

Original Research Article

COMPARISON OF MICROSCOPY, BOECK AND DRBOLAV'S STOOL CULTURE MEDIUM AND BICHRO-LATEX ANTIBODY TEST WITH A REFERENCE ELISA ANTIGEN TEST. FOR *E.HISTOLYTICA* DIAGNOSIS IN CALABAR, NIGERIA

ABSTRACT

Objective: Amoebiasis is a parasitic infection caused by *E.histolytica*, accurate diagnosis of *E. histolytica* is important in the treatment of amoebiasis and to avoid preventable costs. The study objective is to compare different diagnostic methods used in the diagnosis of amoebiasis for detection of *E.histolytica* parasite.

Materials And Methods: Faecal and serum specimens of 200 patients defined as symptomatic (diarrhea and dysentery) and asymptomatic (a case history of *E.histolytica* infection) was used for the study. Stool specimen was examined with microscopy, cultured in Boeck and drbolav's medium and anti-*E. histolytica* antibodies were investigated using a latex slide test. Stool samples were also examined by immunoassay methods for specific antigens which is the reference standard for comparison.

Result: Two hundred (200) samples examined for *E.histolytica* parasite 12(6.0%) were positive in microscopy, 34(17%) in bichro-latex antibody test and 6(3.0%) in Boeck and drbolav's culture medium. The three test methods showed significant detection of *E.histolytica* parasite ($p < 0.05$). Microscopic method detected 100% of *E.histolytica* infection in symptomatic patients and Boeck and Drbolav's culture medium detected 33.3%. However, the method of diagnosis is not associated with the diagnosis *E.histolytica* infection in asymptomatic and symptomatic Patients ($p > 0.05$). The diagnostic precision of the microscopy diagnostic method showed that sensitivity was 40.2%, specificity was 82.3%, PPV 39.6% and NPV 70.4%. The sensitivity was 86.6%, specificity was 70.6% PPV 87.6% and NPV 75.6% for bichro-latex antibody test and The sensitivity was 20.6 %, specificity was 50.6 %, PPV 34.6%, NPV 61.2% for Boeck and Drbolav's culture medium

Conclusion: The result from the comparison of the three diagnostic methods for *E.histolytica* parasite indicate high sensitivity and specificity for bichro-latex antibody test when compared with the other methods. however, in areas where molecular technology such as Polymerase chain reaction and enzyme linked immunosorbent assay is not available, bichro-latex antibody assay is recommended. although, microscopic examination can be used in diagnosis of amoebiasis in geographical areas with technological limitation in health.

Keywords: Amoebiasis, *Entamoeba histolytica*/*E. dispar*, Entamoeba antigens, ELISA, antibody

INTRODUCTION

Amoebiasis is a disease caused by a parasite *Entamoeba histolytica*. Data from world health organization, reported that *E. histolytica* causes approximately 50 million cases and 100,000 deaths annually [1-3]. Amoebiasis is the major cause of parasitic morbidity and mortality in tropical african countries[2]. The majority of these infections are domiciled in the developing countries such as Nigeria [4]. Most infected individuals are asymptomatic carriers; the others show clinical symptoms such as colitis, dysentery and extra-intestinal amoebiasis[3]. Clinical manifestation of extra-intestinal infection is amoebic liver abscess and a deferral in diagnosis and treatment lead to fatalities [4]. Detection of the *E. histolytica* and its distinction from the non-pathogenic *E. dispar* is important in clinical management of the Patient [5]. Laboratory diagnosis of intestinal amoebiasis in tropical countries depend on labour-intensive method including ,staining of stool sample and microscopy. The stool microscopy is characteristically used in diagnosis of *E. histolytica* infection is incapable to distinguish between *E. histolytica* and the non-pathogenic amoeba *E. dispar*[6-7].

Laboratory diagnostic methods for amoebiasis are based on parasitological, immunological and molecular techniques [8]. The parasitological diagnosis is based on recognition of cyst or trophozoites of *E. histolytica* in stool by microscopic examination. This method is used in numerous parasitology diagnostic laboratories, predominantly in tropical countries[3]. However ,the limitation includes; the morphological related nonpathogenic strain *E. dispar*, misdiagnosis and over-treatment are common. Although the appearance of ingested red blood cells most likely implies infection with *E. histolytica*, the morphologies of *E. dispar*, *E. dispar*, and *E. moshkovskii* are indistinguishable. Furthermore, despite the fact that these three species may be distinguished morphologically from the other common amoebas, an inexperienced microscopist will find it difficult to distinguish them. As a result, microscopic examination's diagnostic sensitivity and specificity for detecting *E. histolytica* in stool are reduced [9-13].

