

# Evaluation of a Novel Point-of-Care Cryptococcal Antigen Test on Serum, Plasma, and Urine From Patients With HIV-Associated Cryptococcal Meningitis

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**Background.** Many deaths from cryptococcal meningitis (CM) may be preventable through early diagnosis and treatment. An inexpensive point-of-care (POC) assay for use with urine or a drop of blood would facilitate early diagnosis of cryptococcal infection in resource-limited settings. We compared cryptococcal antigen (CRAG) concentrations in plasma, serum, and urine from patients with CM, using an antigen-capture assay for glucuronoxylomannan (GXM) and a novel POC dipstick test.

**Methods.** GXM concentrations were determined in paired serum, plasma, and urine from 62 patients with active or recent CM, using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA). A dipstick lateral-flow assay developed using the same monoclonal antibodies for the sandwich ELISA was tested in parallel. Correlation coefficients were calculated using Spearman rank test.

**Results.** All patients had detectable GXM in serum, plasma, and urine using the quantitative ELISA. Comparison of paired serum and plasma showed identical results. There were strong correlations between GXM levels in serum/urine ( $r_s = 0.86$ ;  $P < .001$ ) and plasma/urine ( $r_s = 0.85$ ;  $P < .001$ ). Levels of GXM were 22-fold lower in urine than in serum/plasma. The dipstick test was positive in serum, plasma, and urine in 61 of 62 patients. Dipstick titers correlated strongly with ELISA. Correlations between the methods were 0.93 ( $P < .001$ ) for serum, 0.94 ( $P < .001$ ) for plasma, and 0.94 ( $P < .001$ ) for urine.

**Conclusions.** This novel dipstick test has the potential to markedly improve early diagnosis of CM in many settings, enabling testing of urine in patients presenting to health care facilities in which lumbar puncture, or even blood sampling, is not feasible.

Cryptococcal meningitis (CM) is estimated to kill more than 500 000 human immunodeficiency virus (HIV)-infected patients per year in Sub-Saharan Africa [1].

With the evolving HIV epidemic, CM has emerged as the most frequent cause of adult meningitis in much of central and southern Africa [2–4], and outcomes with current optimal antifungal therapy are poor [5–7]. Many deaths from CM may be preventable through early diagnosis and treatment.

The diagnosis of HIV-associated CM is usually made by lumbar puncture (LP) and India-ink testing of cerebrospinal fluid (CSF). However, the presenting symptoms of headache and fever are very nonspecific, and LP is often deferred until the disease is advanced and the prognosis is poor. The alternative of

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immunodiagnosis through detection of cryptococcal polysaccharide capsule glucuronoxylomannan (GXM) in serum by latex agglutination (LA) or sandwich enzyme-linked immunosorbent assay (ELISA) is sensitive and specific and has been commercially available for many years [8, 9]. However, the currently available immunoassays require blood to be sent to a central laboratory with appropriate laboratory infrastructure and trained staff, and immunoassay tests are too expensive for routine use in most African settings and therefore are not widely available.

A point-of-care (POC) immunoassay for cryptococcal antigen (CRAG) would greatly facilitate the early diagnosis of patients presenting with symptoms of CM. Such a test could also be used to prevent CM because CRAG immunoassays are positive prior to the development of clinically apparent disease [10, 11]. Screening patients before initiating antiretroviral therapy (ART), with preemptive antifungal therapy for patients with subclinical infection, could prevent the later development of clinical disease [11, 12]. Currently, up to 70% of all cryptococcal cases in some African centers present after a diagnosis of HIV infection, and approximately 30% present after initiation of ART [6, 13–15].

Current tests have regulatory approval for use on serum and CSF; however, the ability to detect CRAG in other sample types, specifically plasma and urine, is essential for a POC test. In addition, plasma is readily available from samples routinely taken for HIV testing and CD4 cell-count monitoring. Urine is a highly desirable specimen for diagnosis of cryptococcal infection in resource-limited settings [16], particularly where blood sampling is difficult or LPs are not easily performed or accepted. There have been 2 studies of GXM excretion in urine of patients with cryptococcosis [9, 17], both of which supported immunoassay use on urine as an aid to diagnose cryptococcosis. However, neither study determined the quantitative relationship between levels of GXM in serum and urine.

We have evaluated the performance of a quantitative antigen-capture ELISA for GXM and a novel point-of-care lateral flow immunoassay (LFA) using paired serum, plasma, and urine from patients with active or prior CM. Both assays are based on GXM monoclonal antibodies (mAbs) selected to have broad reactivity across the 4 major serotypes of *Cryptococcus neoformans* capsular antigen [18, 19]. The aims were to define the relationship of CRAG levels in serum, plasma, and urine and to test the sensitivity of the novel lateral flow assay in serum, plasma, and urine.

