Title: Evaluation of a Point-of-Care Immunoassay Test Kit 'StrongStep' for Cryptococcal Antigen Detection

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Abstract

Background: HIV-associated cryptococcal meningitis is the leading cause of adult meningitis in Sub-Saharan Africa, accounting for 10%-20% of AIDS-attributable mortality. The development of point-of-care assays has greatly improved the screening and diagnosis of cryptococcal disease. We evaluated a point-of-care immunoassay, StrongStep (Liming Bio, Nanjing, Jiangsu, China) lateral flow assay (LFA), for cryptococcal antigen (CrAg) detection in cerebrospinal fluid (CSF) and plasma.

Methods: We retrospectively tested 143 CSF and 77 plasma samples collected from HIV-seropositive individuals with suspected meningitis from 2010-2016 in Uganda. We also prospectively tested 90 plasma samples collected from HIV-seropositive individuals with CD4 cell count <100 cells/μL as part of a cryptococcal antigenemia screening program. The StrongStep LFA was tested against the Immy CrAg (Immy, Inc., Norman, OK, USA) LFA and CSF culture, when available, for participants with meningitis.

Results: The StrongStep LFA had a sensitivity of 98% (54/55) and specificity of 90% (101/112) in plasma. When tested on CSF, the StrongStep LFA had a sensitivity of 100% (100/100) and specificity of 95% (41/43). Adjusting for the cryptococcal antigenemia prevalence of 9% in Uganda and average cryptococcal meningitis prevalence of 37% in Sub-Saharan Africa, the positive predictive value of the StrongStep LFA was 50% in plasma and 93% in CSF. In lower prevalence settings, a majority of positive results from plasma and serum would be expected to be false positives.

Conclusions: The StrongStep CrAg LFA is a sensitive assay with poor specificity. Human anti-mouse antibodies may be a cause of false positives.

Introduction

HIV-associated cryptococcal meningitis is the leading cause of adult meningitis in Sub-Saharan Africa and accounts for 10%–20% of AIDS-attributable mortality [1-3].The global prevalence of asymptomatic cryptococcal antigenemia (CrAg) in HIV-seropositive individuals averages approximately 6% with an estimated prevalence of 8.8% in Kampala, Uganda [1]. Screening and preemptive treatment of cryptococcal antigenemia is a cost effective method of averting cases of cryptococcal meningitis [4, 5]. Therefore, the World Health Organization and Ugandan National HIV treatment Guidelines recommend screening all HIV-seropositive individuals with a CD4 cell count <100cells/μL for the presence of cryptococcal antigen, followed by preemptive fluconazole therapy [6, 7]. Despite recent advancements in diagnostic tools, CrAg screening, as well as the rapid and accurate diagnosis of cryptococcal meningitis remain a challenge. Due to the unavailability of point-of-care assays and interruptions in the supply chain, there exists unreliable, non-continuous screening and lack of expertise and/or laboratory facilities for CrAg testing in resource-limited settings [4, 8, 9].

In July 2011, a lateral flow immunochromatographic assay (Immy, Inc., Norman, OK, USA) was approved by the US Food and Drug Administration for CrAg detection in CSF and plasma. The Immy CrAg LFA provides a definitive result (positive/negative) in ≤15 minutes and has demonstrated superior diagnostic performance with a sensitivity of 99.3% and specificity of 99.1% in multiple validation studies [10-12].

We evaluated a point-of-care immunoassay, StrongStep (Liming Bio, Nanjing, Jiangsu, China), for CrAg detection in CSF and plasma. The StrongStep LFA was tested against the Immy CrAg LFA (Immy, Inc, Norman, Oklahoma, USA) and CSF fungal quantitative cultures when available for participants with suspected meningitis.

Methods

Study Design

We evaluated the diagnostic performance of the StrongStep CrAg rapid test device by retrospectively and prospectively testing CrAg positive and CrAg negative CSF and plasma samples. Stored samples collected from HIV-seropositive participants between 2010-2016 at Mulago Hospital in Kampala and Mbarara Regional Referral Hospital in Mbarara, Uganda from three cohorts; Timing of Antiretroviral Therapy After Diagnosis of Cryptococcal meningitis (COAT) [13], Predictors of Neurocognitive Outcomes on Antiretroviral Therapy After Cryptococcal Meningitis: a Prospective Cohort Study (NOAT) [14], and Adjunctive Sertraline for the Treatment of HIV-Associated Cryptococcal Meningitis (ASTRO-CM) [15]. We also prospectively tested samples collected from the Integration of Community-Based Cryptococcal Antigen Screening into Routine HIV Care in Uganda (ORCAS 2.0). Cryptococcal antigen detection was confirmed by the Immy CrAg LFA and quantitative CSF culture for participants with meningitis. All participants provided written informed consent to store their samples for future diagnostic meningitis studies. Ethical approval was granted from the Uganda National Council of Science and Technology, Mulago Hospital Research and Ethics Committee, Makerere University Institutional Review Board, and the University of Minnesota.