Antibody testing can be used to diagnose amoebiasis, although the majority of patients with intestinal amoebiasis have been exposed to *Entamoeba histolytica* and have generated IgG antibodies to this parasite, which can last for a long period. As a result of the difficulties in distinguishing between past and current illnesses, exact diagnosis utilising available IgG antibody detection techniques is a problem[14]. Stool culture with isoenzyme analysis was commonly used as reference method to differentiate between *E. histolytica* and *E. dispar* . From the cultured amoeba, isoenzyme analysis is accomplished using zymodeme enzymes as markers to detect the parasite[16].

Isoenzyme analysis, on the other hand, necessitates the use of grown amoeba trophozoites, which is laborious and time-consuming [17-19]. Prior to doing starch-gelelectrophoresis, the trophozoites must be grown to a sufficient volume for four to ten days, and the culture may not always be effective [20]. The success percentage of establishing *E. histolytica* culture in reference laboratories has been estimated to range between 50 and 70% [15]. Isoenzyme analysis of *E. histolytica* cultures obtained from clinical samples frequently yields false-negative results. [21].

Furthermore, an overgrowth of bacteria, protozoa, or fungi during *E. histolytica* cultivation can be a big issue. As a result of its low sensitivity, culture combined with isoenzyme analysis is rarely used in diagnosis [22]. The disadvantages of the traditional parasitological techniques such as Stool microscopy, antibody test and stool culture have led to the current use of ELISAs

for laboratory diagnosis of intestinal amoebiasis[8], ELISAs are important for clinical and epidemiological studies, especially where molecular assays are not practical or available(19). The immunoassay is relatively simple and rapid, and can be performed in most laboratories with least skills. Tech Lab *E. histolytica* II ELISA is the most commonly used antigen detection test. It is the first generation kit in ELISA format produced to specifically detect *E. histolytica* Gal/GalNAc lectin in stool samples [19,21]. This lectin protein is highly immunogenic and conserved and can be used to specifically detect *E. histolytica* due to the antigenic differences between the lectins of *E. histolytica* and *E. dispar*. When examined with stool samples from persons who had diarrhoea, this test had a strong association with nested PCR[21]. Furthermore, when compared to microscopy and culture, this test was revealed to have greater sensitivity (80 to 94 percent) and specificity (94 to 100 percent) [23,24]. A second version of the TechLab ELISA kit, dubbed Tech Lab *E. histolytica* II, was created in response to several limitations found in the previous generation kit. When compared to real-time PCR for the diagnosis of *E. histolytica*, it showed good sensitivity and specificity[25,26]. Although molecular detection techniques are highly sensitive and specific, the cost of using them as a standard laboratory test procedure and conducting research in most endemic areas remains a barrier [22]. The aim of this study is to compare different diagnostic methods in diagnosis of amoebiasis with a reference Tech Lab *E. histolytica* II ELISA with high specificity for *E. histolytica* parasite.

MATERIALS AND METHOD

Study Design

Cross sectional study done in general hospital in Calabar, Cross river state from patients diagnosed of asymptomatic and symptomatic dysenteric patients from January –December, 2013

Enrollment criteria

Stool and blood specimen was collected from patients presenting to the general hospital Calabar with acute and persistent dysentery for symptomatic patients and no clinical manifestation of amoebiasis but a history of the infection for asymptomatic patients within the 12 months period of study were enlisted having consented to participate and fulfilled the research criteria.

Inclusion criteria includes; acute or persistent diarrhoea and dysenteric syndrome for symptomatic and no clinical manifestation but a history of the infection.

Exclusion criteria includes ; patients with diarrhoea or dysentery on antimicrobial agents .

Patients who were admitted to the hospital for causes other than diarrhoea and who had not experienced a diarrhoeal sickness in the previous two weeks served as controls.

Sample collection and processing

This study was carried out with stool and serum samples. Serum samples were obtained from aseptically collected blood from 200 patients clinically diagnosed of asymptomatic and symptomatic diarrhoea or dysentery. One pie size of stool sample from each of the patient was

immediately examined using microscopy and formol ether concentration technique. The remaining stool specimens were stored at -20°C until needed for ELISA antigen tests. For *E. histolytica* antibody assays, the serum was separated by centrifugation of the blood at 3,000 r.p.m for 10 minutes at room temperature to obtain the serum. 3–4 mL of the patients' serum sample were collected and stored at -20°C until required for use.

