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Point of Care Cryptococcal Antigen Screening: Pipetting Finger-Prick Blood Improves Performance of Immuno-Mycologics Lateral Flow Assay

Running head: Method Matters at the Point of Care

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Abstract

Background

Cryptococcal antigen (CrAg) screening at point of care could improve cryptococcal meningitis prevention where laboratory resources are limited. We evaluated the accuracy of Immuno-Mycologics (IMMY, Norman, OK) CrAg lateral flow assay (LFA) using different techniques at point of care.

Setting

Two tertiary-level hospitals in Johannesburg and a community health clinic in Soweto, South Africa.

Methods

A case-control diagnostic validation study, and a prospective clinic-based implementation study using the IMMY CrAg LFA on finger-prick blood. Accuracy, using direct application of LFA to sample, or pipette to transfer sample to diluent, and reading after 10 and 20 minutes, was compared to laboratory-based plasma testing.

Results

The validation study tested 64 CrAg-positive and 152 CrAg-negative patients with no symptoms or signs of meningitis, identified by routine laboratory screening, recruited by convenience sampling. Consecutively-diagnosed HIV-infected adults (n=654) were included in the implementation study. Sensitivity was 82% and 20% when the LFA was read 10 minutes after direct application to finger-prick blood in the validation and implementation

studies respectively. Using a pipette to transfer blood and reading after 20 minutes improved sensitivity to 100%, while retaining 100% specificity, in both studies.

Conclusions

Although the IMMY CrAg LFA performs well when applied directly to finger-prick blood for diagnosing cryptococcal meningitis, this technique may not provide adequate volume to detect low concentrations of CrAg when screening asymptomatic patients. Using a pipette to transfer larger volumes of blood to diluent prior to CrAg LFA testing and reading results after 20 minutes is a more reliable point-of-care method.

Keywords: meningitis, cryptococcal; cryptococcus; antigens; prevention & control; point-of-care systems; diagnostic techniques and procedures; sensitivity and specificity

Introduction

Cryptococcal antigen (CrAg) screening and pre-emptive treatment of CrAg-positive patients with fluconazole, prevents cryptococcal meningitis and death in HIV-infected adults with low CD4+ T-lymphocyte (CD4) counts (1,2). This strategy has been incorporated into HIV management guidelines(3,4) and implemented in several countries where cryptococcal meningitis remains a major problem. However, a predominantly laboratory-based approach to CrAg screening may undermine the potential for earlier detection of disease by introducing a delay between testing and action, particularly where access to laboratory facilities is limited. The IMMY CrAg LFA has been designed for use at the point of care according to WHO 'ASSURED' criteria: affordable,

sensitive, specific, user-friendly tests that are rapid, robust, equipment free, and deliverable to end-users(5). Although accurate when used on finger-prick blood samples of hospitalised patients with suspected cryptococcal meningitis(6), there is limited evidence of accuracy when used to screen patients for cryptococcal antigenaemia attending HIV clinics.

To be effective, CrAg screening programmes must ensure rapid treatment of CrAg-positive patients with antifungal therapy whilst allowing efficient initiation of antiretroviral therapy (ART). In South Africa, blood samples sent to National Health Laboratory Service (NHLS) laboratories are routinely screened for CrAg if the CD4 count is <100 cells/μL. However, around 20% of CrAg-positive patients never receive their result and of those that do, 59% have symptoms of meningitis by the time of assessment (median delay 8 days) (7). Additionally, clinicians must delay starting ART pending CrAg results to avoid risk of cryptococcal immune reconstitution inflammatory syndrome (IRIS). Delayed ART in patients with low CD4 counts increases the risk of progression to AIDS and death (8,9).

The IMMY CrAg LFA could address linkage problems to both antifungal treatment and ART if used on finger-prick blood at the point of care (unlabeled use). We investigated accuracy in this context using a case-control diagnostic validation study (10) of CrAg-positive and –negative patients and a clinic-based implementation study evaluating point-of-care (POC) CrAg screening for newly-diagnosed HIV-seropositive adults.