The CrAg Immunoassay

The CrAg Immunoassay kit, StrongStep, is an immunochromatographic assay impregnated with monoclonal mouse antibodies with the ability to detect the capsular polysaccharide antigen of *Cryptococcus neoformans* and *Cryptococcus gattii.* The StrongStep assay was performed following the manufacturer's instructions. Briefly, two drops (80µl) of CSF or plasma were added to the LFA test well, ensuring that no air bubbles were trapped in the well, and results

were recorded after ten minutes. The Immy LFA was performed following the manufacturer's instructions. One drop (40µl) of specimen was added to the dipstick and inserted into a container containing 40µl of the assay diluent and results were read after 10 minutes.

Qualitative validation

All retrospective CSF and plasma samples that were tested had been stored at -80°C. Frozen samples were completely thawed and kept at room temperature, for no longer than one hour, prior to testing. Prospectively, plasma samples were collected in EDTA coated vacuum containers and stored for <2 days at 4°C prior to testing.

In vitro Analytical Sensitivity

Semi-quantitative titration was performed on the StrongStep CrAg LFA using the Immy CrAg positive control (Glycine-buffered saline spiked with cryptococcal glucuronoxylomannan antigen). Dilutions were prepared with an initial dilution of 1:40, followed by 1:2 serial dilutions to 1:5120. Semi-Quantitative titration was performed simultaneously on the IMMY and StrongStep LFA and repeated in triplicate with results verified by three independent readers. Clinical information was not accessible to the independent readers.

Data analysis

The diagnostic performance (sensitivity, specificity, positive predictive (PPV), and negative predictive values (NPV)) of the StrongStep LFA was compared to the Immy LFA and CSF culture results, when available for persons with meningitis. For plasma samples, the PPV and NPV were calculated to adjust for the cryptococcal antigenemia prevalence of 9% in HIV-seropositive persons with CD4<100 cell/µL in Kampala, Uganda [4]. We also used a pooled average of 37% prevalence of cryptococcal meningitis in Sub-Saharan Africa to adjust for the PPV and NPV of cryptococcal antigen detection in CSF [16]. Adjusted PPV and NPV based on disease prevalence were calculated using Bayes Theorem (PPV=Sensitivity x

prevalence/sensitivity x prevalence + (1-specificity) x (1-prevalence)). We compared the StrongStep LFA to the Immy LFA for all CSF and plasma samples tested. There was CSF culture data available for 119 of 143 CSF samples and 54 of 167 plasma samples tested. One CSF and one plasma sample had a negative test result by Immy LFA, but had a positive CSF culture and a clinical diagnosis of cryptococcal meningitis. Nine CSF samples were CSF culture negative, but CrAg positive by Immy LFA and with a clinical diagnosis of cryptococcal meningitis. The CrAg LFA is noted to be more diagnostically sensitive than quantitative CSF culture and is therefore used as the reference test for early, culture negative cryptococcal meningitis [10]. Data was analyzed using R version 3.2.1 (2015-June-18).

Results

We tested a total of 310 samples (143 CSF and 167 plasma) from 282 participants (28 participants contributed both CSF and plasma samples). There were 155 confirmed cases of cryptococcal disease (100 CSF and 55 plasma) and 155 cases of non-cryptococcal disease (43 CSF and 112 plasma). Of the 236 samples with identifiers, 47.5% (112/236) were male. The median age was 32 years (interquartile range (IQR); 28 to 40) with a median CD4 count of 29 cells/μL (IQR; 9 to 73).

We found that the CrAg Immunoassay, StrongStep had a sensitivity of 98% (54/55) and specificity of 90% (101/112) in plasma. When tested on CSF, the StrongStep had a sensitivity of 100% (100/100) and specificity of 95% (41/43) (**Table 1)**. Adjusting for the cryptococcal antigenemia prevalence of approximately 9% in Kampala, Uganda and cryptococcal meningitis prevalence of 37% in Sub-Saharan Africa, the adjusted PPV of the StrongStep LFA reduced to 50% in plasma and 93% in CSF; the adjusted NPV was 100% in both plasma and CSF.

Table 1: Performance characteristics of the StrongStep LFA in Uganda.

Specimen Type	N	Sensitivity	Specificity	Adjusted PPV	Adjusted NPV
CSF	143	100% (100/100)	95% (41/43)	92.70%	100%
Plasma	167	98% (54/55)	90% (101/112)	49.70%	99.80%

Data are presented as percentage and numerator/denominator. PPV=positive Predictive value, NPV=negative predictive value. The adjusted PPV and adjusted NPV are calculated for a CrAg antigenemia prevalence of 9% in Kampala, Uganda and cryptococcal meningitis prevalence of 37% in Sub-Saharan Africa.

The StrongStep LFA correctly identified all negative CSF samples as true negative results. However, the Strongstep LFA misclassified 2 CSF samples and 11 plasma samples as a false positive result. Two plasma samples were misclassified as a false negative result (**Table 2**).

Table 2: Characteristics of CSF and Plasma specimens misclassified by the StrongStep LFA.