Microscopy

Clinically diagnosed dysenteric and diarrheic specimens from infections or a history of the infection from general hospital Calabar were examined by direct smear method according to the method reported by Cheesbrough [27]. On one end of a slide, a loop of saline is inserted, and on the other end, a drop of iodine is deposited. Using a wire loop a small amount of the faeces is mixed with the normal saline and iodine on the slide and covered with cover slip, then examined systematically with the low and high power ($\times 10$) and ($\times 40$) objectives for trophozoites of *E. histolytica* parasite.

Formol- Ether Concentration Method

Cheesbrough's Formol-Ether concentration method [27] was used to analyse the stool samples. The filtrate from the emulsified faecal samples was transferred to a conical centrifuge tube holding an equivalent volume of ether and centrifuged for 1 minute at 3,000 rpm. The silt was transferred to a clean glass slide and a drop of iodine was added after the faecal particles and ether were discarded. To identify the *E. histolytica* trophozoites, the entire preparation was covered with a cover slip and inspected microscopically under an $\times 40$ objective. **Bichro-Latex**

Antibody Amibe Fumouze Test (Fumouze Diagnostics, Levallois-Perret, France)

Bichro-latex antibody test was done according to manufacturer's instructions, 20 μL of serum from each test were transferred into sterile Eppendorf tubes. The serum samples were diluted in the kit with two drops of diluent. Then, on the test slide, a drop of reagent and a drop of diluted patient serum were added, and the mixture was rotated for 5 minutes in a rotator. Finally, specimens with agglutination were determined to be positive. **Positive and Negative control were included in each test batch for accurate diagnosis.**

The Boeck and Drbohlav's Stool culture medium

The Boeck and Drbohlav's medium was used to culture the dysenteric and diarrhoeic stool with some modifications as described by Sawangjaroen. Calf serum (10%) was used as a substitute of horse serum and bijoux bottle were used as parasite culture tube. Just before culture, a drop of sterilized rice starch (1mg) was included to the medium. Then a small amount of faeces were inoculated in the culture medium and incubated at 37°C for 48 hours. After 48 hours incubation, the culture fluid in the tube was mixed and then observed on a microscope for amoebic growth, the culture was incubated at 37°C and *Entamoeba histolytica* trophozoites along with related bacteria were sub cultured at 48 hours intervals

ELISA Wampole *E. histolytica* II Test (Techlab.)

This is a monoclonal ELISA test that detects *E. histolytica* adhesins (specific antigen) in stools quickly. The specific adhesin for *E. histolytica* was employed in the test, which was a monoclonal antibody-peroxidase conjugate. Before beginning the test, frozen stool samples were

dissolved at room temperature, and the test was carried out according to the directions in the test kit. **Reference Standard**

A reference standard for a positive result was defined as a “Positive” result when *E. histolytica* was detected by antigen testing. They are ELISA Wampole *E. Histolytica* II Test (Techlab.), reference standard for a negative result was defined as a “negative” result by ELISA Wampole *E. Histolytica* II Test (Techlab.)

Statistical Analysis

Correlation of the diagnostic parameters of Microscopy, Bichro-latex antibody and culture with ELISA antigen test for diagnosis of *E. histolytica* test as a gold standard was done using chi-square and kappa's test [17].

RESULT

TABLE 1 Comparison of different methods for diagnosis of *E. histolytica*

Method	NSE	NP	PP(%)
Microscopy	200	12	06
Antibody	200	34	17
Culture	200	06	03

NSE =Number of samples examined NP= number positive PP= percentage positive $X^2=27.456$, df=2, p=0.01

Table 2 comparison of different diagnostic methods in diagnosis of *E. histolytica* infection in symptomatic and asymptomatic patients

Method	NPS	NAS(%)	NSS(%)
Microscopy	12	0 (0.00%)	12(100)
Antibody	34	6(17.6)	28(82.4)

Culture	6	2(33.3)	4(66.66)
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NPS= number positive NAS = Nunnumber of asymptomatic samples NSS= number of symptomatic samples $X^2=3.8, df=2, p=0.150$

Table 3 Diagnostic accuracy of different diagnostic methods for *E. histolytica*

Method	Sensitivity (%)	Spcificity(%)	PPV(%)	NPV(%)
Microscopy	40.2	82.3	39.6	70.4
Antibody	86.6	70.6	87.6	75.6
Culture	20.6	50.6	34.6	61.2

PPV= PositivePredictiveValue NPV = PositivePredictiveValue

The comparison of diagnosis method for diagnosis of *E.histolytica* parasite is shown in Table 1. On the basis of comparison diagnostic method, 6.0% tested positive to the microcopy method, 17.0% tested positive for bichro-latex antibody assay and 3% were positive by Boeck and Drbohlay's culture medium. There was significant difference in the diagnostic method for *E.histolytica* parasite (Table 1). The three test methods showed significant detection of *E.histolytica* parasite. Both chi square and Kappa's test analysis showed that the diagnostic methods significantly detected *E.histolytica* parasite ($p=0.001$).