Methods

For the validation study, we performed POC CrAg tests on CrAg-positive and –negative HIV-infected adults (>18 years) identified by laboratory-based CrAg screening following attendance at Helen Joseph and Tambo Memorial Hospitals in Johannesburg (inpatients or outpatient clinic) between July 2015 and February 2018. Patients with a severe headache or confusion, or who

were on antifungal treatment for previous cryptococcal meningitis were excluded. During an implementation study, we then introduced POC CrAg tests for consecutively-diagnosed HIV-seropositive adults attending a community health clinic in Soweto between September 2016 and February 2018, who were not taking ART or antifungal treatment for previous cryptococcal meningitis.

Ethical approval for the studies was granted by the London School of Hygiene and Tropical Medicine, United Kingdom and the University of the Witwatersrand, South Africa. The initial method used (from July 2015) for testing finger-prick blood samples in both studies involved applying the tip of the LFA strip to finger-prick blood samples obtained using a sterile 1.5 mm lancet. Study personnel were instructed to acquire a finger-prick blood sample of at least 2 mm diameter prior to applying the tip of the LFA strip to absorb the blood. The LFA strip was then added to the diluent. Results were read after the recommended 10-minute incubation time and also after 20 minutes from May 2016. An alternative sampling technique using a disposable 40 µL pipette (HAIN Lifescience, South Africa) to transfer and mix finger-prick blood with diluent was used between July 2017 and February 2018, and results read after 10 and 20 minutes (see supplementary digital content 1, http://links.lww.com/QAI/B163 description of techniques).

For both studies, the reference standard was an IMMY CrAg LFA performed on plasma (used routinely in South Africa) obtained from an ethylene-diamine-tetraacetic acid (EDTA)-containing blood sample drawn on the same day as the POC tests and tested in a laboratory at the National Institute for Communicable Diseases (NICD).

CrAg LFA tests were performed and read by one of two study nurses at point of care in the validation study and a study nurse or phlebotomist in the intervention study, and one of three investigators at NICD. All study personnel received training, and completed verification panels to confirm competence. Study personnel were aware of the CrAg status of patients in the validation study, but blinded to POC CrAg result in the implementation study.

If the reference standard result was different to that obtained at the NHLS laboratory or at point of care, an IMMY CrAg enzyme immunoassay (EIA), using the same monoclonal antibodies for detection as the LFA(11) was performed on the same sample, and the result used to adjust patient management. If the reference standard result differed from the EIA result, this patient was excluded from the study owing to indeterminate CrAg status. Reference standard results were then used to calculate sensitivity, specificity and kappa statistic for each technique.

Results

Of 493 patients screened, 232 patients with CD4 counts <100 cells/µL were enrolled, and 216 patients (64 CrAg-positive and 152 CrAg-negative) were included in the validation study. Of 2130 newly diagnosed HIV-infected adults attending the primary health clinic, we enrolled 679 into the implementation study, of whom 654 were included (Figure 1). Median CD4 count in the implementation study was 236 cells/µl (IQR 98 – 414); 155/614 (25%) had a CD4 count <100 cells/µL. Overall 9 (1.5%) patients were found to be CrAg-positive (3.9%, 6/155 with CD4 count <100 cells µL) by reference standard testing.

The accuracy of the IMMY CrAg LFA using both techniques is reported in Table 1. The LFA had reduced sensitivity compared to the reference standard when read 10 minutes after direct application to finger-prick samples (82%, 95% CI 68%-91%, [40/49] and 20%, 95% CI 0.5%-72% [1/5] in the validation and implementation studies respectively). Using a pipette to test finger-

prick blood and extending reading time to 20 minutes improved sensitivity to 100%, while retaining 100% specificity, in both studies.

In the validation study, median plasma CrAg titre was 1:40 (IQR 1:10 – 1:640); lower among patients who had false-negative than true-positive POC results (median 1:5, IQR 1:1 – 1:10 vs. 1:80, IQR 1:20 – 1:960, p<0.001) when read 10 minutes following direct application of LFA to blood (Supplementary Digital Content 2, http://links.lww.com/QAI/B163 clinical details and photos of POC tests). In the implementation study, CrAg titres performed on fresh plasma were ≤1:40 for three patients with false-negative POC results. For those with true-positive POC results using a pipette, CrAg titres were ≤1:20 for two of four, and >1:2560 for the other two CrAg-positive patients, who had cryptococcal meningitis confirmed by LP. All four of these patients received antifungal treatment on the same day and were alive at one month (Supplementary Digital Content 3, http://links.lww.com/QAI/B163 clinical details and photos of POC tests).

