AMEBIASIS AND COMPARISON OF MICROSCOPY TO ELISA TECHNIQUE IN DETECTION OF *ENTAMOEBA HISTOLYTICA* AND *ENTAMOEBA DISPAR*

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Summary: The analysis of records of amoebal infection in various hospitals in Kilimanjaro indicated frequent occurrence of amebiasis. The population over the age of five years had higher rate of amoebal infection compared to less than that of a five-year-old population; however, both age groups had similar patterns of amebiasis during January 1999 to June 2001. To investigate misdiagnosis of amebiasis, 226 patients (passive cases) in three hospitals and 616 individuals (active cases) from three different localities in Kilimanjaro were examined. In passive cases, the prevalences of *Entamoeba histolytica* and *Entamoeba dispar* were 1% and 7.3%, respectively. Among active cases, 1% were infected with *E. histolytica*, and 15% were infected with *E. dispar*. There were no significant differences in amoebal infection between the male and female populations. A pool of 842 stool samples was used for diagnosis of *E. histolytica* and *E. dispar* by microscopic examination or ELISA kits. The microscopic examination indicated 8.7% amoebal infection; however, using ELISA as the gold standard, the prevalence of *histolytica/dispar* was 0.8% and 7.4%, respectively. This study indicated that *E. dispar* infection was 14.5 times more prevalent than *E. histolytica* infection. (*J Natl Med Assoc.* 2004;96:671–677.)

Key words: misdiagnosis ◆ amebiasis ◆ microscopic and ELISA techniques

INTRODUCTION

In human amebiasis, the differentiation of the invasive parasite *Entamoeba histolytica* from the commensal organism *Entamoeba dispar* is of great concern to the medical community. Amebiasis is transmitted by fecal contamination of drinking water and foods, direct contact with dirty hands or objects, anal sexual contact, and poor sanitation and hygiene. For many years *E. histolytica* and *E. dispar* have been known to be two distinct species. The description of *E. dispar* by Brumpt[1] was dismissed as a synonym of *E. histolytica*. However, later evidence mounted in support of Brumpt's description of *E. dispar* as a separate species.[2-8] *E. histolytica* and *E. dispar* are genetically distinct but closely related protozoan species. Both colonize the human gut, but only *E. histolytica* is able to invade tissues leading to massive and sometimes lethal pathological alterations, such as ulcerative colitis or abscesses of various organs—most commonly, the liver.[9]

Infections of *E. histolytica* and *E. dispar* are often diagnosed by demonstrating cysts or trophozoites in a stool sample. A great number of methods for distinguishing *E. histolytica* from *E. dispar* have now been described in the literature.[2,3,10,11]

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Sargeaunt[10] looked at a database of several thousand isolates from all over the world and grouped all *E. histolytica* into one of two groups: i) pathogenic *E. histolytica* and ii) nonpathogenic *E. histolytica*. This division was further supported by antigenic differences[11] and differences in the DNA.[5] By 1993, a lot of evidence was available to distinguish *E. histolytica* from *E. dispar* and was formally redescribed.[8] The World Health Organization expert committee endorsed the redescription of the two species.[12,13] Since then, an ELISA specific for *E. histolytica* has been developed for fecal-antigen detection. This technique detected all cases of amoebic dysentery.[14] Also, there is a fecal-antigen detection test, which is based on a monoclonal antibody against galactose-inhibitable lectin on the surface of *E. histolytica*.[15] An ELISA-based technique in which PCR products are detected with dioxigenin-labeled primers has been developed to differentiate between *E. histolytica* and *E. dispar*.[16] The tests, based on nucleic acid detection to differentiate the two species, are being used in a routine reference diagnostic service for cyst carriers.[17]

E. histolytica or *E. dispar* infect half a billion people annually,[15,18] with 90% being asymptomatic and the remaining 10% accounting for the third most common cause of death from parasitic disease in the world. Additional health effects of amebiasis are delayed treatment of other related diarrheal diseases due to misdiagnosis of *E. histolytica* when conventional microscopic methods are employed. The immunological diagnostic tests can specifically differentiate between these two related species.

The present study was carried out to examine the prevalence and etiological agent of amebiasis in Kilimanjaro, Tanzania. The main aim of this study was to demonstrate the importance of correctly identifying *E. histolytica* in order to avoid unnecessary treatment costs and delayed treatment of actual infection.

