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Full Length Research Paper

Epidemiology of entamoeba infection in Sudan

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The epidemiology of *Entamoeba histolytica* infection in Sudan is poorly understood. This is due to the inability to differentiate *E. histolytica* from the non pathogenic, *Entamoeba dispar*. Old methods used such as direct microscopy and culturing are insensitive compared to polymerase chain reaction (PCR). In this study, light microscopy and PCR were utilized to study the prevalence of Entamoeba infection in patient attending University of Medical Science and Technology (UMST) hospital, in Khartoum, Sudan. By microscopy 196 stool samples were reported as positive for *E. histolytica*. PCR detected infections caused by *E. histolytica* in 54% (106 of 196), and *Entamoeba dispar* in 51% (100 of 196) of stool samples. By PCR also mixed infections were detected with both *E. histolytica* and *E. dispar* in 5% (10 of of stool samples. All 50 negative stool samples examined by microscopy were negative by PCR. The inability to distinguish *E. histolytica* from the morphologically similar *E. dispar* in stool samples is the main limitation of microscopic methods used mainly all laboratories in Sudan. All the 196 samples tested were reported positive for *E. histolytica* by microscopy but in this study it is shown that only 54% (106 of 196) were positive for *E. histolytica*. The other 51% (100 of 196) were positive for *E. dispar*, which were misdiagnosed as *E. histolytica* infections and mistreated with anti-amoebic drugs. Thus, PCR is recommended for detection and accurate identification of *Entamoeba* species in stool samples.

Key words: Entamoeba histolytica, *Entamoeba dispar*, polymerase chain reaction (PCR), Sudan.

INTRODUCTION

Entamoeba histolytica is a pathogenic amoeba found throughout the world, especially common in the developing world, in area with low socioeconomic status and poor hygiene (Ravdin, 1995; Walsh, 1986). The parasite causes invasive disease in over 50 million people and approximately 100,000 deaths per year are reported, making it one of the leading causes of parasitic death in man (WHO, 1997). Infection caused by *E. histolytica* can lead to asymptomatic colonization, amoebic colitis or extra-intestinal disseminated disease. The most common extra-intestinal disease attributed to *E. histolytica* is liver abscess and infection may spread to other organs, particularly lungs and brains (Haque et al., 2003).

E. histolytica, the pathogenic amoeba is indistinguishable in its cyst and trophozoites stages from those of non-pathogenic *E. dispar* (WHO, 1997), except in rare cases of invasive diseases when *E. histolytica* trophozoites may contain ingested red blood cells (Gonzalez et al., 1999). The diagnosis of Entamoeba infection relies on microscopic examination of fresh and fixed stool samples (Fotedar et al., 2007). However, microscopy cannot differentiate between pathogenic and non pathogenic *Entamoeba.* Also, the accuracy of the microscopy relies on the skills of the technician and has been shown to be less sensitive and less specific compared with other methods (Haque et al., 2003).

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Abbreviations: PCR, Polymerase chain reaction; **UMST,** university of medical science and technology; **PBS,** phosphate buffer saline; **RBCs,** red blood cells.

It is important to improve the methods of diagnosis of *Entamoeba* not only to decrease the morbidity and mortality of ameobiasis, but also to reduce the unnecessary treatment of patients infected with non pathogenic *Entamoeba*. The present study investigated the prevalence of *E. histolytica* and *E. dispar* in patients coming to UMST hospital in Khartoum, Sudan due to gastrointestinal symptoms, and where *Entamoeba* was detected in the stool samples through microscopy.

MATERIALS AND METHODS

Samples information

A total of 246 stool samples were collected during a study period of two months from June to August 2004. This included 196 stool samples collected from patients attending UMST hospital, Khartoum, Sudan, with complains of gastrointestinal discomfort. It also included 50 stool samples, as control from healthy people. These 50 samples were negative for *E. histolytica* and *E. dispar*. Aliquots of fresh unpreserved stool samples were stored at - 20°C until used.

