Title:

Laboratory verification of new commercial lateral flow assays for Cryptococcal antigen (CrAg) detection against the predicate IMMY LFA in a reference laboratory in South Africa

Short title:

CrAg LFA assay verification in South Africa

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LC was responsible for the concept, data collection and analysis and writing of the paper. DK was responsible for the review and editing of the paper.

NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.
Abstract

Background Reflex Cryptococcal antigen (CrAg) testing in HIV-positive patients is done routinely at 47 laboratories in South Africa on samples with a confirmed CD4 count <100 cells/µl, using the IMMY Lateral Flow Assay (LFA) as the standardized predicate method.

Objective This study aimed to verify the diagnostic performance of newer CrAg LFA assays against the predicate method.

Methods Remnant CD4 samples collected between February and June 2019, with confirmed predicate LFA CrAg results, were retested on settled plasma with the (i) IMMY CrAg semi-quantitative (SQ) LFA; (ii) Bio-Rad RDT CryptoPS SQ; and (iii) Dynamiker CrAg SQ assays, within 24 hours of predicate testing. Sensitivity/specificity analyses were conducted comparing predicate versus the newer assays, with McNemar’s test’s p-values reported for comparative results (p values <0.05 significant). Positivity grading was noted for the IMMY SQ and Bio-Rad assays.

Results Of the 254 samples tested, 228 had comparative CrAg results across all assays. The predicate method reported 85 CrAg positive (37.2%), compared to between 35.08 and 37.28% for the Bio-Rad, IMMY SQ and Dynamiker assays. The IMMY CrAg SQ grading (+1 to +5) showed 67% of CrAg positive results had a grading ≥3, indicative of higher CrAg concentration (infection severity). False-negative results across all assays were <2%, with sensitivity >95% for all. False-positive results were highest for the Dynamiker LFA (14%) with a specificity of 77% (p=0.001). IMMY SQ and Bio-Rad assays specificities exceeded 90% (p=0.6 and 0.12). Internal quality control showed 100% accuracy for all assays.

Conclusion Performance verification of newer CrAg LFA assays under typical laboratory conditions varied, with best results by IMMY SQ and Bio-Rad. The high burden of HIV and cryptococcal disease in South Africa requires high specificity and sensitivity (>90%) to prevent unnecessary treatment/hospitalization. The added value of positivity grading for patient management needs confirmation.

WORDS: 298
Introduction

Cryptococcal meningitis (CM) is a highly infectious disease caused by Cryptococcus neoformans, with a high mortality rate, particularly among HIV-positive patients in developing countries such as South Africa [1-3]. Following the inclusion of cryptococcal antigen (CrAg) screening in the 2016 World Health Organization (WHO) HIV guidelines [4], and subsequent inclusion in the South African HIV guidelines [5], a CrAg reflex screening pilot program was launched at selected National Health Laboratory Service (NHLS) CD4 laboratories in South Africa [6, 7]. This program was subsequently extended nationally in June 2017 to 47 CD4 laboratories, where remnant blood samples from HIV-positive patients with a confirmed CD4 count <100 cells/µl (severely immune-compromised) are identified by the laboratory information system (LIS) for reflex CrAg testing [8-10]. The data collected for the program has been used extensively to report on the prevalence of CrAg in South Africa [8, 9, 11], the cost-effectiveness of the local program [12, 13] and the high burden of patients living with HIV with a CD4 count <100 cells/µl (immune-compromised) [8, 9]. Approximately 10% of all CD4 samples tested annually (~230 000/annum) receive a reflex CrAg test with a national CrAg positivity rate of 6.2% [8, 9].