MATERIALS AND METHODS

Study Areas

The study was carried out in the foothills of Mount Kilimanjaro in Tanzania. Residents in this area are mainly engaged in coffee and banana farming. The main source of water is microbial-contaminated furrow water from rivers flowing from the mountain springs. The Kilimanjaro area is considered an endemic area for amebiasis in the tropics. Other diseases in this area from the records of health centers include malaria, upper respiratory infections, diarrheal diseases, and intestinal worms. Three villages (Rundugai, Mabogini, and Mvuleni) with an average population of 5,000 and

Table 1. Detection of amoebal infection (*histolytica/dispar*) among 842 subjects using *Entamoeba* Test (TechLab). The sensitivity (39%) and specificity (96%) were determined using dichotomous approach. The amoebal infection was 8.2%, and infection missed by microscope was 61%.

Test Results	Infected	Not Infected	Total
Positive	27	32	59
Negative	42	741	783
Total	69	773	842

Sensitivity = 39%; Specificity = 96%

Positive predictive value = 46%

Negative predictive value = 95%

Infection missed by microscope:
1- sensitivity = 61%

Fraction unnecessarily treated:
1- specificity = 4%

Fraction with amoebal infection (*dispar/histolytica*) = 8.2%

Table 2. Detection of *E. histolytica* infection among 842 subjects using *E. histolytica*-II Test (TechLab). The sensitivity (43%) and specificity (93%) were determined using dichotomous approach. The *E. histolytica* infection was 0.8%, and infection missed by microscope was 57%.

Test Results	Infected	Not Infected	Total
Positive	3	56	59
Negative	4	779	783
Total	7	835	842

Sensitivity = 43%; Specificity = 93%

Positive predictive value = 5%

Negative predictive value = 99.5%

Infection missed by microscope:
1- sensitivity = 57%

Fraction unnecessarily treated:
1- specificity = 7%

Fraction with *E. histolytica* infection = 0.8%

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one health center were selected for active cases (people who were not sick/no symptoms of infection). Three hospitals (Kibosho, Kilimanjaro Christian Medical College, and Kibongoto) were included in this study for passive cases (people who had symptoms of amoebal infection). Information relating to population structure; disease cases; possible sources of amoebal infection; personal hygiene; history of amebiasis; and the source of drinking, cooking, and washing water was obtained from the health center serving the area.

Data on Amoebal Infection in Hospitals

Data on amoebal infection in various hospitals in Kilimanjaro during January 1999 to June 2001 were collected and analyzed as percent rate of infection. The amoebal infection diagnosis in these hospitals was based on the microscopic examination. On average, 500 patients under and above five years old were examined for amoebal infection.

Collection of Stool Samples

The study population was divided into groups: 1) 616 active cases included individuals who were requested to report to their health center for routine examination of intestinal parasites, and 2) 226 passive cases included individuals who reported in three hospitals with diarrheal problems. The morning stool samples were collected in special stool containers. All stool samples were labeled and brought to the Kilimanjaro Christian Medical College laboratory in cool boxes for examination within 24 hours of collection. For each stool sample, color, consistency, and presence of blood or mucus were recorded. A pool of 842 stool samples was used for diagnosis of *E. histolytica* and *E. dispar* by microscopic examination or monoclonal ELISA kits (TechLab, Corporate Research Park, Blacksburg, VA). Each sample was divided into four parts and used for: 1) microscopic examination, 2) *Entamoeba* Test—*E. histolytica* plus *E. dispar,* 3) *E. histolytica* test, and 4) possible repeat test.

Table 3. Detection of *E. dispar* among 842 subjects using *Entamoeba* Test minus *E. histolytica*-II Test (TechLab). The sensitivity (39%) and specificity (96%) were determined using dichotomous approach. The *E. dispar* infection was 7.4%, and infection missed by microscope was 61%.

Test Results	Infected	Not Infected	Total
Positive	24	35	59
Negative	38	745	783
Total	62	780	842

Sensitivity = 39%; Specificity = 96%

Positive predictive value = 41%

Negative predictive value = 95%

Infection missed by microscope:
1- sensitivity = 61%

Fraction unnecessarily treated:
1- specificity = 4%

Fraction with *E. dispar* infection = 7.4%

Microscopic Examination

Lugol's iodine was added to the stool smear and covered with a cover slip and examined within five- to 15 minutes using the 100X objective. Detailed processing and examination procedures were as described by Bailey.[19] During examination, all observed parasites were recorded.