## METHODS

### Participants and Procedures

Sample collection was performed between March 2009 and November 2010 at GF Jooste Hospital, a public-sector adult referral hospital in Cape Town, South Africa, and approved by

the Research Ethics Committee of the University of Cape Town. Paired blood and urine samples were collected from adult patients ( $\geq 21$  years), with a history of laboratory-confirmed HIV-associated cryptococcal disease within the preceding 2 years (CSF India ink or CRAG positive, titers  $\geq 1:1024$ , Meridian Cryptococcal Latex Agglutination System, Meridian Bioscience, and confirmed on CSF culture in all but 4 patients in whom culture results were unavailable), who were either under follow-up in the outpatient clinic or admitted to the medical wards. Written informed consent was obtained from each participant prior to study enrollment. Samples were collected over a broad range of follow-up times from original diagnosis, and from sufficient numbers of patients, so that a wide range of antigen titers was obtained. Blood and urine samples were frozen for later analysis. Basic demographic and laboratory data (age, sex, CD4 cell count), details of CM episode (timing, basis of diagnosis, and treatment received), and details of antiretroviral exposure and secondary fluconazole prophylaxis were recorded.

### Quantitative Sandwich Enzyme-Linked Immunosorbent Assay

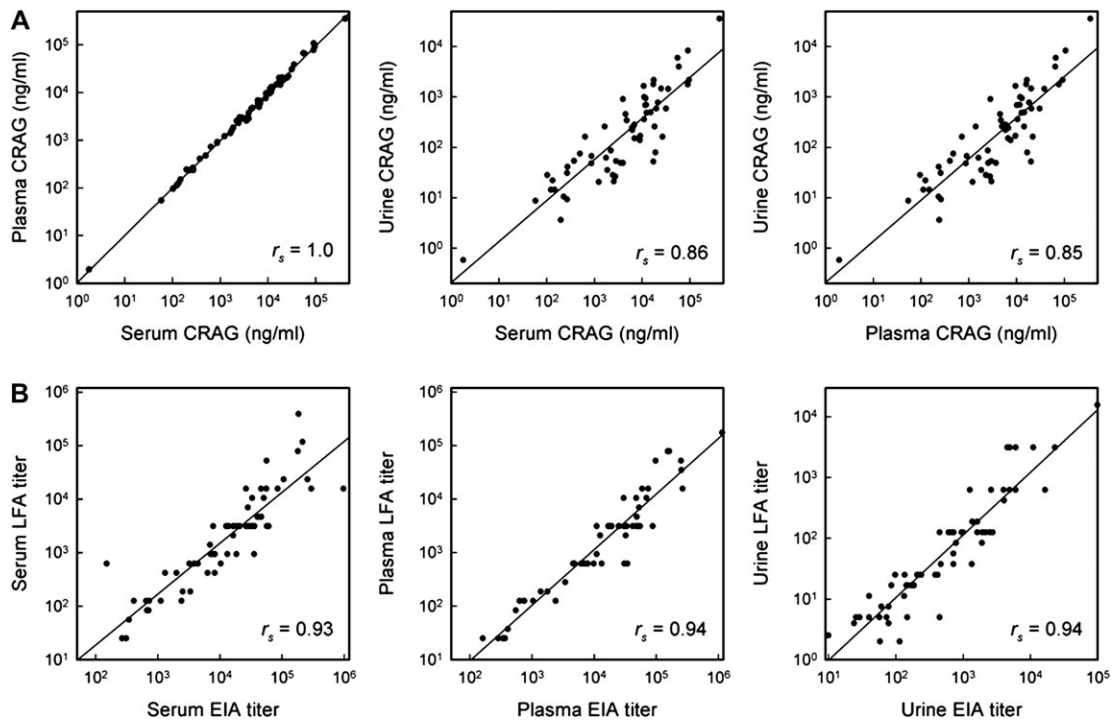
GXM concentrations were determined in each sample by use of a quantitative sandwich ELISA that was constructed using the GXM mAbs F12D2 and 339 [18, 19]. In this ELISA, microtiter plates were coated overnight with an optimized 50:50 mixture of GXM mAbs F12D2 and 339 for the capture phase. After plates were washed and blocked, the wells were incubated for 90 minutes with serial dilutions of serum, plasma, or urine in phosphate-buffered saline–Tween. The plates were washed, incubated for 90 minutes with an optimized 50:50 cocktail of horseradish peroxidase-conjugated mAbs F12D2 and 339 for the indicator phase, and washed and incubated with substrate solution. Purified serotype A GXM isolated from strain CN-6 was used as a standard. The results are reported as the concentration of GXM in nanograms per milliliter.

