Journal of Clinical Microbiology Mini Review

Cryptococcal Meningitis Diagnostics and Screening in the Era of Point-of-Care Laboratory Testing

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Abstract

Over the past ten years, standard diagnostics for cryptococcal meningitis in HIV-infected persons have evolved from culture, to India ink, to detection of cryptococcal antigen (CrAg) with the recent development and distribution of a point-of-care lateral flow assay. This assay is highly sensitive and specific in cerebrospinal fluid (CSF), but is also sensitive in the blood to detect CrAg prior to meningitis symptoms. CrAg screening in HIV-infected persons in the blood prior to development of fulminant meningitis, and preemptive treatment for CrAg-positive persons is recommended by the World Health Organization and many national HIV guidelines. Thus, CrAg testing is occurring more widely, especially in resource-limited laboratory settings. CrAg titer predicts meningitis and death, and could be used in the future to customize therapy according to burden of infection.

Introduction

Globally cryptococcal meningitis causes 15% of AIDS-related deaths, with an estimated 181,100 deaths annually.(1) HIV-infected persons with advanced HIV disease are at highest risk of infection. While efforts are underway to increase access to antiretroviral therapy (ART) globally, persons who do not start, or who default ART remain at risk of cryptococcal meningitis.(2) The majority of cases of cryptococcal meningitis occur in sub-Saharan Africa where diagnostic facilities, access to optimal antifungal medications, and access to intensive hospital-based treatments are limited.(3) Thus, 6-month mortality from cryptococcal meningitis in hospital settings, despite standard of care antifungal therapy, ranges from 40 to 60% in resource-limited settings.(2, 4) Over the last 10 years, drastic improvements in the development and distribution of a point-of-care lateral flow assay (LFA) for cryptococcal antigen (CrAg) has dramatically improved prevention efforts and diagnosis of this lethal infection.

Diagnostics for cryptococcal meningitis

Diagnosis of cryptococcal meningitis requires CSF culture, India ink or CrAg testing.

Culture

CSF culture is considered the gold standard for diagnosis of cryptococcal meningitis. Unfortunately, diagnosis can take days, up to 1-2 weeks for definitive results. Thus, other diagnostic methods have been used to expedite diagnosis and treatment. In research settings, quantitative CSF cultures have been utilized which provide valuable clinical information.(5) These CSF quantitative cultures are easily performed in any microbiology laboratory. The simple technique uses 100 µL input volume of CSF with five 1:10 serial dilutions in water, plating on Sabouraud dextrose agar, and then quantitative culture counting on the plate with the least growth.(5) Increasing quantitative culture burden is a risk factor for 2-week mortality with a ~40% increase in odds of mortality per $\log_{10}$ CFU/mL CSF increase in *Cryptococcus* growth.(6) The change in quantitative culture growth with subsequent serial lumbar punctures gives clinicians valuable feedback on the rate of CSF *Cryptococcus* clearance, and when the CSF is likely to be sterile.

India ink

India ink microscopy has historically been a quick, low-resource method to detect *Cryptococcus* in the CSF.(7) The stain fills the background field, but is not taken up by the thick *Cryptococcus* capsule, forming a halo of light by which it can be visualized using a light microscope. While simple, and readily accessible in resource-limited settings, where the burden of cryptococcal infection is the greatest, unfortunately sensitivity is low, at 86% in expert hands, meaning 1 in 7 diagnoses are missed by India ink microscopy.(8) For persons presenting early in disease process with lower burden of infections, India ink's sensitivity is only 42% when the CSF *Cryptococcus* colony forming units (CFUs) <1000 per mL of CSF.(8) CSF centrifugation can likely increase the sensitivity of microscopy; however, microscopy is less sensitive than testing for CrAg.(8)