Entamoeba Test Versus *E. Histolytica* II Test

The *Entamoeba* Test and the *E. histolytica* II Test (gold standard) were used. These two tests are based on a monoclonal antibody against galactose adhesions distinct epitopes of *E. histolytica* or *E. dispar*, which do not cross-react serologically and are used for the rapid detection of the parasites in stools. The *Entamoeba* Test (sensitivity and specificity of 87.7% and 98.3%, respectively) is designed to detect but not differentiate the antigens of *E. histolytica* and *E. dispar* in stool. The *E. histolytica* II Test (sensitivity and specificity of 96.9% and 100%, respectively) is designed to detect specifically *E. histolytica* in stool. The *Entamoeba* Test was performed on all the stool specimens. The *E. histolytica* II Test was performed only on those specimens which were positive with the *Entamoeba* Test. As specified earlier, a total of 616 (active cases) and 226 (passive cases) stool samples (less than 24 hours old) were analyzed.

Each stool sample was thoroughly mixed prior to performing the assay. This included vortexing of the stool sample prior to transfer to the diluent, and complete mixing of the diluted stool sample prior to transfer to the microwell. The diluent was formulated to stabilize the adhesin in the stool sample and minimize degradation. The diluted stool sam-

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ple was stored at 2–8°C until the test was performed. It has been found that under these conditions, the stool sample remained positive when tested daily over a period of five days.

Preparation of the Stool for Monoclonal ELISA

One vial for each sample to be tested was set up. A 0.4-ml diluent was added to each vial labeled directly on the side and vortexed to ensure adequate mixing. For formed stools 0.15–0.20 g and for liquid stools, 0.4 ml were used. The stools were vortexed before being transferred to their respective labeled vials. The vials were vortexed for 10 seconds and stored at 2–8°C until ELISA was performed. The specimens were vortexed again before transferring the diluted specimen to the wells.

The test procedure for monoclonal ELISA was performed as described in the TechLab manual. A test sample was considered positive if it had an obvious yellow color when compared to the negative control well. A test sample was considered negative if the reaction was colorless. If the yellow was not distinct, the test was repeated.