Microscopic examination of stool

Saline and iodine wet mounts of fresh unpreserved stool samples were examined microscopically for demonstrating *E. histolytica* or *E. dispar* or any other Entamoeba cysts and trophozoites as previously described (Parija et al., 2001). Briefly the wet preparations were made by mixing approximately 3 mg of stool with a drop of saline on a glass slide and placing a cover glass over the stool suspension. Same as the saline, iodine wet mounts were prepared by adding 3 mg of stool to a drop Logo's iodine (diluted 1:5 with distilled water) on a glass microscope slide and placing a cover glass on the stool suspension. These wet amounts were examined using a low power field (10x) and high power field (40x) objective of a light microscope. The preparations were read immediately and viewing at least 100 fields per slide. Each stool sample was screened by two well trained technologists before reporting negative results.

Deoxyribonucleic acid (DNA) extraction

DNA was extracted from all samples using the Qiagen DNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. An initial washing step was added; about 5 mg of the stool sample was washed once with phosphate buffer saline (PBS). After centrifugation for 5 min at 4000 X g, the sediment was re-suspended in an equal amount of saline and 200 µl was used for the extraction.

Deoxyribonucleic acid (DNA) amplification

In this amplification two reactions were performed. In the first reaction two primers sets were used. One set, the forward primer Psp 3` and the reverse primer Psp 5` for *E. histolytica* and at the second reaction the forward primer NPsP 3` and the reverse primer NPsp 5` for *E. dispar* described by Diamond and Clark (1991). PCR were carried out for the both reactions in a final volume of 50 µl containing each primer at a concentration of 0.3 mM, 1.0x PCR golden buffer, 200 mM deoxyribonucleoside triphospate, 1.5 mM $MgCl_2$, 1.25 U/50 ml of Ampli Taq Gold (Sigma).

Gel analysis of polymerase chain reaction (PCR) product

PCR condition were based on these describes: 32 cycles of 94°C (denaturation) for 1 min, 58°C (annealing) for 0.30 min, and 72°C for 1 min (extension). PCR products were analyzed by electrophoresis on 2% agarose gel in 0.5x TBE buffer (Tri base, boric acid and EDTA [pH 8.0]). (The gel was stained in 0.5 mg/ml ethidium bromide bath, visualized by ultraviolet (UV) translumination, and photographed using Polaroid films). DNA fragment 880 bp for *E. histolytica and E. dispar* were obtained in both reactions.

RESULTS

The 196 stool samples out of 246 were found to be positive by microscopic for *Entamoeba* and the negative control samples as were prepared for PCR. The PCR was positive in all 196 samples giving a sensitivity of 100%. All the 50 negative control stool samples were negative by PCR thus showing a specificity of 100%. The probability of negative PCR results in a control samples due to PCR inhibitors was ruled out by spiking the DNA of negative samples with control DNA of Entamoeba followed by PCR.

The PCR detected single infection with *E. histolytica* in 54.1% (106 of 196), and *E. dispar* in 51% (100 of 196) of stool samples. The PCR also detected mixed infections by both *E. histolytica* and *E. dispar* in 5.1% (10 of 196) of samples. Analysis of sex and age showed that 43.9% (86 of 196) infected by Entamoeba were male and 56.1% (110 of 196) infected were female. No significant difference between age groups could be disclosed.

According to presented symptoms it was shown that 66% (70 of 103) of diarrhoea patient were infected by *E. histolytica* while 93.4% (99 of 101) of stool containing red blood cells (RBCs) were infected by *E. histolytica* (Table 1).

DISCUSSION

The true prevalence and incidence of disease and infection caused by *E. histolytica* is unknown for most part of the world including Sudan. Methods that will differentiate between pathogenic *E. histolytica* and non pathogenic *E. dispar* are not available in routine diagnostics. The epidemiology of Entamoeba in Sudan is thus poorly understood. Our aim was to elucidate the proportions of *E. histolytica* and *E. dispar* in a population with known *Entamoeba* infections having symptoms and among healthy individuals.