Cryptococcal Antigen

CrAg can be identified using latex agglutination, which historically has a sensitivity and specificity of >99% in blood and CSF.(8) More recent comparisons have reported sensitivities of 97-98% and specificities of 86-100% dependent on the specific manufacturer.(8) Results can be qualitative, or semi-quantitative with titers by 1:2 serial dilution. The latex agglutination detects polysaccharide antigens of the *Cryptococcus* capsule, but the process of performing the test requires testing in a laboratory environment, thus skilled laboratory workers, steady electricity, heat inactivation, cold-chain shipping, and refrigeration of reagents.(9) While feasible in high-income country laboratories, these requirements are frequently prohibitive where the majority of cryptococcal infection occurs. The major expense of the test in high-income country is laboratory labor. In low-income countries, the major expense is cold chain shipping and storage. Thus, clinicians historically have relied on India ink for diagnosis in low-income countries, despite the lower sensitivity. Overall, CrAg latex agglutination is now an archaic test as latex agglutination is more expensive, more labor intensive, less sensitive, less specific, and requires cold-chain shipping/storage. (8)

The CrAg lateral flow assay (LFA), approved by the US Food and Drug Administration (FDA) in 2011 (Immy, Norman, OK), is an immunochromatographic dipstick assay that also detects antigen with qualitative or semi-quantitative results.(9) If CrAg is present in the drop of serum, plasma, or CSF sample, it will bind to the gold-conjugated, anti-cryptococcal antibodies on the test strip to cause a visible line. This FDA approved point-of-care dipstick test has changed the diagnostic landscape of cryptococcal meningitis, as it does not require laboratory infrastructure. Semi-skilled healthcare workers without laboratory training can perform this test in clinics, or at the patient bedside. No refrigeration is required, and results are available after 10 minutes. Conversely, laboratory based CrAg testing using the latex agglutination takes approximately 5 hours from test ordering to availability of results in high-income settings.(8)

In a large validation study in South Africa and Uganda, 832 HIV-infected persons underwent diagnostic testing for cryptococcal meningitis. The CrAg LFA performed best, with a sensitivity of 99.3% and specificity of 99.1% for CSF (**Figure 1**). CrAg testing by either latex agglutination or LFA was more sensitive than CSF culture, which has historically been considered the gold standard for diagnosis. Importantly the LFA identified 6 additional persons with cryptococcal meningitis that were not detected by any other means.(8)

In 2018, there are five manufacturers of CrAg LFAs. The first CrAg LFA by Immy is FDA-approved, (European Conformity) CE-marked in Europe, and has been used among hundreds of thousands of persons globally with large multi-site validation studies. Three other CrAg LFAs have more limited validation data and none are FDA-approved. Second is the Biosynex CryptoPS (Biosynex, Paris, France), which is CE-marked in Europe. This CryptoPS is also marketed by Bio-Rad (Hercules, California, USA). To date, a single site validation study has been performed testing 186 serum/plasma and 23 CSF samples from Cameroon.(10) In comparison to the FDA-approved Immy CrAg LFA, the CryptoPS LFA had 78% (11/14) sensitivity in serum, 92% (11/12) in plasma, and 100% (4 of 4) in CSF.(10) Specificity was excellent (100%) in all sample types.(10) In the initial validation study, among serum specimens positive by Immy CrAg LFA with Immy titers <1:100, only 2 of 5 were positive by Biosynex CrAg, missing titers positive at 1:10 and 1:20 dilution using the Immy LFA.(10) More validation is needed among serum specimens with low titers and among CSF specimens.

A third test is the StrongStep CrAg (Liming Bio, China). In a two-site validation study in Uganda, 143 CSF and 167 plasma samples were tested in comparison to the Immy CrAg LFA and CSF culture.(11) The StrongStep performed well in CSF with 100% (101/101) sensitivity and 98% (41/42) specificity.(11) However, the specificity was only 90% (101/112) in plasma.(11), with 98% sensitivity (54/55). The limited specificity of plasma in the setting of CrAg screening, and 9% CrAg prevalence rate equates to a positive predictive value of only 50%.(11) This test appears to be highly sensitive; however, there are substantial challenges with specificity.(11)

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Two other manufactured tests exist. The Dynamiker CrAg LFA and FungiXpert Cryptococcal Capsular Polysaccharide K-Set are manufactured in China. No published validation studies exist for either test, although Dynamiker has clinical validation studies ongoing in 2018. Neither test is approved in Europe or the United States.

