JCM
Journals.ASM.org

Rapid Diagnosis of Cryptococcal Meningitis by Use of Lateral Flow Assay on Cerebrospinal Fluid Samples: Influence of the High-Dose "Hook" Effect

André Lourens,[a] Joseph N. Jarvis,[b,c,d] Graeme Meintjes,[e] Catherine M. Samuel[a]

Division of Medical Microbiology, Stellenbosch University, National Health Laboratory Services, Tygerberg Hospital, Cape Town, South Africa[a]; Department of Clinical Research, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom[b]; Botswana-University of Pennsylvania Partnership, Gaborone, Botswana[c]; University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA[d]; Institute of Infectious Disease and Molecular Medicine and the Division of Infectious Diseases and HIV Medicine, Department of Medicine, University of Cape Town, Cape Town, South Africa[e]

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

Cryptococcal meningitis (CM) is a frequent HIV-related opportunistic infection caused by *Cryptococcus neoformans* (serotypes A and D) and *Cryptococcus gattii* (serotypes B and C) (1, 2). It is the main cause of adult meningitis in sub-Saharan Africa (SSA) (3–5) and is a major cause of HIV-related mortality, accounting for between 13 and 44% of deaths in HIV-infected cohorts in resource-limited countries (3). Case fatality rates remain unacceptably high. Locally, the 30-day mortality rate is 33 to 41% in routine settings (6, 7), possibly related to delayed diagnosis and the commencement of appropriate combinations of antifungal therapy (3, 8, 9). Despite the high case fatality ratio (7, 9, 10), these patients may have good long-term survival rates, if they are able to overcome the acute phase of the illness (8). Key factors influencing survival are the fungal burden at presentation and the rate of sterilization of cerebrospinal fluid (CSF) with combination treatment (6, 8, 9, 11, 12). The rapid and accurate laboratory diagnosis of CM is thus important to enable the timely use of appropriate medication and prevent diagnostic delays contributing to increased CSF fungal loads and poor clinical outcomes (6, 11, 13, 14).

The rapid detection of *Cryptococcus* has previously been hampered by the lack of a point-of-care (POC) test for CM. The standard diagnostic methods include India ink staining, the conventional cryptococcal 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 (SA) with subsequent delays in the return of results to the sites of patient management. The CLAT is labor intensive, and sample batching may further delay the turnaround time. Cultures may be negative or slow to grow for patients with low fungal burdens or those already receiving 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) (IMMY, Norman, OK, USA), a commercially available rapid diagnostic test that detects capsular polysaccharide antigens of the four major cryptococcal serotypes (A and D for *C. neoformans* and B and C for *C. 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-cryptococcal antigen (anti-CrAg) monoclonal antibodies and gold-conjugated control antibodies on the test membrane.

The LFA 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 samples, but published data on the sensitivity and specificity compared to those of the CLAT and culture are limited (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 that of the CLAT and culture, which together with microscopy are the current diagnostic tests employed at the National Health Laboratory Services (NHLS) laboratories in SA.

MATERIALS AND METHODS

Clinical samples. Consecutive CSF samples from patients with suspected CM referred to the NHLS microbiology laboratory at Tygerberg Hospital, Cape Town, SA, were prospectively collected during October 2012. Patients included those with clinically suspected or confirmed HIV infection with signs and/or symptoms suggestive of meningitis. Samples were included in the study if there was a sufficient amount for analysis after routine laboratory investigations had been performed. The study was ap-

Received 23 June 2014 **Returned for modification** 9 July 2014
Accepted 8 September 2014
Published ahead of print 17 September 2014
Editor: D. W. Warnock
Address correspondence to Adré Lourens, adre.lourens@gmail.com.

doi:10.1128/JCM.01683-14

TABLE 1 Results of the lateral flow assay and conventional cryptococcal latex agglutination test performed on 465 CSF samples before and after CSF dilution

LFA[a] result	CLAT[b] result (no. of indicated result)		
	Positive	Negative	Total
Predilution			
Positive	30	1[c]	31
Negative	3	431	434
Total	33	432	465
Postdilution[d]			
Positive	33	1[c]	34
Negative	0	431	431
Total	33	432	465

[a] LFA, lateral flow assay.
[b] CLAT, cryptococcal latex agglutination test.
[c] This sample was from a patient known to have had prior CM and who most likely had low-level antigenemia undetectable by the CLAT with a negative culture due to prior antifungal therapy.
[d] 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 for samples which were LFA negative but contained yeast cells on microscopy.