### Lateral Flow Assay

A novel LFA was constructed from the same mAbs F12D2 and 339 used in the quantitative sandwich ELISA. The test is in dipstick format. Forty microliters of specimen was mixed with 1 drop of sample diluent, the dipstick was inserted into the diluted sample, and the test strips were read after 10 minutes by 4 different observers. Tests were considered positive if all 4 observers read the strip as positive and equivocal if some but not all observers read the strip as positive. LFA titers were determined by diluting patient samples in LA diluent solution and assessing reactivity as described above. The highest sample dilution that produced a positive result when read by 4 observers was recorded as the LFA titer.

### Statistical Methods

Correlation coefficients were calculated using the Spearman rank-order correlation. GXM concentrations were log transformed,



**Figure 1.** A, Correlation of glucuronoxylomannan concentrations in paired serum and plasma, serum and urine, and plasma and urine measured by quantitative enzyme-linked immunosorbent assay (ELISA). B, correlation of cryptococcal antigen (CRAG) titers from the quantitative ELISA and lateral flow assay (LFA) in serum, plasma, and urine from 62 subjects with recent or current cryptococcal meningitis. Correlations were calculated using Spearman rank-order correlation. Abbreviation: EIA, enzyme immunoassay.

and geometric means with 95% confidence intervals (CIs) are presented where appropriate.

## RESULTS

Paired blood and urine samples were obtained from 62 patients. The median age was 34 years (interquartile range [IQR], 28–37), 40% were male (25 of 62), and the median CD4 cell count was 45 cells/ $\mu$ L (IQR, 22–100). Sixty-one percent of patients (38 of 62) had samples collected during their acute episode of CM, and 39% (24 of 62) during follow-up visits, a median of 189 days (IQR, 98–376) after initial CM presentation. All episodes of CM were treated with amphotericin B (1 mg/kg per day) for 14 days. Of the follow-up patients, 92% (22 of 24) were on fluconazole secondary prophylaxis. Overall, 56% (35 of 62) were on ART at the time of sample collection, 92% (22 of 24) of follow-up patients and 34% (13 of 38) with acute CM.

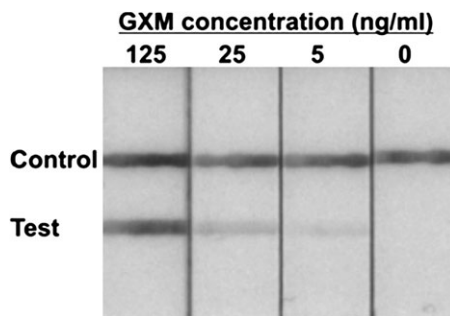
### Quantitative Immunoassay for Glucuronoxylomannan in Patient Samples

All 62 patients had detectable GXM in serum, plasma, and urine using quantitative ELISA. The mean (95% CI) GXM concentrations were 3800 (2100–6600) ng/mL in serum, 3600 (2100–6300) ng/mL in plasma, and 170 (100–280) ng/mL in urine. A comparison of immunoassay results of paired serum and

plasma showed identical results with the 2 sample types (Figure 1). There was a very strong correlation between GXM concentrations in serum and plasma ( $r_s = 1.00$ ;  $P < .001$ ). There were also strong correlations between levels of GXM in serum and urine ( $r_s = 0.86$ ;  $P < .001$ ) and in plasma and urine ( $r_s = 0.85$ ;  $P < .001$ ). The actual levels of GXM in urine were lower, on average 22-fold lower in urine than in serum or plasma. The ratio of GXM in serum/urine was independent of the concentration of GXM in serum ( $P = .77$ ) or urine ( $P = .36$ ).

### Lateral Flow Assay

The lateral flow assay produced a consistently positive result with purified GXM at concentrations  $\geq 5$  ng/mL (Figure 2). The LFA was positive in the serum, plasma, and urine in 61 of 62 patients (Table 1). The remaining patient had extremely low concentrations of GXM detectable in the serum, plasma, and urine by quantitative ELISA testing (2 ng/mL, 2 ng/mL, and 1 ng/mL respectively); on LFA testing, the results for this patient were equivocal on serum and plasma, and negative in the urine. LFA titer results, calculated by serial dilution, correlated strongly with GXM concentrations measured by the quantitative ELISA method. The Spearman rank-order correlations between the 2 methods were 0.93 ( $P < .001$ ) for serum, 0.94 ( $P < .001$ ) for plasma, and 0.94 ( $P < .001$ ) for urine (Figure 1).



**Figure 2.** Signal strength of lateral flow dipstick with purified serotype A glucuronoxylomannan (GXM) at various concentrations.