All reflexed CrAg testing is performed using the lateral flow assay (LFA) from IMMY (Immuno-Mycologics, IMMY, Norman, OK, USA) [14]. The IMMY LFA was the only commercially available assay at the time of the national roll-out and was recommended in the WHO HIV guidelines from 2018 [15]. Several studies described the performance of this assay against the ELISA and latex agglutination assays on serum, plasma, cerebrospinal fluid and urine [14, 16-30]. Since 2018, additional CrAg LFA products have been developed and introduced worldwide and became available in South Africa [31-33].
This study set out to verify the diagnostic performance of the newer CrAg LFA assays against the predicate method (IMMY LFA) for accuracy, sensitivity and specificity under typical laboratory conditions. The high burden of HIV and cryptococcal disease in South Africa requires an assay with high specificity and sensitivity (>90%) to prevent unnecessary treatment/hospitalization. This will enable the accurate distinction of positive and negative results to ensure appropriate early initiation of antifungal therapy to reduce mortality.

**Materials and Methods**

**Ethics clearance**

Ethics clearance was obtained from the University of the Witwatersrand (M1706108, approved for 5 years from 13/07/2017 to 07/2022). No patient consent was required as random remnant blood samples were used as per ethics clearance after laboratory predicate results were reported. The study results were for assay verification purposes only. No patient information was available to either the testing staff or the authors of this paper.

**CrAg Testing methods**

All reagents and test consumables were supplied by local diagnostic suppliers, with training provided for each assay for two medical technologists. The assays verified in this study are CE/IVD approved (Conforms to European Union Requirements/in vitro diagnostics). The manufacturer instructions defined in the package insert were used for reagents storage, quality control (positive and negative internal controls), testing (sample volume, incubation time), interpretation of results, detection limitations and result reporting. All safety precautions were adhered to as part of good laboratory
practice (GLP). Results were reported as positive, negative, or invalid (no control line visible), with concentration ratings noted for specific assays as per their package inserts (IMMY SQ and Bio-Rad). Samples with faint positive lines were verified by a senior staff member or retested if the result was inconclusive. All CrAg testing and retesting were done on settled plasma, with no titrations performed as this is not the practice for CrAg reflex screening.

**Laboratory predicate method**

The predicate method is the LFA by IMMY (Immuno-Mycologics, Norman, Oklahoma, United States) [34]. This assay is an immunochromatographic test for the qualitative and semi-quantitative detection of the capsular polysaccharide antigen of *Cryptococcus* species in plasma and serum, using specimen wicking and gold-conjugated anti-CrAg antibodies. Currently, the predicate method is used only as a qualitative assay for reflex testing. Samples were prepared according to the manufacturer's instructions and the national internal standard operating procedure.

**Newer generation CrAg LFA methods**

This IMMY CrAg SQ dipstick sandwich immunochromatographic assay is a newer commercial IMMY CrAg dipstick (Immuno-Mycologics, Norman, Oklahoma, United States) [33]. The principle is similar to the original IMMY CrAg LFA assay, with the exception that there is one control line and two test lines (T1 and T2) on a wicking strip [33, 35]. Positive results will always have a control line, with either T1 and/or T2 lines visible. The intensity (concentration) of CrAg binding is interpreted from a scoring scale of 1+ to 5+ as per package insert.

The Bio-Rad RDT CryptoPS (Biosynex CryptoPS) assay is a single-use rapid semi-quantitative CrAg strip test (BIOSYNEX S.A., Illkirch–Graffenstaden, France) [36]. Sample and diluent are dispensed into the well and test lines (T1 and T2) will form
depending on the concentration in the specimen [31, 36]. T1 represents concentrations lower than 25ng/ml with T2 detecting concentrations up to 2.5µg/ml.

Dynamiker CrAg LFA (Dynamiker Technology, Tianjin Eco-City, Tianjin, China) [32] is an immunochromatographic test using conjugated Cryptococcus antibodies to gold particles. CrAg will form complexes that appear as a visible line, while the free antibodies bind to form a control line [32].