TABLE 2 Performance of the lateral flow assay and conventional cryptococcal latex agglutination test on 465 CSF samples before and after CSF dilution

Comparator	Total no. of CSF samples in study	Sensitivity (%)	Specificity (%)	PPV[b] (%)	NPV[c] (%)	Kappa
LFA[a] predilution						
CLAT[d]	465	91	99.8[e]	96.8	92.9	0.9329
Culture	465	88.4	98.1	74.1	99.3	0.7945
LFA postdilution[f]						
CLAT	465	100	99.8[e]	97	100	0.9839
Culture	465	100	98.1	76.4	100	0.8576

[a] LFA, lateral flow assay.
[b] PPV, positive predictive value.
[c] NPV, negative predictive value.
[d] CLAT, cryptococcal latex agglutination test.
[e] The specificity of the LFA test was 100% when a single sample from a patient known to have had prior CM and who most likely had low-level antigenemia undetectable by the CLAT and culture due to prior antifungal therapy was assumed to be a true-positive result.
[f] 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 for samples which were LFA negative but contained yeast cells on microscopy.

proved 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 min (18). A positive result was reported if two visible lines developed over the control and test areas, and a negative result was reported if a single control line was present (18). If the LFA was negative but Gram staining 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.

Gram stains and bacterial and fungal cultures were performed on CSF samples according to the laboratory protocols. CSF samples were referred for additional investigations according to clinicians' requests (e.g., tuberculosis [TB] or VDRL test). Selective agar (Sabouraud dextrose) for yeast isolation was utilized and incubated aerobically at 35°C 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. The cryptococcus antigen latex test (CLAT) (Remel, Inc., Lenexa, KS, USA) for detecting capsular polysaccharide (CPS) antigens of C. *neoformans*, using murine IgM monoclonal antibodies, was performed according to the manufacturer's instructions (20). Pronase and heat-inactivation treatments were not performed on the CSF samples prior to performance of 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 those of the CLAT. Results were reanalyzed using culture as a reference standard. Agreements between the diagnostic tests were summarized using kappa statistics and analyzed using McNemar's test of equality of paired proportions.

RESULTS

A total of 465 CSF samples were included in the study over a period of 1 month. The samples included CSF obtained from adult and pediatric patients.

Analysis using the CLAT as a 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 the 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 staining and were LFA positive after a further 1:2 sample dilution. When a positive CLAT result was used as a gold standard, the sensitivity and specificity for the LFA on undiluted CSF samples were 90.9% and 99.8%, respectively (Table 1). After sample dilution, the sensitivity improved to 100% (Table 2). Clinical data from these 3 patients were retrieved and are summarized in Table 3. Of the 31 LFA-positive samples, 30 CSF samples were CLAT positive; thus, one additional case, for which the culture yielded no growth, was detected using the LFA. This sample was from a patient known to have had prior CM and who most likely had low-level antigenemia undetectable by the 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 the reference standard. *C. neoformans* was cultured on selective medium 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 of having a high organism load were diluted before the assay was performed. However, when performed on undiluted CSF samples, the LFA had a sensitivity of only 90.9% compared to that of the CLAT as the reference standard. This reduced sensitivity could possibly be due to the high-dose "hook" effect (18, 21) (also referred to as prozoning), one of the potential limitations of the LFA described by the man-

TABLE 3 Summary of the CSF findings for the 3 patients with false-negative LFA results who had cultures positive for *C. neoformans*

Patient	Age (yr)	HIV status[a]	CSF findings				
			CD4 count (no.)	Polymorphonuclear cells (mm^3)	Lymphocytes (mm^3)	Erythrocytes (mm^3)	Protein (g/liter)[b]
A	59	Pos	217	44	50	17	1.62
B	25	Pos	Unknown	8	2	0	0.44
C	55	Pos	125	3	17	53	0.91

[a] Pos, positive.
[b] Normal range, 0.15 to 0.45 g/liter.

ufacturer (18). This occurs when excess analyte, in this case high concentrations of the cryptococcal antigen, resulted in decreased visual intensity of the test lines or, as also in this case, yielded 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 the culture grew the organism after a mean incubation period of 48 h. These false negatives were 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 is 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 retesting using serial dilutions of the CSF sample. Use of the LFA for antigen detection in CSF samples in conjunction with staining and microscopy should, therefore, diagnose all cases of CM with a sensitivity that is equivalent or superior to the CLAT.

