that since there are different strains of *Entamoeba histolytica* in the environment and that humans are continuously exposed to them in a similar manner, it is possible for a single host to be infected by more than one strain at the same time. Multiple bands could then result from infection of a single patient with several different strains of *Entamoeba histolytica.* This can be from the same point source or different sources infected at the same time or different times. Besides parasite mutations during infection can produce modified strains capable of being distinguished from the original strain and thus resulting in multiple bands in the same patient.

In conclusion, Entamoeba infection in Soroti, Eastern Uganda is more frequently due to *E. dispar /moshkovskii* (13.3%) the non-pathogenic forms than to *E. histolytica,* the pathogen (7.31%).

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