**Patient samples and study settings**

Remnant samples collected in Ethylenediaminetetraacetic acid (EDTA) submitted for CD4 testing at the Charlotte Maxeke Academic Hospital (CMJAH) and Tambo Memorial laboratories between February and May 2019, with a confirmed count of <100cells/µl, received onsite reflex CrAg testing using the IMMY CrAg LFA predicate method. These laboratories are accredited by the South African National Accreditation System (SANAS), adhere to good laboratory practice (GLP) and International Organization for Standardization (ISO/ICE 15189:2014) guidelines [37]. In addition, they are enrolled in the local external quality assessment (EQA) [38]. Patient management was based on the predicate CrAg result reported.

Reagents for 300 tests were provided from local service providers for IMMY and Dynamiker (only 280 tests available from Bio-Rad). Before comparative testing commenced, four tests per assay were reserved for training and ten tests for possible duplicate/repeat testing. Daily quality-control tests at two levels (negative and positive) were done per assay/testing day (n=32 tests/assay in total) (Fig 1).

**Figure 1: Summary of verification samples tested:** Patient samples tested across three new CrAg LFA assays, including quality control (QC), duplicate testing and rejections, using the available tests provided by suppliers. (Authors own work).
Following predicate testing, samples were delivered to the CMJAH CD4 research unit for re-testing within 24 hours. The project coordinator collated CrAg results in an Excel spreadsheet and batched available samples for blind testing. The batch size was restricted to a maximum of 25 samples (n=75 tests across 3 assays) per day (≤3 hours per testing day per trainer) and included all positive samples collected from the testing laboratories on the testing day with random negative samples. Results for the IMMY CrAg SQ LFA [33], Bio-Rad RDT CryptoPS Assay (Biosynex CryptoPS) [31, 36], and Dynamiker CrAg LFA assays [32] were recorded on printed worklists against anonymized sample ID numbers and collated by the project coordinator into the project Excel file.

The verification criteria for sensitivity and specificity levels were set at >90% to correlate with the current IMMY CrAg LFA predicate method. The Standards for Reporting of Diagnostic Accuracy Studies (STARD) statement checklist was used for transparency of result reporting [39, 40].

**Statistical analysis**

Statistical analysis and the graphic display were done with GraphPad Prism Software version 7 (GraphPad Software, San Diego, United States). Assay performance statistics included: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy. These parameters were calculated using the predicate IMMY CrAg LFA as the reference method. The MedCalc software and online calculator were used for this purpose [41], with McNemar's test's for paired nominal data used to assess significance between the predicate and each new CrAg assay, with p-values calculated (p<0.05 regarded as significant) [42].
Results

Assay-specific positive and negative controls were analyzed with every batch of samples retested with 100% accuracy for all assays over the test period. Of the 10 tests per assay set aside for duplicate or re-testing, only 4 repeat tests were performed with the Bio-Rad assay. Repeat test results were not reported as the outcome did not change from the original result observed.

Performance of reference and test methods

Reference methods results

In total, 254 patient samples were tested using the IMMY LFA SQ and Dynamiker SQ assays. Only 230 samples could, however, be retested using the Bio-Rad assay due to import challenges and the available timeline of the project. With 2 samples excluded due to receipt >72 hours after predicate testing, only 228 samples could be used for comparison across the three new assays (Fig 1). Of these 228 results, the predicate LFA CrAg reference method identified 143 CrAg negative (62.72%) and 85 CrAg positive results (Table 1).

Of all samples tested, 183/228 (80.3%) showed equivalent results across all testing assays versus predicate; 102 negative (71.3% of total negative samples) and 81 positive samples (95.3% of total positive samples). The remaining 45/184 samples showed discrepant results for one or more assays against the reported predicate result (41 and 4 reported a negative and positive result respectively) (Fig 2).