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 (1, 22). Furthermore, the LFA is known to detect antigens of *C. gattii* infection (1, 20) with a higher sensitivity than the current latex agglutination tests (23). In our study, the species was not identified 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 with the LFA in a small number of CSF samples with visible cryptococci on Gram stains, the LFA remains an attractive test for use in most settings. The LFA is stable at room temperature, has a rapid turnaround time with results available within 10 min, and does not require sample pretreatment or processing *except* in cases suspected of having 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 are often overworked, the major advantage of the 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 are limited data on the POC use with CSF samples (24). Potential difficulties in achieving widespread implementation of the LFA as a POC diagnostic test for CM include the need for supplementary microscopy results with 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 rates 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 reliable detection of *Cryptococcus neoformans* antigens in CSF samples, provided a microscopy result is available, and might 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 1:2 dilution could be routinely performed for all undiluted samples from HIV-positive patients or patients with a high suspicion of disease that tested negative, but further assessment in larger cohorts with antigen quantification (titers) is necessary to evaluate whether 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.

ACKNOWLEDGMENTS

We acknowledge the microbiology staff at Tygerberg Hospital for their assistance in sample collection and processing, Justin Harvey for his assistance with the statistical analysis, and our reviewers for their substantial input.

J. N. Jarvis has received research funding from Gilead in the form of an investigator-initiated award. The remaining authors declare no conflicts of interest.

This work was supported by the Harry Crossley Foundation and the NHLS K-Fund. IMMY sponsored part of the kits but had no influence on study design, data collection, analysis, interpretation, or preparation of the manuscript.

REFERENCES

1. **Vijayan T, Chiller T, Klausner JD.** 2013. Sensitivity and specificity of a new cryptococcal antigen lateral flow assay in serum and cerebrospinal fluid. MLO Med. Lab Obs. **45:**16, 18, 20.
2. **Springer DJ, Chaturvedi V.** 2010. Projecting global occurrence of *Cryptococcus gattii*. Emerg. Infect. Dis. **16:**14–20. http://dx.doi.org/10.3201/eid1601.090369.
3. **French N, Gray K, Watera C, Nakiyingi J, Lugada E, Moore M, Lalloo D, Whitworth JAG, Gilks CF.** 2002. Cryptococcal infection in a cohort of HIV-1-infected Ugandan adults. AIDS **16:**1031–1038. http://dx.doi.org/10.1097/00002030-200205030-00009.
4. **Jarvis JN, Meintjes G, Williams A, Brown Y, Crede T, Harrison TS.** 2010. Adult meningitis in a setting of high HIV and TB prevalence: findings from 4961 suspected cases. BMC Infect. Dis. **10:**67. http://dx.doi.org/10.1186/1471-2334-10-67.
5. **Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG,**

Chiller TM. 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS **23:**525–530. http://dx.doi.org/10.1097/QAD.0b013e328322ffac.

