Rapid diagnosis of cryptococcal meningitis using the lateral flow assay
on CSF samples: the influence of the high dose “hook” effect.

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ABSTRACT

Cryptococcal meningitis is the most frequent cause of meningitis and a major cause of mortality in HIV-infected adults in Africa. This study evaluates the performance of the lateral flow assay (LFA) against existing diagnostic tests on CSF samples for the diagnosis of cryptococcal meningitis. LFA performed on 465 undiluted CSF samples had a sensitivity of 91%. When paired with Gram stain, a sensitivity of 100% was achieved after implementation of a dilution step for samples with negative LFA results and yeasts on microscopy. Microscopy is essential in preventing the reporting of false negative results due to the high dose “hook” effect.

Key words: Cryptococcus neoformans, meningitis, diagnosis, lateral flow assay, high dose hook effect
Cryptococcal meningitis (CM) is a frequent HIV-related opportunistic infection caused by *Cryptococcus neoformans* (serotype A and D) and *Cryptococcus gattii* (serotype B and C). It is the main cause of adult meningitis in Sub-Saharan Africa (SSA) and is a major cause of HIV-related mortality accounting for between 13 - 44% of deaths in HIV-infected cohorts in resource-limited countries. Case fatality rates remain unacceptably high. Locally the 30-day mortality rate is 33 - 41% in routine settings, possibly related to delayed diagnosis and commencement of appropriate combination anti-fungal therapy. Despite the high case fatality ratio, these patients may have good long-term survival rates, if able to overcome the acute phase of illness. Key factors influencing survival are fungal burden at presentation and the rate of sterilization of cerebrospinal fluid (CSF) with combination treatment. Rapid and accurate laboratory diagnosis of CM is thus important to enable timely use of appropriate medication and prevent diagnostic delays contributing to increased CSF fungal loads and poor clinical outcomes.

Rapid detection of *Cryptococcus* has previously been hampered by the lack of a point-of-care (POC) test for CM. Standard diagnostic methods include India ink staining, the conventional latex agglutination test (CLAT), and culture of CSF which is generally performed by trained technical staff, predominately at centralized laboratories. Samples are referred from peripheral hospitals and clinics in South Africa.
Africa (SA) with subsequent delay in the return of results to sites of patient management. CLAT testing is labour intensive and sample batching may further delay turn-around time. Cultures may be negative or slow to grow in patients with low fungal burdens or those initiated on treatment. Prolonged fungal culture often results in bacterial contamination and further delays as the isolate is purified. Given the high mortality rate of CM it is clear that initiation of treatment cannot be delayed pending culture results \(^{(15)}\).

The recent development of the Cryptococcal Antigen Lateral Flow Assay (LFA) (Immuno Mycologics Inc, Norman, USA), a commercially available rapid diagnostic test that detects capsular polysaccharide antigens of the four major cryptococcal serotypes (A and D for \textit{var neoformans} and B and C for \textit{var gattii}), has addressed some of the limitations of the current diagnostic tests \(^{(1)}\). The LFA is essentially a sandwich immunochromatographic assay adapted to operate along a single axis to suit the dipstick (test strip) format. The test uses specimen wicking to capture gold-conjugated, anti-CrAg monoclonal antibodies and gold-conjugated control antibodies on the test membrane.

It has proved to be inexpensive, stable at room temperature and easy to perform. Early demonstration studies have shown that the LFA has a high level of agreement with conventional antigen tests when performed on urine and serum samples \(^{(16)}\). The LFA has been FDA approved for use on CSF, but there is limited published data.
available on the sensitivity and specificity when compared to CLAT and culture (1,17).

The aim of this study was to evaluate the performance of the LFA on CSF samples for the diagnosis of CM compared to CLAT and culture, which together with microscopy are the current diagnostic tests employed at the National Health Laboratory Services (NHLS) laboratories in SA.

**METHODS**

**Clinical samples.** Consecutive CSF samples referred to the NHLS microbiology laboratory at Tygerberg Hospital, Cape Town, SA, from patients with suspected CM were prospectively collected during October 2012. Suspects included clinically suspected or confirmed HIV-infected patients with signs and/or symptoms suggestive of meningitis. Samples were included in the study if there was sufficient sample for analysis after routine laboratory investigations had been performed. The study was approved by the Health Research Ethics Committee of the Faculty of Health Sciences at Stellenbosch University.

