Evaluation of rapid diagnostic methods for the diagnosis of cryptococcal meningitis in HIV positive patients in a health facility, Nairobi-Kenya

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ABSTRACT

Background: Cryptococcal meningitis is a fatal opportunistic infection in immune-compromised patients. Lack of simple, affordable, rapid and specific methods for diagnosis in many government health facilities in Kenya has aggravated patients' conditions.
Objective: This study was aimed at determining the performance of India ink (Microscopy), latex agglutination test (LAT) and enzyme immunoassay (EIA) in the diagnosis of cryptococcal meningitis in a Kenyan population.
Design: Laboratory based cross-sectional study.
Setting: A high-volume government health facility based in Nairobi.
Subjects: One hundred and thirteen CSF samples from HIV positive patients with signs and symptoms of cryptococcal meningitis sent to the laboratory for routine analysis.
Methodology: The study was approved by the KEMRI Scientific Committee/Ethics Review Committee and informed consent sought from the patients. The test for the agreement between the test methods and the gold standard (Culture) was calculated using the non-parametric McNemar's test using SPSS version 17 (SPSS Inc., Chicago, IL) at 5% significant level.
Results: Twenty one point six percent of all samples tested positive on LAT while 9% were positive on microscopy, EIA and culture. The sensitivity, specificity, positive and negative predictive values for microscopy and EIA were: 90%, 99%, 90% and 99% respectively. By LAT, the values were: 100%, 86.1%, 41.7% and 100% respectively. Both EIA and microscopy had an agreement of 89% whereas that of LAT was 52.8% with the gold standard (C.S.F culture).
Conclusion: From the results of this study, it is evident that Latex Agglutination Test was the most sensitive among the study methods. Therefore, LAT is an appropriate diagnostic test but requires confirmatory testing. EIA could be an appropriate confirmatory test but is limited because it is not available in our setup. India ink though available is user-dependent, a major limitation. The use of test combinations did not increase the sensitivity of neither India ink nor enzyme immunoassay and is therefore not significant in the diagnostic work up for cryptococcal meningitis.
Recommendations: Local settings may consider frequent and unlimited use of LAT as a sensitive diagnostic tool for cryptococcal meningitis. It is also important to note that positive LAT tests are accompanied with a confirmatory test due to unfavourable specificities as evidenced from the research findings. In areas where cost is not a limitation, EIA may be used as a confirmatory test. Future research of comparing lateral flow immunoassay (LFA) and the current test methods using CSF in our hospital set up is warranted.

Key words: Enzyme Immunoassay, Latex Agglutination Test, India ink, Culture, HIV/AIDS and Cryptococcal meningitis

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INTRODUCTION

Cryptococcal meningitis (CM) is a fungal disease caused by *Cryptococcus species*. It is the most common form of meningitis and a leading cause of death among HIV/AIDS positive persons in sub-Saharan Africa[1]. This condition is also a common opportunistic infection in immune-compromised patients, particularly in southeast Asia and Africa[2].

Current data indicates that there is an increased incidence of cryptococcal meningitis associated with a higher mortality than meningococcal meningitis caused by *Neisseria meningitides*[3]. Mortality from HIV-associated CM is said to be 10-44% worldwide[4].

Currently, diagnosis of cryptococcosis in government health facilities in Kenya includes: microscopy (India ink) and/or culture-based methods, or occassionally and limited, detection of cryptococcal antigen (CrAg) in body fluids using latex agglutination test (LAT) in non governmental organisation (NGO) supported laboratories. India ink is simple to perform and rapid, however, it has been proven to have poor sensitivity of about 50% to 80% and a lower detection limit of approximately 1,000 yeast cells per millilitre of cerebral spinal fluid (CSF) [5]. Culture technique-though considered a gold standard method of diagnosis-is cumbersome, labour-intensive and time-consuming resulting in the deaths of patients yet to be diagnosed. It has also been known to show poor sensitivity[6].

LAT has a high sensitivity of over 90%[7]. However, this test has unacceptably high rates of false negativity and it is difficult to interpret the borderline cases[8]. In addition, LAT cross reacts with other fungal agents such as *Candida* and *Trichosporon* species as evidenced in most researches[8]. The EIA test is a rapid test that provides visual and numeric results in less than an hour. The test has low cross-reactivity with other micro-organisms[4]. It has been shown to have a higher sensitivity of 100% and is less expensive (almost half the cost of latex agglutination test)[9].

In this study, we sought to determine the performance of India ink, LAT and EIA against that of culture in the diagnosis of cryptococcal meningitis among HIV/AIDS positive patients in one of the government health facilities in Nairobi.