Microscopic method detected 100% of *E.histolytica* infection in symptomatic patients, 82.4% was detected in Bichro-latex antibody assay for symptomatic patients and 17.6% in asymptomatic subjects and 66.66% in symptomatic patients and 33.3% for asymptomatic subject in Boeck and Drbohlay's culture medium (Table 2). However, the method of diagnosis is not associated with the detection of *E.histolytica* infection in asymptomatic and symptomatic Patient ($p=0.150$).

The diagnostic accuracy of the microscopy diagnostic method showed that sensitivity was 40.2%, specificity was 82.3%, positive predictive value PPV 39.6% and negative predictive value 70.4%.

The sensitivity was 86.6%, specificity was 70.6%, positive predictive value 87.6% and negative predictive value of 75.6% for bichro-latex antibody assay. The sensitivity was 20.6%, specificity was 50.6%, positive predictive value 34.6%, negative predictive value 61.2% for Boeck and Drbohlay's culture medium. (Table 3)

DISCUSSION

Amoebiasis, an enteric disease caused by *Entamoeba histolytica*, is a public health issue in many tropical countries [28]. Identification of the pathogenic *E. histolytica* and its distinction from the non-pathogenic *Entamoeba sp.* is very significant in the clinical management of patients[28]. Laboratory diagnosis of intestinal amoebiasis in tropical countries is labour-intensive and insensitive methods. The manifestation of ingested RBCs in the cytoplasm of the trophozoites is regarded as diagnostic of *E. histolytica* infection. However, Haque *et al*, 1998 found that 16% of *E. dispar* isolates had ingested RBCs; thus, this distinction between the two species is not absolute [21].

In the present study, different diagnostic methods of microscopy, antibody and culture was compared against ELISA antigen techniques for the detection of *E.histolytica* parasite. The detection of *E.histolytica* was 6%, 17% and 3% from microscopy, antibody and Culture diagnostic methods, respectively. This is in contrast to another study, which found *E. histolytica*/*dispar* cysts and/or trophozoites in 2.2 percent of stool samples by direct inspection using the saline-iodine method and 0.7 percent by ELISA method[31]. *E.histolytica* parasite was detected in 1.3% using saline-iodine preparation methods after sedimentation[32] which is lower than the result from this study. A study of stool samples of patients using Microscopic preparation from iodine and saline, detected low *E.histolytica* parasite detection compared to other test and underlined the necessity of working with specific ELISA for *E. histolytica* detection. In a study, specific *E. histolytica* antigen positivity in 21.7% of cases using ELISA and microscopy positivity in 26.4% was reported [34], which is higher than *E.histolytica* reported from the same diagnostic method in this study, the difference may be attributed to study population and environmental factors. However, in a study in 2014, *E.histolytica* positivity was seen in 54.7% of the samples by seeing suspected amoeba cysts/trophozoites using direct microscopy, 15.5% using ELISA and in 7.1% using culture.[35] These results is greater than previously reported, the Increased detection of *E.histolytica* parasite might be due to the huge sample size as our study was limited to only 200 sample for the three diagnostic methods. According to these two investigations, the prevalence of *E. histolytica*/*E. dispar* ranged from 0.2 to 45.9%. They advised employing combination approaches and examining them alongside clinical findings in the laboratory diagnosis of patients with amoebiasis in various regions between 2008 and 2013. The significant difference in the three diagnostic methods for detection of *E.histolytica* parasite indicate that the three methods can be used in diagnosis of the parasite.