Figure 2: CrAg test results compared to the predicate LFA method: A summary of CrAg test results by the predicate method, indicating the number of results that were equivalent across the four LFA assays versus the numbers of discrepant results, where one or more assays had opposite results to the predicate result reported. (Purple bars represent CrAg negative results and blue bars CrAg positive results).
Newer commercial CrAg LFA test assay results

Excellent agreement was noted for the number/percentage positive samples identified by the IMMY SQ, Bio-Rad and Dynamiker assays against the predicate method. The agreement for negative CrAg results, however, showed greater variability with the Bio-Rad and Dynamiker assays. False-positive results were observed with all assay comparisons to the predicate reference method used and ranged from 3 to 32 (1.31 to 14.03%) of the 228 samples tested, while false-negative results (compared to the reference method) of <2% were reported across the test assays (Table 1). The highest number of discordant results was reported for the Dynamiker assay, against the reference predicate method (p-value <0.001, McNemar’s test’s test). Of the 32 samples classified as false-positive, 11 had very faint positive bands, while 21 samples had clear positive bands as reported by two independent observers. Further analysis of Dynamiker assay performance excluding the 11 samples with faint positive results, only managed to reduce the percentage of false-positive samples by 3%, i.e. false positivity rates remained greater than 9% (data not shown).

IMMY SQ results showed an excellent correlation with the predicate method (Table 1; McNemar’s test’s test p=0.62). Of the 87 reported positive CrAg results, 20 recorded a 1+ reading (including the 3 false-positives compared to the predicate), with 20 a 2+, 28 a 3+, 18 a 4+ and only one a 5+ result, confirming that 67.85% of samples had a score of 3+ or more, i.e. an elevated concentration of CrAg associated with a high burden of disease. With Bio-Rad (McNemar’s test’s test p=0.12), of the reported 92 positive samples, 51 had a T1 result, of which 50 (98.03%) correlated with an IMMY SQ of a 1+ or 2+ result, while 41 Bio-Rad tests had a T2 result, of which 37 (90.24%) correlated with an IMMY SQ results of ≥3+.  

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Each of the newer CrAg assays was assessed against the predicate IMMY CrAg LFA. All results are reported as a percentage with the 95% confidence interval in brackets. Data were collected from February to June 2019 at two testing laboratories in South Africa.

### Table 1: Summary of all CrAg assay results.

| IMMY Predicate CrAg LFA as Reference | IMMY SQ        | BioRad   | Dynamiker |
|-------------------------------------|---------------|----------|-----------|
| Positive number (% of total)        | 84 (36.84)    | 81 (35.52) | 85 (37.28) |
| Negative number (% of total)        | 140 (61.40)   | 132 (57.89) | 111 (48.68) |
| False-positive number (% of total)  | 3 (1.31)      | 11 (4.82)  | 32 (14.03) |
| False-negative number (% of total)  | 1 (0.43)      | 4 (1.75)   | 0 (0.0)    |
| p-value (McNemar test)              | 0.617         | 0.123     | <0.001***  |
| **TOTAL**                           | **228**       | **228**   | **228**    |

The total test number and number of positive and negative samples were recorded as well as the number of false-positive and false-negative samples for each CrAg assay compared to the predicate CrAg LFA assay as reference method (n=85 CrAg positive and 143 CrAg negative samples). Data is reported as test results recorded and the percentage of total tests (n=228), with a p-value of the McNemar’s test’s for paired nominal data comparison (<0.05 statistically significant).

### Sensitivity and Specificity analysis

Sensitivity and specificity analyses were done, using the predicate IMMY CrAg LFA as the reference method on 228 samples (Table 2). Specificity was greater than 95% for IMMY SQ assay, while 92.36% and 77.62% were reported for the Bio-Rad and Dynamiker assays respectively. Sensitivity ranged from 95.24% to 100% for the Bio-Rad and Dynamiker assays. An accuracy of 85.95% was reported for Dynamiker, compared to >90% reported for IMMY SQ and Bio-Rad assays.