Discussion

The results of both studies show that directly applying the IMMY CrAg LFA to finger-prick blood resulted in reduced sensitivity compared to the reference standard of laboratory-based CrAg LFA on plasma. However, introduction of a pipette to transfer and mix finger-prick blood with the diluent prior to testing, and reading the result after 20 minutes improved sensitivity to 100% in both studies, whilst maintaining specificity at 100%.

Direct application of LFA to finger-prick blood has previously been found to be a suitable technique for diagnosing cryptococcal meningitis at the point of care. A case-control diagnostic accuracy study in South Africa tested 158 patients with cryptococcal meningitis and 99 without using this technique and found a sensitivity of 99% compared to cerebrospinal fluid culture or CrAg latex agglutination(6). A diagnostic accuracy study in Uganda used this technique to test

207 HIV-infected adults with suspected meningitis and found 100% agreement between finger-prick sample and serum or plasma for identifying CrAg(12).

However, while patients with cryptococcal meningitis in these prior studies were likely to have CrAg concentrations well above the published detection limit of the IMMY CrAg LFA, (12,13) the asymptomatic or minimally-symptomatic individuals enrolled in our studies had low CrAg titres (median 1:40). Improved accuracy of the CrAg LFA with a pipette and extended reading time is likely due to testing a larger volume of blood for a time period which is adequate to detect lower concentrations of CrAg in the context of screening.

Previous studies using the IMMY CrAg LFA for POC screening have also found variation using different methods. A prospective screening study in Cape Town found the IMMY CrAg LFA had 100% concordance with plasma or serum when used on whole blood from 192 HIV-infected adults with CD4 counts <100 cells/μL(2). However, venous (and not finger-prick) blood was used for a majority of these tests. In Tanzania, CrAg screening (applying LFA directly to finger-prick blood) was implemented in seven HIV clinics, and results compared to laboratory-based plasma testing(14). Of 12 CrAg-positive patients identified, 3 (25%) had false-negative results using finger-prick blood (Gladys Mbwanji, Ifakara Health Institute, personal communication). In Zimbabwe, CrAg screening using the same technique was introduced for asymptomatic patients with CD4 counts ≤100 cells/μL in 20 HIV clinics. Sensitivity was 48% (61/128) compared to laboratory-based serum testing and as in our study, sensitivity improved when testing patients with higher CrAg titres (82% if titre ≥160)(15). No studies to date have assessed the accuracy of the IMMY CrAg LFA for screening patients using a pipette to transfer finger-prick blood. However, in Lesotho, POC CrAg screening (without a laboratory-based reference standard) using a pipette identified 14/129 (11%) CrAg-positive patients with a CD4 count <100 cells/mm^3, 12 of whom were asymptomatic at the time of testing(16).

These studies investigated accuracy of the IMMY CrAg LFA and applicability of our findings to other CrAg LFAs is not known. However, the mechanistic problem of detecting lower CrAg titers with smaller sample volumes in the context of POC CrAg screening is likely to have a similar effect on other LFAs. Case-control diagnostic accuracy studies are limited in determining accuracy since the prevalence of disease is higher than in the intended screening population. In addition, readers were not blinded to the result of the index test. The clinic-based implementation study addressed these limitations by performing CrAg screening on all newly-diagnosed HIV-seropositive patients, with no prior CrAg testing and regardless of CD4 count. However, the number of patients tested was relatively small. Additionally, the prevalence of cryptococcal antigenaemia in this population (1.5%) was below that in most screening programmes where a CD4 cut-off of <100 cells/μL is used.

Despite these limitations, these studies highlight significant loss of accuracy when the IMMY CrAg LFA is applied directly to finger-prick samples at the point of care, which improved when using a disposable 40μL pipette and reading the test strip after 20 minutes. Our study also demonstrated that POC CrAg screening performed by a trained nurse or phlebotomist in a busy urban community health clinic was feasible and resulted in all CrAg-positive patients receiving assessment and appropriate antifungal treatment on the same day as screening. Although pipettes may not always be readily available in low-resource settings, many POC tests available in HIV clinics use capillary tube systems, which could also be used to deliver the correct volume of blood for CrAg testing(17). An additional benefit of using a graduated pipette or microcapillary tube would be the ability to perform serial dilutions, to measure titer or to exclude false negative results due to a high-dose 'hook' effect previously described(18). These additional tests would have important management implications(19,20), however were not investigated as part of this study.