CrAg testing in the blood to detect meningitis

Management of cryptococcal meningitis is complex and resource-intensive. Specifically, persons with cryptococcal meningitis frequently have elevated intracranial pressure, caused by the large polysaccharide capsule of the cryptococcal organism plugging the arachnoid villa, and obstructing CSF outflow.(12) This elevated intracranial pressure is associated with increased mortality.(13) Therapeutic lumbar punctures are therefore needed to release this pressure and reduce mortality in persons with cryptococcal meningitis.(14, 15) In resource-limited settings, where access to lumbar punctures is difficult, combining the diagnostic lumbar puncture with the first therapeutic lumbar puncture would streamline care.(16) Thus, using peripheral blood for a rapid point-of-care diagnosis of cryptococcal meningitis, allows clinicians to remove large amounts of CSF and reduce intracranial pressure with the first lumbar puncture, as well as confirming the diagnosis.

Performance of the CrAg LFA as a fingerstick point-of-care test has been evaluated in this context.(16) Specifically, for those with suspected meningitis, a point-of-care fingerstick CrAg LFA was performed prior to lumbar puncture and compared to subsequent plasma and CSF CrAg LFA. The positive predictive value of fingerstick LFA for the detection of CrAg in the blood was 100%, and 93% for cryptococcal meningitis (**Figure 2**). Those (7%) that had a positive fingerstick but negative CSF CrAg were found to have serum/plasma CrAg positive; thus these weren't false positives, but actual cryptococcal infection the blood. Fingerstick had 100% concordance with serum or plasma CrAg results, and 100% negative predictive value for excluding cryptococcal meningitis. Fingerstick CrAg testing does have limitations in asymptomatic populations with low fungal burdens, where false negatives can occur in comparison to serum or plasma testing.(17) Pipetting fingerstick whole blood onto the LFA improves diagnostic performance over direct application of blood to the CrAg LFA sample pad.(17)

Other Diagnostics

Other available diagnostics in high-income country settings include PCR. The FilmArray® Meningitis/Encephalitis panel (Biofire, Salt Lake City, Utah) is a multiplex PCR assay that detects 14 meningitis-causing pathogens (bacteria, viruses, and fungi), including *Cryptococcus*. FilmArray PCR detected *Cryptococcus* in 96% (95%CI, 83%-99%) of CSF when there were >100 *Cryptococcus* colony forming units per mL of CSF, and specificity was 100%.(18) The expense of multiplex-PCR does not make this an ideal cryptococcal-assay, but the multiplex component does make this very nice as an overall meningitis assay for common

causes of community-acquired meningitis. As well, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) has also been reported to detect *Cryptococcus* in clinical specimens.(19)

CrAg-based screening for cryptococcal meningitis

Early Cryptococcal Meningitis

The majority of persons with cryptococcal meningitis present with signs and symptoms of meningitis (headache, neck stiffness, fever), and are found to have positive CSF CrAg with diagnostic lumbar punctures and peripheral blood CrAg through fingerstick or venipuncture. However, there is a population with symptoms of meningitis, who are negative for CSF CrAg and negative by CSF culture but CrAg positive in the blood.(20) In one cohort in Uganda, this represented 4.3% of those HIV-infected with suspected meningitis. This population (blood CrAg positive, symptoms of meningitis, but CSF CrAg negative) was presumed to have early cryptococcal CNS infection, and in-hospital mortality was 39%, which was similar to in-hospital mortality of those with fulminant cryptococcal meningitis (32%). Larger studies are needed to further characterize the natural history of this population.

Cryptococcal Antigen Screening

CrAg is detectable in blood weeks to months before onset of meningitis symptoms.(21) Prevalence of asymptomatic cryptococcal antigenemia varies from 1% to 15% among HIV-infected persons with advanced HIV disease.(1, 22) In high-income countries the average prevalence of asymptomatic CrAg is 2.6%.(1) Asymptomatic CrAg positivity is an independent predictor of meningitis and death.(21, 23) Preemptively treating those with cryptococcal antigenemia with high dose fluconazole before symptoms of meningitis develop prevents mortality. This has been evaluated most rigorously in a randomized controlled trial of 2000 persons with advanced HIV disease in sub Saharan Africa, that demonstrated a 28% survival benefit with CrAg screening and preemptive treatment, alongside adherence support (24). Given this clear survival benefit, the World Health Organization and numerous national HIV guidelines now recommend CrAg screening those with advanced HIV disease, and preemptively treating those CrAg+ with high dose fluconazole (25).