### Table 2: Summary of specificity and sensitivity analyses.

| Predicate IMMY CrAg LFA | Sensitivity and Specificity parameters as percentage (95% CI) | IMMY CrAg SQ LFA | Bio-Rad CrAg LFA | Dynamiker CrAg LFA |
|-------------------------|---------------------------------------------------------------|-------------------|------------------|---------------------|
|                         | Specificity                                                    | 97.90 (93.99-99.57) | 92.36 (86.74-96.13) | 77.62 (69.90-84.16) |
|                         | Sensitivity                                                    | 98.82 (93.32-99.97) | 95.24 (88.25-96.65) | 100 (95.75-100.00)  |
|                         | Positive Predictive Value (PPV)                               | 96.55 (90.13-98.85) | 87.91 (80.44-92.78) | 72.65 (66.19-78.28) |
|                         | Negative Predictive Value (NPV)                               | 99.29 (95.23-99.9) | 97.08 (92.73-98.86) | 100                 |
|                         | Accuracy                                                       | 98.25 (95.57-99.52) | 93.42 (89.38-96.27) | 85.96 (80.77-90.20) |

Each of the newer CrAg assays was assessed against the predicate IMMY CrAg LFA. All results are reported as a percentage with the 95% confidence interval in brackets. Data were collected from February to June 2019 at two testing laboratories in South Africa.
Discussion

This study set out to verify the diagnostic performance of the newer CrAg LFA assays against the predicate method (IMMY LFA) for accuracy, sensitivity and specificity under typical laboratory conditions.

Of the newer commercial CrAg assays, IMMY SQ faired best, with sensitivity and specificity exceeding 95%, while Bio-Rad results were also acceptable with sensitivity and specificity greater than 90%. Similar acceptable outcomes have been reported for the IMMY SQ assay by Tenforde et al. and Temfack et al [43, 44].

The lower sensitivity of between 78-88% and specificity of more than 90% [43-46] have been reported for the Bio-Rad CryptoPS assay (also marketed as Biosynex CryptoPS) as well as lower specificity in samples with a low fungal burden (missed positivity) in these samples [43, 44] The verification results of this current South African comparison, confirmed the slightly lower sensitivity and specificity of the Bio-Rad CryptoPS assay, however still within local acceptable criteria of sensitivity and specificity of >90%. A possible explanation previously reported mentioned the differences described in the limit of detection between IMMY LFA (5ng/ml) vs. Bio-Rad (25ng/ml) [46].

Our local study reported acceptable sensitivity of more than 98% for the Dynamiker CrAg assay, but specificity rates of 77% (vs. predicate LFA). The Dynamiker assay reported the highest number of false-positive results, mainly due to weak positive bands reported by two independent observers. Even with these removed from all calculations, the false positivity rate remained at ~10%. Similar high false-positive results were reported by Kwizera et al, where they showed comparable sensitivity with IMMY LFA, but poor specificity in serum and plasma samples [35]. This may in part
be due to the lower detection limit of Dynamiker (1.25ng/ml vs. 1.75 ng/ml for IMMY LFA). A small study (n=25) published in 2018, reported that Dynamiker had a brighter intensity of a positive result than comparative results with the IMMY LFA, and the authors suggested that a faint positive result with Dynamiker should be reported as CrAg negative with this assay [47]. This is, however, impractical in a typical laboratory setting where only one assay kit is used and laboratory staff is trained to report all positive results (faint or clear) as such. No verification testing is done routinely in the NHLS CrAg reflex program and comparative testing may only be available in research facilities for specific projects. The observed higher positivity could be attributed to the differences in methods compared to IMMY and other LFA strips, in that there is no diluent used. A comparative study on cryopreserved serum samples (n=162) reported acceptable specificity of 89% of the Dynamiker assay versus IMMY LFA [48]. Their study had only a small number of positive samples (n=14; 8.6% of total tests), but reported an equivalent number of false-positive results (n=15; 9.3% of total tests), which they contribute to the lower detection threshold of the Dynamiker assay.