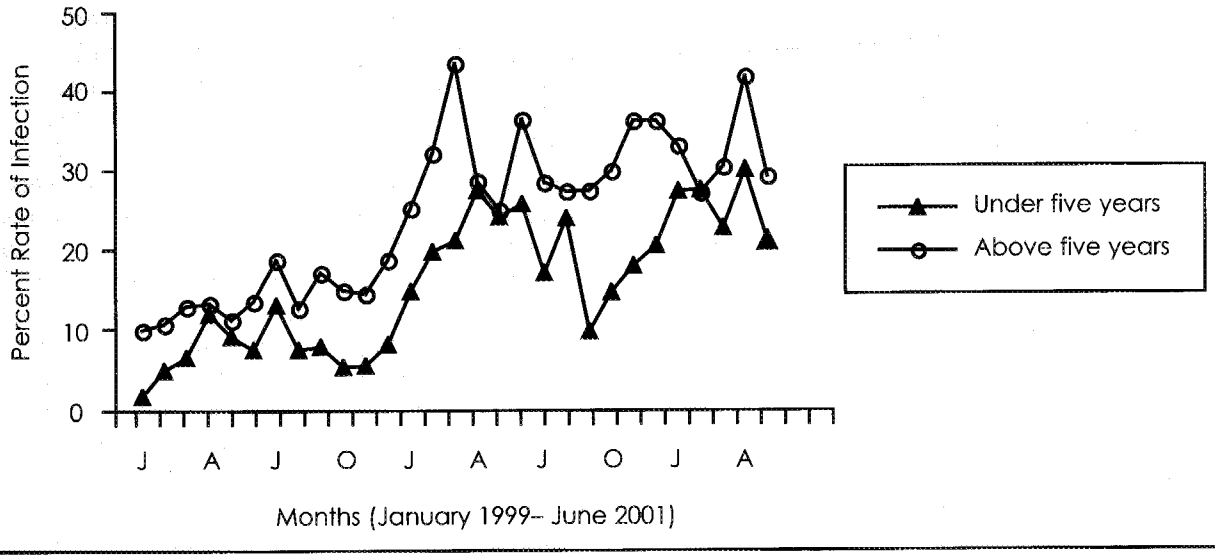
STATISTICAL ANALYSIS

To compare the percentage differences among various groups, the mean percent rate of infections was calculated. To compare the microscopic test and ELISA tests, the sensitivity and specificity, positive predictive value (PPV) and negative predictive values (NPV) were computed—assuming that the ELISA test can adequately serve as a gold standard, although there is no specific ELISA test for *E. dispar* alone. It can be determined from the results of both ELISA tests on a stool sample. So the total number of those with *E. dispar* is the number which test positive on the *Entamoeba* Test minus the number which test positive on the *E. histolytica*-II Test. Since the examination by microscopy cannot distinguish between the two amoeba, therefore, the total number of positive and negative microscopic diagnosis is the same for *E. histolytica* infection. Using these facts, the sensitivity and specificity, PPV, and NPV for *E. histolytica* and *E. dispar* have been computed using a dichotomous approach.[20]

RESULTS

The analysis of the records of amoebal infection in various hospitals in Kilimanjaro indicated frequent occurrence of amebiasis. The results of this study indicated that the population over the age of five years had a higher rate of amoebal infection compared to the population under the age of five years. However, both age groups had similar patterns of amoebal infection throughout the study period (Figure 1). This study indicated that females over the age of five had a higher rate of infection

Figure 1. Percent rate of amoebal infection in the different age groups among the people of Kilimanjaro, Tanzania. The rate of amoebal infection was higher in persons older than five years. However, the infection rate pattern was the same in both groups.



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during February–March of 2000. However, during February–March of 2001, males over the age of five had a higher rate of infection, which remained high until the middle of the year; otherwise, there was no significant difference in the amoebal infection between the male and female populations (Figure 2).

The frequency of *E. histolytica* infection in both passive and active cases was 1%, while the prevalence of *E. dispar* was 7.3% among passive cases and 15% among active cases.

The detection of amoebal infection with the microscopy was compared to the ELISA test. The sensitivity and specificity of microscopy technique were 39% and 96%, respectively, while the PPV and NPV were 46% and 95%, respectively. The total amoebal infection was 8.2%, and the detection of amoebal infection missed by microscopy was 61% (Table 1). In detecting only *E. histolytica* infection, microscopy sensitivity and specificity were 43% and 93%, respectively. The PPV and NPV were 5% and 99.2%, respectively. The total *E. histolytica* infection was only 0.8%, and the detection of *E. histolytica* infection missed by microscopy was 57% (Table 2). In detecting only *E. dispar*, the sensitivity and specificity were 39% and 95%, respectively. The PPV and NPV for *E. dispar* were 41.1% and 94.7%, respectively. The total *E. dispar* infection was 7.4%, and the detection of *E. dispar* infection missed by microscopy was 61%. (Table 3). This study indicated that *E. dispar* infection was 14.5 times more prevalent than *E. histolytica* infection, and the proportion of *E. histolytica* infection in the population is 7%.