MATERIALS AND METHODS

Clinical samples and processing: One hundred and thirteen HIV/AIDS positive patients admitted in a health facility in Nairobi, with informed consent, requested to participate in the study. Three millilitres of CSF were collected in prelabeled sterile bijou bottles and transported to KEMRI Mycology laboratory. Upon arrival of the samples in the laboratory, the specimens were centrifuged at 3000 rpm for 10 minutes and pellet obtained. The pellet and supernatant were aliquoted into separate sterile tubes and stored at 4-8^{o}C until the time for fungal isolation by culture, fungal detection by India ink and antigen detection by cryptococcal latex agglutination test (LAT) using IMMY Kit (Immuno-Mycologics, Inc. Norman U.S.A) and enzyme immunoassay (EIA) using PREMIER cryptococcal antigen kit (Meridian Bioscience, Inc. Cincinnati, Ohio, and U.S.A). For LAT, the supernatant was inactivated by placing in boiling water bath (100^{0}C) for 5 minutes prior to each test to limit non-specific interference. The standard operating procedure (SOP) for the processing of samples was followed with minor adjustments in the volume(s) of sample and reagent.

Culture: Primary isolation was done on Sabouraud dextrose agar (Oxoid) plates, incorporated with 2% chloramphenical. The cultures were incubated at 37^{0}C for two weeks and for a further three weeks where there was no growth observed from the initial incubation. The coded *cryptococci* isolates were characterized phenotypically and aliquot samples stored as water cultures at room temperature and at -80°C. The yeast isolates from Sabouraud agar were plated on ChroMagar Candida agar (ChroMagar Frane, Paris, France) incubated aerobically at 30°C for preliminary differentiation and for detection of mixed yeast colonies**.** Plates on ChroMagar Candida Medium were read after 42 to 48 hours. Pink colonies suspected to be *Cryptococcus* were sub-cultured on analytical profile index medium for confirmation of species.

India ink (Microscopy): Fifty microlitres of the CSF pellet were placed on a clean slide and fifty microlitres of India ink reagent added and cover slipped and observed under low power, X10 using Olympus microscope (BH, Japan). A refractile capsule in a dark background indicated a positive test while the absence of a refractile capsule was interpreted as a negative test[8].

Latex agglutination test: This was performed with IMMY Kit (Immuno-Mycologics, Inc. Norman U.S.A). The test was performed according to the manufacturer's instructions with minor adjustments in volumes of reagent and specimen used. 20 µl of Cryptococcus Antigen Positive Control (REF CB0010), Negative Control (REF N80110) and each heat-treated CSF were placed onto separate rings of the ring slide. A new pipette tip for each reagent and specimen was used. 20 µl of Cryptococcal Latex (REF CG0020) was added to each ring. Separate applicator sticks, were used to mix the contents of each ring. The ring slide was then placed on a rotator set to 100 rpm for 5 minutes at room temperature. The reactions were read immediately.