**Laboratory investigations.** The LFA was performed on CSF samples according to the manufacturer’s instructions; 40 µl of sample diluent was mixed with 40 µl of CSF in a disposable tube, followed by insertion of the LFA test strip. Results were read after 10 minutes (18). A positive result was reported if two visible lines developed over the control and test area and negative if a single control line was present (18). If the LFA was negative but Gram stain demonstrated the presence of yeasts, the LFA
was repeated with a dilution of the CSF; 40 µl of sample diluent was mixed with 40 µl of CSF plus 40 µl of titration diluent (1:2 dilution). The LFA test strip was then inserted and read as above. Test strips from 10 different allotments were used during this study.

If the LFA was negative but Gram stain demonstrated the presence of yeast, the LFA was repeated with a dilution step – 1 drop of titration diluent was added to the CSF sample prior to the addition of the sample reagent and test strip.

Gram stain, bacterial and fungal culture were performed on CSF samples according to laboratory protocols. CSF was referred for additional investigations as per clinicians’ request (TB, VDRL). Selective agar (Sabouraud-dextrose) for yeast isolation was utilized and incubated aerobically at 35 degrees Celsius for 14 days to ensure optimal growth (15). *Cryptococcus* species were identified on the automated Vitek 2 platform (BioMérieux, France) (19) or Auxacolor 2 (Bio-Rad, Marnes-la-Coquette, France) colorimetric sugar assimilation test. *Cryptococcus Antigen Latex Test* (Remel Inc, Lenexa, USA), to detect polysaccharide antigens (CPS) of *C. neoformans*, using murine IgM monoclonal antibodies, was performed according to the manufacturer’s instructions (20). Pronase and heat-inactivation treatment was not performed on the CSF samples prior to performing the CLAT. Laboratory testing was conducted in an ISO-accredited facility. The investigators who performed the LFA testing were blinded to the CLAT and culture results.

**Data Analysis.** The performance of the LFA was assessed by determining the sensitivity and specificity of the test in comparison to CLAT. Results were re-analyzed...
using culture as a reference standard. Agreement between diagnostic tests was summarized using kappa statistics and analyzed using McNemar’s test of equality of paired proportions.

**RESULTS**

Four hundred and sixty five CSF samples were included in the study over a period of one month. Samples included CSF obtained from adult and paediatric patients.

**Analysis using CLAT as reference standard:** The lateral flow assay performed on 465 undiluted CSF samples detected cryptococcal antigen in 31 samples (6.7%). In contrast, cryptococcal antigen was detected by CLAT in 33 (7.1%) samples. Three (0.6%) samples were CLAT positive but LFA negative. All three samples showed numerous yeast cells on Gram stain, and were LFA positive after further 1:2 sample dilution. Using a positive CLAT result as a gold standard, the sensitivity and specificity for LFA on undiluted CSF samples was 90.9% and 99.8% respectively (Table 1). After sample dilution, the sensitivity improved to 100% (Table 2). Clinical data from these 3 patients was retrieved. This is summarised in Table 3. Of the 31 LFA positive samples, CLAT was positive in 30 CSF samples, thus one additional case was detected using the LFA of which the culture yielded no growth. This sample was from a patient known to have had prior CM and most likely had low-level antigenemia undetectable by CLAT and a negative culture due to prior antifungal therapy. Assuming therefore that this positive LFA is a true positive result yields an LFA specificity of 100%.
Analysis using CSF culture as reference standard: *C. neoformans* was cultured on selective media in 26 (5.6%) CSF samples sent for analysis. All culture-positive samples were CLAT positive and 23 were LFA positive. The three discordant samples were those described above, which after sample dilution, were also positive using the LFA. Of the 439 patients with a negative culture result, 7 (1.6%) were CLAT positive and 8 (1.8%) were LFA positive.