## CONCLUSIONS

These results indicate that serum and plasma can be used interchangeably for CRAG testing. The ability to use plasma as a clinical sample will reduce the logistical difficulty and cost of CRAG testing in patients where plasma is already collected as part of other clinical tests, most notably for determination of CD4 cell count after HIV diagnosis and before ART. Thus, laboratories can automatically forward plasma from patients with a CD4 cell count of  $<100$  cells/ $\mu$ L for CRAG screening.

The study also demonstrates the potential value of testing urine for CRAG. Urine is a noninvasive sample that is easily obtained in environments with limited resources [7]. GXM concentrations in urine were significantly lower than in serum/plasma (approximately 20-fold). However, despite these lower levels, there was 100% sensitivity in testing of urine relative to serum or plasma when the highly sensitive enzyme immunoassay (EIA) was used. Moreover, the sensitivity limit of the LFA (approximately 5 ng GXM/mL) was well below the lower end of the 95% CI for GXM concentration in urine (approximately 100 ng/mL). The LFA found readily detectable CRAG in urine from 61 of 62 patients.

Patients with HIV-associated CM usually have high organism burdens, and in this study of patients with current or recent CM, levels of antigen are likely to have been higher than are found on screening of asymptomatic patients prior to ART [11]. However, all samples with detectable GXM by quantitative EIA were also positive on LFA testing, with the exception of a single patient who

had very low antigen titers in the serum, plasma, and urine. There were also very close correlations between GXM levels on ELISA and LFA titers in serum, plasma, and urine. The ability of the POC test to reliably detect antigen at concentrations  $\geq 5$  ng/mL in this study suggests that sensitivity of the POC test in urine may also be sufficient in asymptomatic patients with early subclinical infection, but this needs confirmation in prospective studies.

This POC test has the potential to markedly improve the early diagnosis of CM in many settings, enabling the testing of urine in patients presenting with symptoms of CM to primary health care facilities or rural clinics where LP, or even blood sampling, may not be feasible. The POC test will be a great advantage in diagnostic laboratories in much of the developing world, where erratic electricity supplies and lack of basic equipment may preclude the use of conventional CRAG assays. This technology also has potential applications in the developed world, both in HIV-infected patients and in other patient groups such as solid organ transplant recipients.

Furthermore, the findings have important implications for the feasibility of CRAG screening interventions in ART programs. At a programmatic level, using the excess plasma from CD4 count measurement for CRAG testing would prevent the need for collection of additional serum samples and prevent any delay in initiation of ART, especially in the majority of patients who test antigen negative. Indeed, routine CRAG testing of all CD4 cell count samples in which the CD4 cell count is  $<100$  cells/ $\mu$ L at laboratory level has been proposed as part of the introduction of CRAG screening in South Africa [20].

The relatively small sample size means that these results require validation in larger studies, and a limitation of the current study is that although the sensitivity of CRAG detection in serum, plasma, and urine by ELISA and LFA has been demonstrated, no inference about the specificity of the tests can be drawn from these data. The extremely close correlation between CRAG titers in the blood and urine and between the results from the ELISA and LFA tests suggest that specificity of CRAG urine testing and the novel LFA should be as good as conventional immunodiagnostic testing. Nevertheless, prospective screening studies are needed and are under way to further validate the specificity of the POC test. Work is also ongoing to validate modifications to the LFA that provide a quantitative result, and data from these ongoing prospective studies will be used to determine the most useful cut-points for any such quantification. Widespread availability and use of such a POC test has the potential to substantially lower the global burden of cryptococcal disease.

## Notes

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**Table 1.** Lateral Flow Assay Results in Serum, Plasma, and Urine

	Serum	Plasma	Urine
CRAG LFA+	61	61	61
CRAG LFA +/-	1	1	0
CRAG LFA -	0	0	1
Sensitivity of LFA	100%	100%	98%
95% CI	94%–100%	94%–100%	91%–100%

Abbreviations: CI, confidence interval; CRAG, cryptococcal antigen; LFA, lateral flow assay.

**Potential conflicts of interest.** S. B. is president of Immuno Mycologics (IMMY). J. P. is an employee of IMMY. The University of Nevada, Reno, has licensed the mAbs used for immunoassay construction to IMMY. All other authors report no conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**Author contributions.** J. N. J., T. K., and T. S. H. conceived and designed the study. J. N. J., G. N. W., and N. L. performed the patient recruitment and clinical sample collection. T. K. conceived and designed the mAbs and the quantitative sandwich ELISA. A. P. performed the ELISAs. S. B. and T. K. designed and produced the LFA test kit. LFA testing was performed in the United States by S. B. and J. P., and in Cape Town, South Africa, by G. M., G. N. W., N. L., and T. S. H. J. N. J. and T. K. analyzed the data. J. N. J. wrote the manuscript, with input from T. K. and T. S. H. All authors read and approved the final version of the manuscript.

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