In 2011, a study employing the antigen test demonstrated that *E. histolytica*/*E. dispar* was found in 7% of stool samples from patients with clinical gastroenteritis symptoms. They also stated that, because direct microscopy has a limited sensitivity, antigen detection procedures such as ELISA should be used to confirm diagnosis in individuals with suspected amoebiasis[36]. According to a 2012 study, persistent trichrome staining is the preferred approach of *E.histolytica* detection since it enables for later examination of faeces for identification of the protozoa's internal structure[37]. In 2014, researchers discovered that stool antigen testing by ELISA for the diagnosis of amoebiasis has 100% sensitivity and 100% specificity, and they advised that this technology be used as a diagnostic test[38]. A comparison of the IHA, latex agglutination, and the ELISA test, on the other hand. The tests were reported to have a sensitivity

and specificity of 97.6% and 97 percent for IHA, 90.7 percent and 95 percent for latex agglutination, and 93 percent and 100 percent for ELISA, respectively[39]. In a 2009 study, stool samples were separated into two groups: trophozoites and erythrocytes were detected using direct microscopy, and cysts and trophozoites were detected using trichrome and/or lugol staining. They reported the presence of trophozoites in red blood cells that differentiates *E. histolytica* from *E. dispar*. According to Tanyüksel and Petri (2005) [30], sensitivity and specificity for microscopy were about 60% positive and between 10% and 50% for microscopy, 95 percent for an ELISA test based on the antigen in the stool, and between 90% and 85% for an ELISA test based on the antibody in serum. Microscopy and PCR are the gold standard reference procedures, according to Goi et al.,2009[41]. They discovered 17.1 percent and 96.6 percent for antigen testing sensitivity and specificity, and 24.4 percent and 97.5 percent for ELISA, respectively, in their experiments that used microscopy as the gold standard. Tüzemen and Dogan,2014 [35] took multiplex PCR for a reference, and they found sensitivity and specificity at 66.7% and 77.4% for direct microscopy, 44.4% and 83.5% for trichrome staining and 11.1% and 91.3% for ELISA, respectively. The non significant difference between the three diagnostic methods indicates that either of both methods can be used in diagnosing the parasite.

The results from this study discovered 12(100%),28(82.4%),and 4(66.7%) in symptomatic patients in microscopy, antibody and culture diagnostic methods which is higher than the *E.histolytica* parasites detected in asymptomatic subjects. However, *E.histolytica* parasite was not detected in microcopy diagnostic method these is explained that trophozoite /parasite presents in stool there must be active infection [42]. Asymptomatic infection, symptomatic infection without tissue invasion, and symptomatic infection with tissue invasion are all possible clinical manifestations of *E. histolytica*/*E. dispar* infection. The majority of *E. histolytica*/*E. dispar* infections are asymptomatic. Individuals with such infections will have a negative or weak serologic response[42] this accounts for decrease in detection of *E.histolytica* parasite in antibody diagnostic method. The study also revealed that the three diagnostic methods was not significantly associated with the detection of *E.histolytica* parasite in asymptomatic and symptomatic patients.The implication from this results is that the three methods can only detects the parasite in symptomatic patients significantly.

The sensitivity of 86.6 % the antibody diagnostic method was high ,when compared to the two other diagnostic method .This is in line with Kraoul *et al*,[24] who reported 90.7% latex agglutination for antibody and 93.0 % for ELISA antibody .although ,slightly higher than the result. The difference is as a results diagnostic method. The specificity was higher 82.3% in microscopy diagnostic method; this is in contrast to other studies Singh *et al*,2009 Tanyüksel and Petri,2005. Goñi *etal*. 2012 [41]. Tüzemen and Dogan, 2014 [35] which reported lower specificity. This is explained by the stool concentration technique for microscopy in this study. The positive predictive value and negative predictive value 87.6% and 75.6% respectively was highest for the antibody diagnostic method. The results suggest that antibody diagnostic method showed good performance in detecting *E.histolytica* parasite.

The clinical implications of this study are significant since. Therefore, most patients identified with *E. histolytica*/*E. dispar* complex infection by microscopy in Calabar received unnecessary therapy. Use of simple cost effective latex agglutination antibody test would allowed for a

specific diagnosis and remove the need for unnecessary chemotherapy with its attendant costs, risk of side effects, danger of drug resistance, and potential mistreatment of another disease. Although previous studies suggest that the rate of false-positive results for serology is higher [21] serological based antibody test may help identify *E. histolytica*–infected patients

CONCLUSION

In conclusion, we suggest that latex agglutination be used in routine laboratory screening tests and epidemiological studies in areas where amoebiasis is endemic and where ELISA and PCR facilities are not available, due to its speed, simplicity, and low cost, as well as its better to moderate accuracy and specificity. However, microscopy can also be used in resource limited countries.

CONSENT According to laid down international standards written informed consent was obtained from the patient (or other approved parties) for publication of this study. Written informed consent was obtained from all study participants

ETHICAL APPROVAL Ethical approval was sought and obtained from the appropriate ethics committee. All tests were performed in accordance with laid down standards. Ethical clearance was sought and obtained from the ethical committees of the University of Calabar Teaching Hospital.

DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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