**DISCUSSION**

Our study found that the LFA had a sensitivity of 100% for the diagnosis of CM when samples suspected to have a high organism load were diluted prior to performing the assay. However, when performed on neat CSF samples, the sensitivity of the LFA was only 90.9% compared to CLAT as the reference standard. This reduced sensitivity could possibly be due to the high dose “hook” effect (18, 22) (also referred to as prozoning), one of the potential limitations of the LFA described by the manufacturer (18). This occurs when excess analyte, in this case high concentrations of cryptococcal antigen, result in decreased visual intensity of the test lines, or as in this case even yielded a negative test results (18). Exceedingly high concentrations of unbound CrAg may out-compete the gold-labeled antibody-antigen complex that normally wicks up the membrane to interact with the test line which has the immobilized anti-CrAg monoclonal antibodies. The latter reaction will result in a visible test line but unbound CrAg interacting avidly with the monoclonal antibodies will result in no line. This effect may be negated by changing the dilution of the assay.
The 3 false negative LFA results showed numerous yeast cells on microscopic examination of the CSF Gram stain and cultured the organism after a mean incubation period of 48 hours. These false-negatives corrected after a 1:2 dilution allowing the sensitivity of the diagnostic LFA to reach 100%.

This is the first report of a probable high dose “hook” effect with cryptococcal LFA testing of CSF, and a limitation that clinicians and diagnostic laboratory staff need to be aware of. A negative LFA test, with a Gram or India Ink stain demonstrating yeast cells, should prompt LFA re-testing using serial dilutions of the CSF sample. Using the LFA for antigen detection in CSF in conjunction with staining and microscopy should, therefore, diagnose all cases of CM with equivalent or superior sensitivity to CLAT testing.

The calculated specificity of the LFA in this study should be interpreted with caution, as the CLAT is not an ideal reference test. The LFA has been reported to detect lower levels of antigen than the conventional latex agglutination tests. Furthermore, the LFA is known to detect antigens of C. gattii infection with a higher sensitivity than current latex agglutination tests. In our study species identification could not be ascertained in the single discordant case (with the positive LFA but negative CLAT result) due to the negative culture.

Despite the possibility of the high dose “hook” effect observed when using the LFA in a small number of CSF samples with visible cryptococci on Gram stain, the LFA remains an attractive test for use in most settings. The LFA is stable at room
temperature, has a rapid turn around time with results available within 10 minutes, and does not require sample pre-treatment or processing except in cases suspected with a high organism load, where serial dilution is required. Minimal equipment is necessary to perform this relatively inexpensive test, and in settings where laboratory staff is often overworked, the major advantage of LFA over the current latex agglutination or previous enzyme immunoassays, is its ease of use. A further potential advantage of the LFA is its utility as a POC test, but there is limited data on the POC use on CSF \(^{21}\). Potential difficulties in achieving widespread implementation of the LFA as a POC diagnostic test for CM include the need for supplementary microscopy results on CSF samples and the need to ensure proper quality control of the assay in the hospital setting. Much progress has been made in introducing similar assays for the diagnosis of malaria at POC facilities. Given the scale of the HIV pandemic and the substantial morbidity and mortality associated with CM, particularly in peripheral hospitals, a similar approach for the diagnosis of CM, should be undertaken.

In summary the LFA is a simple, inexpensive test that allows for reliable detection of Cryptococcus neoformans antigens in CSF provided a microscopy result is available, and could replace the traditional diagnostic method of latex agglutination. A negative test with a positive microscopy finding should prompt repeat testing using serial dilution. Alternatively, a routine 1:2 dilution could be performed on all samples from HIV positive or highly suspicious patients that tested negative on the neat sample, but further assessment in larger cohorts with antigen quantification (titers)
are necessary to evaluate if a 1:2 dilution would detect all positive patients. Nonetheless, it is important for researchers, microbiologists and clinicians to be aware of the potential for the high dose “hook” effect.