Different methods have been reviewed for detection of *E. histolytica* and *E. dispar* (Ali et al., 2003; Haque et al., 1998; Huston et al., 1999; Roy et al., 2005). The present study showed that PCR which is cheap and available developing countries like Sudan can be used to detect and differentiate *E. histolytica* and *E. dispar* directly in the stool samples of patients is reliable. A study by Hamzah et al. (2006) showed that, out of 27 stools sampled

Table 1. Cross tabulation between presenting symptoms and diagnosis (Statistically significant, P value < 0.05).

Symptoms	Positive *E. histolytica* by microscopical diagnosis (%)	Amoebiasis by clinical diagnosis (%)
Diarrhea (n =103)	70(66)	43 (43)
No diarrhea (n 93)	36(34)	57 (57)
RBCs (n = 101)	94(93)	12 (12)**
No RBSs (n =95)	106(100)	88 (88)
Total		

* 10 patients (23.2%) have cross infection *E. histolytica,* prevalence of diarrhea patients with cross infection =36.7; ** 10 patients (83.3%) have cross infection with *E. histolytica*, prevalence.

positive for *Entamoeba* sp. by microscopy, only 7 were successfully identified at species level by PCR, which included 1 positive for *E. histolytica* and 6 for *E dispar*. In contrast, our study showed that all the stool samples which were positive by microscopy also were positive by PCR even at species level. A detailed comparative study between these two techniques may give useful information especially in the field of molecular-based diagnosis of amoebiasis.

In the present study, by using PCR, it was shown that the highest rate of infection was with *E. histolytica.* It was demonstrated in 106 out of 196 stool samples (54.1%) amongst patients diagnosed by microscopy having an *Entamoeba* infection attending UMST hospital. The study also shows that the rate of co-infection with *E*. *histolytica* and *E. dispar* was 10 patients out of 196 which is similar to the occurrence of co-infection with *E. histolytica* and *E. dispar* in the stool samples documented by several studies earlier (Haque et al., 1998; Newton-Sanchez et al., 1997; Nunez et al., 2001; Parija and Khairnar, 2005; Romero et al., 1992).

In this study, the PCR appears to be more useful for simultaneous detection of the two species, *E. histolytica* and *E. dispar* when performed directly on the stool samples, which is the main advantage of this test. The importance is due to the fact that there is an increasing number of a reported case of *E. dispar* from different parts of the world (Ali et al., 2003; Hamzah et al., 2006; Haque et al., 1998; Huston et al., 1999; Tanyuksel and Petri, 2003). The existence of non-pathogenic *E. dispar* as single infection among the investigated subjects showed an increased possibility of miss-diagnosis when identification of *E. histolytica* was based primarily and only on morphology by microscopic examination of stool. The high number of PCR positive samples for *E. dispar* among the study population supports the point theory that humans are true hosts for this amoeba (Ali, et al., 2003).

The inability to distinguish *E. histolytica* from the morphologically similar *E. dispar* in stool samples is the main limitation of direct microscopy. All the 196 samples here tested were reported positive for *E. histolytica* by microscopy but in this study only 56.1% (106 of 196) were positive for *E. histolytica* while 51% (100 of 196) were positive for *E. dispar*, resulting in samples wrongly diagnosed as positive for *E. histolytica* infections and accordingly the patients were mistreated with anti-amoebic drugs. Thus, the recommendation is to use PCR for proper detection and accurate identification of *Entamoeba* sp. in stool samples.

Few studies have shown the correct incidence and prevalence of *E. histolytica* and *E. dispar* in Africa (Adams and MacLeod, 1977; Omer et al., 1981; Stauffer et al., 2006). Studies in South Africa, Egypt and Sudan regarding *E. histolytica* as a common protozoan infection (Adams and MacLeod, 1977; Omer et al., 1981; Stauffer et al., 2006), although asymptomatic, harmonises with the present finding that the *E. histolytica* is a common protozoa among subjects hospitalised at the UMST hospital. In order to report a more complete picture of the epidemiology of *E. histolytica* infection and illuminate the disease in Sudan further epidemiological studies from other part of Sudan need to be done.

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