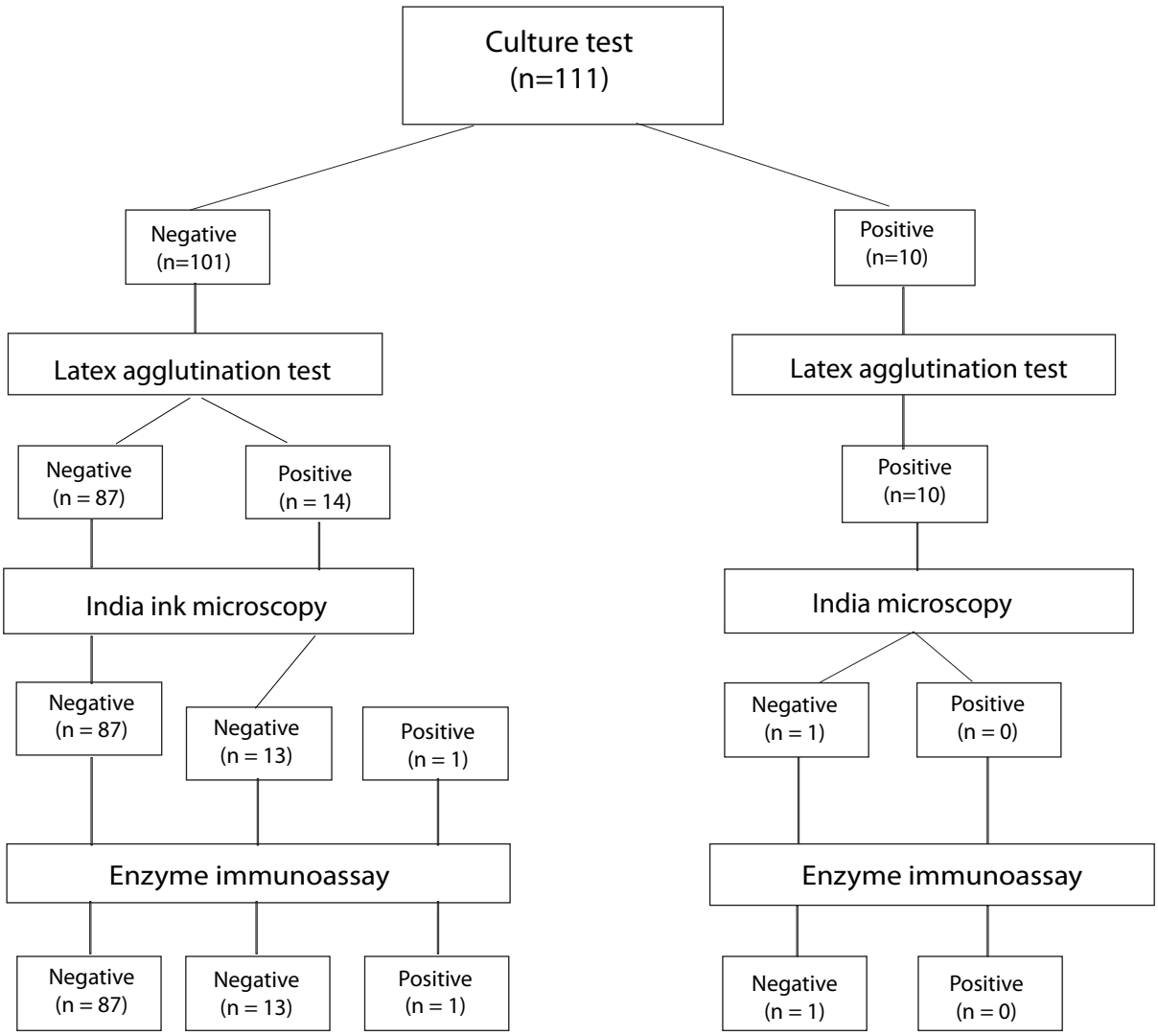
Enzyme immunoassay: Enzyme immunoassay was performed using the PREMIER Cryptococcal antigen kit (Meridian Bioscience, Inc. Cincinnati, Ohio, and U.S.A). The procedure was carried out as described by Saha *et al*[6] . Fifty microlitres of CSF were added to anticryptococcal polyclonal antibody coated microtitre plates for ten minutes, followed by addition of fifty microlitres of horseradish peroxidase-conjugate monoclonal antibody for ten minutes. The micro wells were then washed with wash buffer four times and a substrate added and incubated for ten minutes. The reaction was stopped with 2 Normal solution of sulphuric acid (2N H_2SO_4). Results were

read visually and spectrophotometrically at 450 nm within fifteen minutes after adding stop solution.

RESULTS

A comparative evaluation of the various diagnostic tests was done in 111 CSF samples by taking CSF culture as the gold standard (2 samples were excluded from the study; one was grossly blood-stained and the other was positive for *Rhodotorulla* species hence reducing the sample size from 113 to 111). Of the 111 samples, 10 (9%) were positive for *Cryptococcus neoformans* (*CN)* while 101 (91%) were negative by microscopy, EIA and culture. Twenty four (21.6%) of the samples were LAT positive. Of the 101 culture negative, 14 (13.9%) were positive by LAT whereas 1 (0.99%) were positive by both microscopy and EIA. Of the 10 culture positive, 100% were positive by LAT while 9 (90%) were positive by both India ink (microscopy) and EIA (Figure 1).

Figure 1: *Flow chart of the results from the three methods under study in comparison with C.S.F culture results*



Based on CSF culture as the gold standard (for a comparative analysis), test sensitivities were as follows: LAT 100%; Microscopy and EIA, 90%. The specificities of each test were; 86.1%, and 99% respectively. LAT had 41.7% positive predictive values (PPV) while India ink and EIA had 90%. Negative predictive values (NPV) were; 100% by LAT and 99 % by India ink and EIA respectively (Table 1).

Table 1: *Performance characteristics of specific tests against C.S.F culture as the gold standard*

Tests	Sample Size	Sensitivity (%)	95%CI	Specificity (%)	95%CI	NPV (%)	PPV (%)
Latex agglutination test	111	100	74.1-129.4	86.1	77.8-92.2	100	41.7
Enzyme	111	90	55.5-99.7	99	94.6-99.9	99	90
Immunoassay	111	90	55.5-99.7	99	94.6-99.9	99	90

CI = Confidence interval, (%) = Percentage

Kappa Statistic for each test was: LAT 52.8% (0.53), Microscopy 89% (0.89) and EIA 89% (0.89). Using McNemar test at 5% level of significance, with exact probability p = 1.000, there was no significant difference amongst microscopy (India ink), EIA and culture method of diagnosing cryptococcal meningitis. However, there was a highly significant difference between LAT and culture ($p < 0.001$).