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20. CLAT REMEL package insert: http://www.oxoidshop.com/sap%28cz1TSUQjM2FBTk9OJTNhbXlzDFhMDFfQzExXzAwJTNhczVtbWIHOFPFaigzS19KR2xIbDNXUXJYbk51SXjrRvp6UWZNV51qXY1BVFO=%29/bc/gui/sap/its/zqc42R/~f1NUQVRFPTI3ODk1LjAwNC4wMi4wNA==?qcwww-selcharg=D&qcwww-selmatnr=++++2&qcwww-selwerks=REM&qcwww-kunnr=106343&~okcode=CERT


<table>
<thead>
<tr>
<th></th>
<th>CLAT Positive</th>
<th>CLAT Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA Positive</td>
<td>30</td>
<td>1†</td>
<td>31</td>
</tr>
<tr>
<td>LFA Negative</td>
<td>3</td>
<td>431</td>
<td>434</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>33</strong></td>
<td><strong>432</strong></td>
<td><strong>465</strong></td>
</tr>
</tbody>
</table>

(b) Post-dilution*

<table>
<thead>
<tr>
<th></th>
<th>CLAT Positive</th>
<th>CLAT Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA Positive</td>
<td>33</td>
<td>1†</td>
<td>34</td>
</tr>
<tr>
<td>LFA Negative</td>
<td>0</td>
<td>431</td>
<td>431</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>33</strong></td>
<td><strong>432</strong></td>
<td><strong>465</strong></td>
</tr>
</tbody>
</table>

CLAT, Cryptococcal latex agglutination test; LFA, Lateral flow assay

* All samples were analyzed using the LFA according to the manufacturer’s instructions – 1 drop of sample reagent was added to 40 µl of CSF. A further 1 in 2 dilution step was performed in samples which were LFA negative, but contained yeast cells on microscopy.

† This sample was from a patient known to have had prior CM and most likely had low-level antigenemia undetectable by CLAT with a negative culture due to prior antifungal therapy.
TABLE 2. Performance of the lateral flow assay and conventional cryptococcal latex agglutination test on 465 CSF samples (a) before and (b) after CSF dilution

(a) Lateral flow assay (LFA) pre-dilution

<table>
<thead>
<tr>
<th>Comparator</th>
<th>n</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLAT</td>
<td>465</td>
<td>91%</td>
<td>99.8%*</td>
<td>96.8%</td>
<td>92.9%</td>
<td>0.9329</td>
</tr>
<tr>
<td>Culture</td>
<td>465</td>
<td>88.4%</td>
<td>98.1%</td>
<td>74.1%</td>
<td>99.3%</td>
<td>0.7945</td>
</tr>
</tbody>
</table>

(b) Lateral flow assay (LFA) post-dilution†

<table>
<thead>
<tr>
<th>Comparator</th>
<th>n</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLAT</td>
<td>465</td>
<td>100%</td>
<td>99.8%*</td>
<td>97%</td>
<td>100%</td>
<td>0.9839</td>
</tr>
<tr>
<td>Culture</td>
<td>465</td>
<td>100%</td>
<td>98.1%</td>
<td>76.4%</td>
<td>100%</td>
<td>0.8576</td>
</tr>
</tbody>
</table>

CLAT, Cryptococcal latex agglutination test; PPV, positive predictive value; NPV, negative predictive value

*The specificity of the LFA test was 100% when a single sample from a patient known to have had prior CM and most likely low-level antigenemia undetectable by CLAT and culture due to prior antifungal therapy, was assumed to be a true positive result.

†All samples were analyzed using the LFA according to the manufacturer’s instructions – 1 drop of sample reagent was added to 40 µl of CSF. A further 1 in 2 dilution step was performed in samples which were LFA negative, but contained yeast cells on microscopy.
Table 3: Summary of the CSF findings on the 3 patients with false negative LFA results, that cultured C.neoformans.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>HIV status</th>
<th>CD4 Count</th>
<th>Polymorphonuclear cells (cu mm)</th>
<th>CSF Lymphocytes (cu mm)</th>
<th>CSF Erythrocytes (cu mm)</th>
<th>CSF Protein (g/l) (0.15-0.45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>59</td>
<td>Pos</td>
<td>217</td>
<td>44</td>
<td>50</td>
<td>17</td>
<td>1.62</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>Pos</td>
<td>Unknown</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>0.44</td>
</tr>
<tr>
<td>C</td>
<td>55</td>
<td>Pos</td>
<td>125</td>
<td>3</td>
<td>17</td>
<td>53</td>
<td>0.91</td>
</tr>
</tbody>
</table>