6. **Jarvis J, Meintjes G.** 2011. Cryptococcal meningitis—a neglected killer: editorial. S. Afr. Med. J. **101:**244–245.
7. **Lessells RJ, Mutevedzi PC, Heller T, Newell M-L.** 2011. Poor long-term outcomes for cryptococcal meningitis in rural South Africa. S. Afr. Med. J. **101:**251–252.
8. **Bicanic T, Meintjes G, Wood R, Hayes M, Rebe K, Bekker LG, Harrison T.** 2007. Fungal burden, early fungicidal activity, and outcome in cryptococcal meningitis in antiretroviral-naive or antiretroviral-experienced patients treated with amphotericin B or fluconazole. Clin. Infect. Dis. **45:**76–80. http://dx.doi.org/10.1086/518607.
9. **Bicanic T, Wood R, Meintjes G, Rebe K, Brouwer A, Loyse A, Bekker LG, Jaffar S, Harrison T.** 2008. High-dose amphotericin B with flucytosine for the treatment of cryptococcal meningitis in HIV-infected patients: a randomized trial. Clin. Infect. Dis. **47:**123–130. http://dx.doi.org/10.1086/588792.
10. **Harrison TS.** 2009. The burden of HIV-associated cryptococcal disease. AIDS **23:**531–532. http://dx.doi.org/10.1097/QAD.0b013e328322ffc3.
11. **Anekthananon T, Manosuthi W, Chetchotisakd P, Kiertiburanakul S, Supparatpinyo K, Ratanasuwan W, Pappas PG, Filler SG, Kopetskie HA, Nolen TL, Kendrick AS, Larsen RA, BAMSG 3-01 Study Team.** 2011. Predictors of poor clinical outcome of cryptococcal meningitis in HIV-infected patients. Int. J. STD AIDS **22:**665–670. http://dx.doi.org/10.1258/ijsa.2011.010538.
12. **Jarvis JN, Bicanic T, Harrison TS.** 2007. Treatment of HIV-associated cryptococcal meningitis in South Africa: the case for amphotericin B over conventional dose fluconazole for initial therapy. S. Afr. J. HIV Med. **8:**36–39.
13. **Jarvis JN, Bicanic T, Loyse A, Namarika D, Jackson A, Nussbaum JC, Longley N, Muzoora C, Phulusa J, Taseera K, Kanyembe C, Wilson D, Hosseinipour MC, Brouwer AE, Limmathurotsakul D, White N, van der Horst C, Wood R, Meintjes G, Bradley J, Jaffar S, Harrison T.** 2014. Determinants of mortality in a combined cohort of 501 patients with HIV-associated cryptococcal meningitis: implications for improving outcomes. Clin. Infect. Dis. **58:**736–745. http://dx.doi.org/10.1093/cid/cit794.
14. **Mora DJ, da Cunha Colombo ER, Ferreira-Paim K, Andrade-Silva LE, Nascentes GA, Silva-Vergara ML.** 2012. Clinical, epidemiological and outcome features of patients with cryptococcosis in Uberaba, Minas Gerais, Brazil. Mycopathologia **173:**321–327. http://dx.doi.org/10.1007/s11046-011-9504-9.
15. **Barenfanger J, Lawhorn J, Drake C.** 2004. Nonvalue of culturing cerebrospinal fluid for fungi. J. Clin. Microbiol. **42:**236–238. http://dx.doi.org/10.1128/JCM.42.1.236-238.2004.
16. **Jarvis JN, Percival A, Bauman S, Pelfrey J, Meintjes G, Williams GN, Longley N, Harrison TS, Kozel TR.** 2011. Evaluation of a novel point-of-care cryptococcal antigen test on serum, plasma, and urine from patients with HIV-associated cryptococcal meningitis. Clin. Infect. Dis. **53:**1019–1023. http://dx.doi.org/10.1093/cid/cir613.
17. **Lindsley MD, Mekha N, Baggett HC, Autthateinchai R, Sawatwong P, Harris JR, Park BJ, Chiller T, Balajee SA, Poonwan N.** 2011. Evaluation of a newly developed lateral flow immunoassay for the diagnosis of cryptococcosis. Clin. Infect. Dis. **53:**321–325. http://dx.doi.org/10.1093/cid/cir379.
18. **IMMY.** 2012. CrAg LFA package insert. IMMY, Norman, OK. http://www.immy.com/products/cryptococcal-antigen-lateral-flow-assay-lfa/.
19. **Graf B, Adam T, Zill E, Göbel UB.** 2000. Evaluation of the VITEK 2 system for rapid identification of yeast and yeast like organisms. J. Clin. Microbiol. **38:**1782–1785.
20. **Remel, Inc.** 2009. CLAT package insert. Product no. R30851501. Remel, Inc., Lenexa, KS.
21. **Qin Z, Chan WCW, Boulware DR, Akkin T, Butler EK, Bischof JC.** 2012. Significantly improved analytical sensitivity of lateral flow immunoassays by using thermal contrast. Angew. Chem. Int. Ed. Engl. **51:**4358–4361. http://dx.doi.org/10.1002/anie.201200997.
22. **Gates-Hollingsworth MA, Kozel TR.** 2013. Serotype sensitivity of a lateral flow immunoassay for cryptococcal antigen. Clin. Vaccine Immunol. **20:**634–635. http://dx.doi.org/10.1128/CVI.00732-12.
23. **Hansen J, Slechta ES, Gates-Hollingsworth MA, Neary B, Barker AP, Bauman S, Kozel TR, Hanson KE.** 2013. Large-scale evaluation of the immuno-mycologics lateral flow and enzyme-linked immunoassay for detection of cryptococcal antigen in serum and cerebrospinal fluid. Clin. Vaccine Immunol. **20:**52–55. http://dx.doi.org/10.1128/CVI.00536-12.
24. **Boulware DR, Rolfes MA, Rajasingham R, Von Hohenberg M, Qin Z, Taseera K, Schutz C, Kwizera R, Butler EK, Meintjes G, Muzoora C, Bischof JC, Meya DB.** 2014. Multisite validation of cryptococcal antigen lateral flow assay and quantification by laser thermal contrast. Emerg. Infect. Dis. **20:**45–53. http://dx.doi.org/10.3201/eid2001.130906.