Performing CrAg screening tests at the point of care for HIV-infected adults initiating ART could improve the efficient delivery of antifungal medicines and ART to those in urgent need, and reduce preventable disease and death caused by cryptococcosis. CrAg screening using a pipette to sample finger-prick blood and reading the result at 20 minutes should be implemented in areas which lack laboratory infrastructure to provide results that are not only rapid, robust and affordable but also accurate at the point of care.

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Figure legend

Figure 1 Flow charts to show enrolment and testing procedures in the a) case-control diagnostic accuracy study and b) clinic-based implementation study. Abbreviations: CrAg = cryptococcal antigen; LFA = lateral flow assay; POC = point of care; IMMY = Immnomycologics; EIA = enzyme immunoassay.

*All were CrAg-positive on routine screening, but CrAg-negative at point of care and when the reference standard CrAg LFA was repeated at enrolment and/or on confirmatory EIA (median 17 days after routine screening, IQR, 8 – 26, 12 had received fluconazole, all of 8 LPs performed were negative)

**CrAg LFA result indeterminate at point of care, reference standard CrAg LFA positive, confirmatory CrAg EIA negative (no delay or treatment between tests)

Table 1 Accuracy and kappa statistic for IMMY CrAg LFA performed at point of care by direct application of finger-prick blood samples to the LFA strip and by transferring blood to diluent using a disposable pipette, compared to the reference standard of IMMY CrAg LFA performed on settled plasma in the laboratory in a) a case-control diagnostic accuracy study and b) a clinic-based implementation study

	No. tested	Sensitivity %, (no.), 95% CI	Specificity %, (no.), 95% CI	Kappa value (95% CI)
a) Case-control Diagnostic Accuracy Study				
IMMY CrAg LFA strip applied to finger-prick blood sample (July 2015 – June 2017)				
10 minutes	176	82% (40/49), 68% – 91%	100% (127/127), 97% - 100%	0.87 (0.78 – 0.95)
20 minutes	74	100% (14/14), 77%-100%	100% (60/60), 94%-100%	1.0 (1.0 – 1.0)
IMMY CrAg LFA performed on finger-prick blood sample added to diluent with a disposable pipette (July 2017 – February 2018)				
10 minutes	27	95% (14/15*), 68% - 100%	100% (25/25), 86% - 100%	0.9 (0.8 – 1.0)
20 minutes	27	100% (15/15), 78% - 100%	100% (24/24), 86% - 100%	1.0 (1.0 – 1.0)
b) Clinic-based POC CrAg Screening Implementation Study				
POC IMMY CrAg LFA strip applied to finger-prick blood sample (September 2016 – June 2017)				
10 minutes	420	20% (1/5), 0.5% - 72%	100% (415/415), 99% - 100%	0.33 (-0.15 – 0.82)
20 minutes	420	20% (1/5), 0.5% - 72%	100% (414/414), 99%-100%	0.33 (-0.15 – 0.82)
POC IMMY CrAg LFA performed on finger-prick blood sample added to diluent with a disposable pipette (July 2017 – February 2018)				
10 minutes	235	100% (4/4), 40% – 100%)	100% (230/231†), 98% - 100%	0.89 (0.7 – 1.0)
20 minutes	235	100% (4/4), 40% – 100%	100% (230/231†), 98% - 100%)	0.89 (0.7 – 1.0)

Abbreviations: CI = confidence interval; POC = point of care; IMMY = Immunomycologics; CrAg = cryptococcal anigen; LFA = lateral flow assay. Exact binomial 95% confidence intervals are given.m
*CrAg titer for the false negative CrAg tests when using a pipette to transfer finger-prick blood to diluent, and read after 10 minutes was 10. This was read as positive at 20 minutes.
†No reason was found for the false positive result at point of care. A repeat POC test three days later was negative.