Culture from these samples showed the presence of *Rhodotorulla* species in one specimen while the rest were *cryptococcus* species. Among the *Cryptococcus* species identified; 6 (60%) were *Cryptococcus neoformans*, 3 (30%) *Cryptococcus laurentii* while 1 (10%) was *Cryptococcus albidus.*

DISCUSSION

Reliable diagnostic and screening tests are valuable tools in making a fast diagnosis to guide disease management. There are notable challenges facing diagnosis of cryptococcal meningitis in most government health facilities in Kenya because of lack of reliable, cheap and rapid diagnostic methods. This study sought to determine the test performance of India ink (Microscopy), LAT, EIA and Culture techniques in the diagnosis of cryptococcal meningitis in patients attending a health facility in Nairobi, Kenya.

In this study, both microscopy and EIA had higher overall accuracy in the detection of the cryptococcal infection among the studied population with sensitivity and specificity of 90% and 99% respectively and a NPV and PPV of 99% and 90% respectively compared to Culture. Comparable results have been reported elsewhere: sensitivity ranging from 93.5% to 100% of India ink method and a varying specificity ranging from 62.5% to 100%[5,10]. These two studies had comparable PPV and NPV to ours (100% versus 90%) and 90.9% versus 99%) respectively. Contrary reports also exist. Sato *et al*.[11] noted that though India ink was a rapid test, the technique is quite insensitive and strongly depends on the operator's skills. Other studies have shown a modest India ink sensitivity of 60%, worse than a clinical determination based on low CD4 count plus coma and advocated for increased availability of cryptococcal antigen tests[12]. Saldanha *et al*.[13] revealed that India ink staining though rapid and specific (specificity: 100%) was not sensitive (sensitivity: 50%) compared to antigen detection which in their study proved to be both a sensitive and specific method. It is worth noting that the results obtained by India ink method strongly depend on the reader rather than the weakness of the test in terms of its performance per se.

Enzyme immuno assay (EIA) though not routinely available locally gave comparable results with culture. In other studies by Sekhon *et al*.[14] and Illnait *et al*.[15], EIA has been considered as a rapid serological test for *C. neoformans* in CSF providing visual and numeric results in less than an hour and with a low cross-reactivity with other microorganisms. EIA has potential advantages over LAT as it provides a clear discrimination of positive from negative results (cut-off), and higher sensitivity and specificity. Despite notable positives, the test has a limitation of unavailability. LAT, considered the most rapid method for diagnosis of cryptococcosis for several months after infection, gave a sensitivity and specificity of 100% and 86.1% respectively against culture[16.] Other studies found a high sensitivity 100% and varied specificity 33.9 to 86.7% associated with the apparent subjectivity of reading and grading the LAT, especially in borderline cases and the kits variation depending on the manufacturers[5,10,17]. From this study, it is apparent that LAT would be appropriate for diagnosis but requires a confirmatory test if positive.

One unique aspect of this study was the ability to compare performance of the three (India ink, LAT and EIA) tests combined against Culture as the gold standard. This model involved testing all the samples first tested by Latex Agglutination Test then retesting all the positive samples by India ink microscopy and enzyme immunoassay. Only triple positives (positive by three tests) were counted as positive; sample positive by latex agglutination but negative by either India ink microscopy or enzyme immunoassay were counted as negative. Sensitivity and specificity of this three test combination was 90% and 99% compared with culture while PPV was 90% and NPV was 99%. While the combination improved the specificity (99% versus 86.1%) and PPV (99% versus 41.7%) of Latex Agglutination Test, this test combination did not improve the sensitivity of India ink or enzyme immunoassay Tests.

CONCLUSIONS

From the results of this study, it is evident that Latex Agglutination Test was the most sensitive among the study methods. Therefore, LAT is an appropriate diagnostic test because of the high sensitivity but requires confirmatory testing. EIA would be an appropriate confirmatory test but is limited because of its unavailability in our setup. India ink though available is user dependent and this is a major limitation. Therefore future research of comparing Lateral Flow immunoassay (LFA) and the current test methods using CSF in our hospital set up is warranted.

Use of test combinations did not increase the sensitivity of India ink nor enzyme immunoassay and is therefore not significant in the diagnostic work up for cryptococcal meningitis.

Local settings may consider frequent use of LAT as a sensitive diagnostic tool for cryptococcal meningitis to minimize cases of patients dying undiagnosed.

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