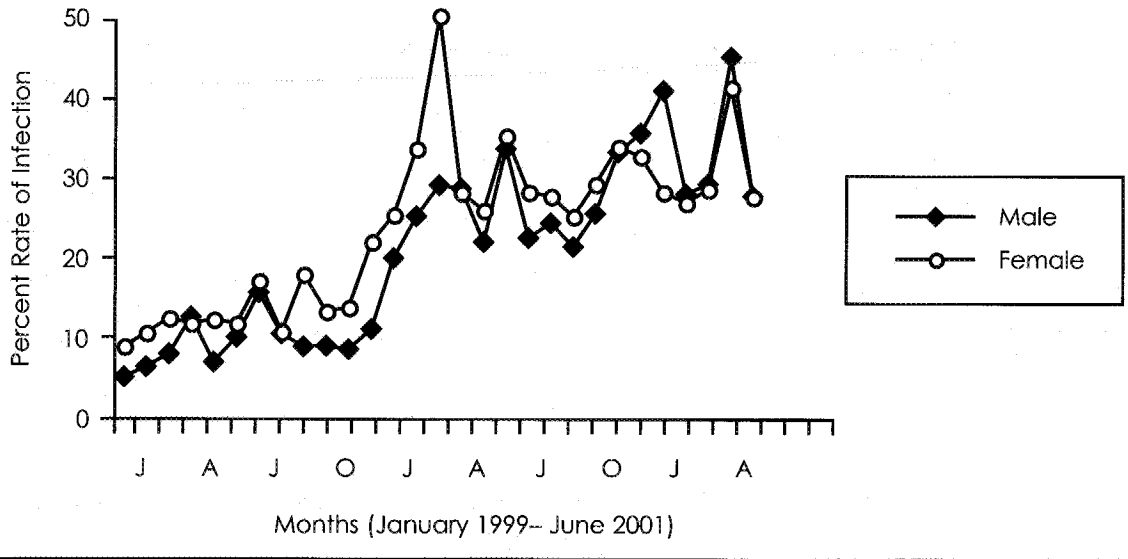
DISCUSSION

Since microscopic examination cannot distinguish with certainty between *E. dispar* and *E. histolytica* parasites, the amoebal infection detected with the microscope wrongly overestimated the number of people infected with *E. histolytica*. Even if the microscope test is positive for *E. histolytica*, it is still highly likely (1-PPV=95%) that the patient does not have *E. histolytica* infection. However, when microscopic examination revealed negative results, then *E. histolytica* infection is very unlikely.

The ELISA technique is considered an ideal gold standard with which microscopy technique is compared. Although the ELISA technique cannot perfectly distinguish between *E. histolytica* and *E. dispar*, it has excellent sensitivity, specificity, PPV, and NPV. On the other hand, microscopic examination gives more false positives, and has low sensitivity (50%) and exceedingly low PPV (3.6%).

The observed prevalence of 0.8% and 7.4% for *E. histolytica* and *E. dispar*, respectively, for amebiasis in Kilimanjaro confirms the postulated idea

Figure 2. Showing the differences in the percent rate of amoebal infection between male and female populations of Kilimanjaro, Tanzania. Both sexes had the same pattern of amoebal infections. The amebiasis was more prevalent during 2000 and 2001,[5] compared to 1999.



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by Clark[18] that the pathogenic amoeba (*E. histolytica*) is only about 10% of all amoebal infections. Although metronidazole is effective in treating *E. histolytica* infection, it has side effects and is expensive. In addition, there is a possibility of developing resistance to the drug through widespread and unnecessary use. The use of metronidazole is intended only for elimination of tissue-invasive organisms and is not effective against intestinal lumen infections.[21] Metronidazole was given for the treatment of nonpathogenic *E. dispar*, which also delayed the treatment for the actual cause of illness. The cost of this unnecessary treatment was calculated in one of the Kibosho's hospitals for passive cases. The total number of *E. dispar* infected patients was 3,539 over the period of three years (1,416 patients per year), and the cost of metronidazole (twice a day for seven days) in U.S. dollars was $16.19 per patient. Therefore, the cost of treating *E. dispar* infection at Kibosho hospital was $22,918.60 per year, which was considered very expensive in the country where the majority of people earn less than $1 U.S. per day. The question is, would it be cost-effective to switch to the ELISA test? The answer is not clear. However, it will reduce unnecessary treatment. The experts on amebiasis[22] supported development and use of alternative diagnostic methods for both clinical and epidemiological studies—most of which have been rendered absolute by differentiation of the two similar species. The expert committee further recommended that, ideally, *E. histolytica* should be specifically identified and treated.

The cases reported for amebiasis are usually a mixture of pathogenic *E. histolytica* and nonpathogenic *E. dispar*, resulting from reliance of conventional microscopic diagnostic methods. Simple, inexpensive diagnostic tests for distinguishing *E. histolytica* infections from those with *E. dispar* are needed to reduce unnecessary drug prescription and to allow for collection of accurate prevalence and incidence data. The prevalence of *E. histolytica* infection is often quoted at 10% of the world population or 500 million infections.[23] This is obviously misleading, if 90% of these infections are due to *E. dispar*.

Why is *E. dispar* important despite the fact that it does not cause disease? Part of the answer lies in the realm of diagnosis. In endemic areas, *E. dispar* is by far the more prevalent species by a ratio of perhaps as much as 10:1.[18,24-27] In Europe and North America, where invasive amebiasis is rare, almost all infections previously ascribed to *E. histolytica* were in fact due to *E. dispar*.[10,14,28,29] The significance of this is that in most cases, there is no need for antiparasitic agents to be administered. Indeed, the new WHO recommendation[12,13] specifically states that drug treatment is not recommended unless *E. histolytica* is unequivocally shown to be present or if there is strong reason to suspect that the patient may be carrying *E. histolytica* and not *E. dispar*.

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