Specimen	Cohort	N	StrongStep CrAg LFA result	Immy CrAg LFA result (CSF/Plasma)	Quantitative CSF culture result	Clinical Diagnosis	Classification
CSF	ASTRO	1	+	-/NA	NA	Non-Cryptococcal Meningitis	False Positive
CSF	COAT	1	+	-/NA	-	Non-Cryptococcal Meningitis	False Positive
Plasma	NOAT	1	-	+/-	+	Cryptococcal Meningitis	False Negative
Plasma	NOAT	2	+	-/-	-	Non-Cryptococcal Meningitis	False Positive
Plasma	ORCAS	9	+	NA/-	N/A	Cryptococcal Antigenemia	False Positive

CSF; cerebrospinal Fluid, N; number of specimens, CrAg; cryptococcal antigen, LFA; lateral flow immunochromatographic assay,(-); negative, (+); positive, NA; not available

We also performed the semi-quantitative titration procedure on the StrongStep LFA in order to determine the degree of sensitivity in detecting cryptococcal antigen at various dilutions. The StrongStep LFA and the Immy LFA were tested simultaneously on three separate titrations and using the same dilution specimen. We found that the StrongStep assay repeatedly gave

positive test results up to dilutions of 1:1280 as compared to the Immy LFA, which gave positive test results up to dilutions of 1:160 (**Table 3**).The StrongStep LFA read as high as a dilution of 1:5120 on one replicate, while the Immy LFA read as high as 1:320 on one replicate.

Table 3: Semi-Quantitative Titration of the StrongStep LFA compared to the Immy LFA.

Test	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Immy 1	+	+	+	-				
Immy 2	+	+	+	+	-			
Immy 3	+	+	+	-				
StrongStep 1	+	+	+	+	+	+	-	
StrongStep2	+	+	+	+	+	+	-	
StrongStep 3	+	+	+	+	+	+	+	-

Semi-Quantitative titration was performed using the Immy CrAg positive control with an initial dilution of 1:40, followed by 1:2 serial dilutions up to 1:5120. +; positive test result, -; negative test result; blank; dilution not done.

Discussion

This study evaluated the diagnostic performance of the StrongStep CrAg LFA, compared to the Immy CrAg LFA, in detecting the presence of cryptococcal antigen in both CSF and plasma specimens collected from HIV-infected participants in Uganda. The StrongStep LFA was found to have a sensitivity of 100% and specificity of 95% in CSF with a PPV of 98% and NPV of 100%. However, in plasma, the StrongStep LFA was found to have a sensitivity of 98% and specificity of 90%. When adjusting for disease prevalence, the PPV of the StrongStep in plasma was only 50%, whereas the PPV only slightly reduced in CSF to 93%. Notably, we demonstrated that the StrongStep LFA has higher analytical sensitivity than the Immy CrAg LFA at the cost of specificity with frequent false positives.

The implementation of a national CrAg screening program for early diagnosis and preemptive antifungal therapy is of utmost importance given the high mortality rates associated

with cryptococcal meningitis [1]. Widespread access to point of care diagnostics is paramount for the rapid and accurate diagnosis of cryptococcal disease, including cryptococcal antigenemia and cryptococcal meningitis. The CrAg Immunoassay, Strongstep, gives results in 10 minutes, is easy to use, needs no special laboratory equipment, does not require a diluent, and can be kept at room temperature, thus fulfilling the World Health Organization (WHO) ASSURED criteria as a point of care test [6, 17]. However, its problems with specificity are problematic.

Within Kampala, Uganda, the predictive value of the StrongStep LFA was considerably reduced when the prevalence of cryptococcal antigenemia was taken into account. As a screening test to be used nationally, there is concern that a positive test result on plasma has only a 50% probability of being correctly identified as a true positive result. In an already strained healthcare system, the high rate of false positivity, especially in plasma, would subject people to unnecessary medical therapy and overburden an already strained healthcare system.

One explanation for the reduced specificity of the StrongStep LFA is the possible interference of human anti-mouse antibodies with the lateral flow assay, resulting in a false positive result. The prevalence of anti-animal antibodies, the most common being human anti-mouse antibodies (HAMA), ranges between <1% to 80% in the general population [18]. The exact prevalence in Sub-Saharan Africa is unknown. Other human antibodies with the ability to interfere with the lateral flow assay, such as Rheumatoid Factor, could have also contributed to a false positive test result [19]. Blocking strategies using active or passive blockers added to either a diluent or the test well were not employed in this study.

Through this study, we were able to evaluate the diagnostic performance of the StrongStep LFA in detecting the presence of cryptococcal antigen in the CSF and plasma of HIV-seropositive individuals in Kampala and Mbarara, Uganda. The StrongStep LFA did not diagnostically perform well in plasma and would be a problematic assay to be used for a

nationwide screening program in Uganda. However, the StrongStep LFA did show promise in cryptococcal antigen detection in the CSF and can be used reliably for the diagnosis of meningitis. Further work is needed to determine if there are interfering antibodies that could result in a false positive test result and if there are methods to improve the specificity of the StrongStep LFA. Improving the specificity of the StrongStep LFA would be the first step in establishing whether it would be more cost effective than other point-of-care assays for cryptococcal antigen screening.

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