All findings of our study were shared with the local service provider and subsequently, a refined version of the assay was provided for testing. Unfortunately, the issue of faint false-positive results persisted (data not shown)

Our study showed that the performance of the IMMY SQ and Bio-Rad assays were comparable to IMMY LFA results with both sensitivity and specificity greater than 90% These results confirm earlier reported verification results with this CrAg assay [43, 44, 46], where there was a good correlation with LFA titer results, where the grading from 1+ to 5+ showed correlation with titers [43, 44, 46]. This was further confirmed in a study on cerebrospinal fluid (CSF), where increasing IMMY SQ grades were associated with greater LFA titer and quantitative culture results/colony forming units
The risk stratification offered by the IMMY SQ and Bio-Rad assays may be important from a clinical perspective to identify the severity of meningitis and the infiltration of the central nervous system (CNS) associated with elevated CrAg concentrations [43, 44, 46]. Patients with a grade of ≥3 may have a higher risk of poor outcomes, as reported by Tadeo et al, 2021 [49].

**Limitations**

This verification study of the diagnostic performance of newer LFA CrAg test methods was done on typical laboratory samples, tested by qualified technologists. In-house unpublished reproducibility verifications performed on fresh EDTA samples over time using the IMMY LFA method showed reproducible results up to 48 hours on samples kept at room temperature. Assay recommendations indicate testing within 24 hours with samples stored at room temperature or up to 48 hours if refrigerated [32-34, 36]. Manufacturers need to take into consideration the time delay from sample collection to testing to ensure this does not affect the outcome of the test results, i.e. ensure the robustness of their assay to produce a reliable result even on samples older than 24 hours. Although the LFA technology is ideal for point-of-care testing at a clinic level, this would delay patient testing while waiting for a confirmation of a CD4 count, with most patients typically only returning for test results within 7-14 days [15].

Operator feedback highlighted extra preparation steps needed with some assays and expressed challenges with result interpretation where more than two bands were present, i.e. particularly with assays like Bio-Rad and IMMY SQ with multiple lines to read manually, even though all tests were read independently by two LFA trained members of staff.
Typical laboratory challenges with CrAg LFA testing include mislabeling and transcription errors to the worksheet and onto the LIS. Automated cassette readers could be used to ensure direct transfer of the strip result to the LIS. Some strip readers were tested locally, though the challenges were either it making use of an intermediary program (like Excel) for result reporting (no direct interface with laboratory information system), or single sample reading of results that may not necessarily reduce turn-around-time of CrAg result reporting or hands-on time by operators.

The positivity percentages reported here do not represent the incidence of CrAg in South Africa [50, 51] as samples were collected from two facilities for assay performance specifically. Due to fairly low positivity rates at these facilities, all positive samples available were retested. Care was taken to include as many positive samples for statistical comparisons between assays, within the limitations of suitable samples, available reagents and time of operators to conduct testing.

Conclusion

The local verification of performance of newer commercial CrAg assays is necessary to confirm accurate result reporting as there is inevitable variability between assays. Additional information provided by some assays like the Bio-Rad and IMMY SQ i.e. the intensity of antigen detected should be further investigated to assess its value to clinicians in deciding on whether a lumbar puncture is needed or a CNS infiltration is suspected. The relevance of this added information may, however, depend on the local treatment guidelines i.e. in South Africa all CrAg positive patients will get a lumber puncture currently [15, 52] and cost-effectiveness of graded assays, especially in large national CrAg screening programs.
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Total tests provided (4 tests for training, 10 tests set aside for duplicate testing where indicated)

- IMMY SQ (n=300)
  - 254 Patient samples
  - 32 QC samples
  - 2 Rejected (age)
  - 4 QC samples per testing day

- BioRad LFA (n=280)
  - 230 Patient samples
  - 32 QC samples
  - 2 Rejected (age)
  - 4 QC samples per testing day
  - 4 duplicate tests (not resulted)

- Dynamiker LFA (n=300)
  - 254 Patient samples
  - 32 QC samples
  - 2 Rejected
  - 4 QC samples per testing day

Fig 1