CrAg Titer

Both latex agglutination and CrAg LFA can be semi-quantified using titers. CrAg LFA titers are performing by the same serial dilution, and the titer is the last positive test before the dilution turns negative. Titers across LFA manufacturers are not comparable, and even the titer between Immy latex-agglutination and Immy LFA; the median difference was 2.5-fold (IQR, 1.25 to 5-fold) higher with the CrAg LFA.(8) This difference in titer confirms the better

analytical sensitivity of the CrAg LFA over latex agglutination, but the titer difference is variable.

CrAg titer is predictive of meningitis and death.(26-29) Plasma CrAg titers ≤1:80 by Immy CrAg have an exceedingly low probability of meningitis. (26, 27) As serum or plasma CrAg titers rise from 1:160 to 1:320 to 1:640, the probability of CSF involvement becomes increasingly probable.(27) CrAg titers of ≥1:1280 have near universal central nervous system (CNS) involvement.(26, 27) The Biosynex CrAg LFA provides a semi-quantification with both a low and high titer test lines. The high CrAg titer band equates to approximately 1:1024 Immy CrAg LFA titer.

There have been four published cohorts of asymptomatic CrAg+ persons investigating CrAg titer versus outcome, each relatively small.(26-29) We combined these cohorts to summarize the effect of CrAg titer on survival when preemptive fluconazole monotherapy is given to CrAg-positive persons. Of 415 records, 287 had plasma CrAg titers measured at time of starting fluconazole. Survival was measured for those with low CrAg titers (<1:160), medium titers (1:160 to 1:2560), and high titers (>1:2560). Survival decreased as the plasma CrAg titer increased (Log-rank P<0.0001) (**Figure 3**). Among asymptomatic CrAg+ persons, CrAg titers ≥1:160 are associated with increased mortality despite receiving fluconazole preemptive therapy.(28, 29)

How to screen for CrAg

There are two possible methods for implementing CrAg screening into laboratories. The first is reflexive laboratory testing. With this method, those with a CD4 cell count result ≤100 cells/μL would routinely have a CrAg performed on the remaining plasma specimen from the CD4 test. Thus, the ordering provider would not be responsible for initiating this test, but testing would occur via a laboratory protocol. The result would be presented with the CD4 lab test result with a brief explanation of what to do if the CrAg result is positive.

The alternative is to depend on healthcare providers to order a CrAg test when a CD4 lab value returns ≤100 cells/μL. While a seemingly simple task, in settings that are already overburdened and understaffed, asking providers to remember to order this lab test has proved challenging. In one South African evaluation, only 27% of eligible persons were CrAg screened using a provider-initiated approach (30). Conversely, with reflex laboratory testing >95% of eligible persons were screened (30). Additionally, by having a provider order the test, there is a delay in testing. A new CrAg positive result in an untreated patient is a critical laboratory result, requiring urgent action to prevent progression to meningitis and death.

The difficulties with laboratory based screening are that there is often a delay between receipt of the lab result and bringing the patient back to the clinic for results and potential treatment. Conversely with provider initiated screening, there is less uptake of initial screening, but if done in the clinic room with the patient, the results are potentially available in 10 minutes,

and the patient can initiate therapy immediately, if needed. In the United States, CrAg testing is not a U.S. Clinical Laboratory Improvement Amendments (CLIA)-waived test, so provider point-of-care testing would not be allowable. In most high prevalence settings where CrAg screening occurs, a reflexive laboratory-based approach has been adopted. South Africa performs reflexive CrAg screening at national laboratories where CD4 testing is performed.

Future Implications & Conclusions

Given the importance of CrAg titer in predicting meningitis and/or death, CrAg titer will likely be used in the future to customize therapy both for prevention and treatment of cryptococcal meningitis. For example, if someone is found to be asymptomatic with a low CrAg titer (<1:160), they could be treated with fluconazole preemptive therapy, per current standard of care. However, given the high mortality despite high dose fluconazole in asymptomatic persons with a high CrAg titer, more intensive therapies should be evaluated to improve survival. Such therapies may include liposomal amphotericin and/or flucytosine. It is also possible that in those with fulminant meningitis, those with high titers may benefit from longer duration of therapy compared to those with low titers. Thus, titer will likely play a significant role in the management of cryptococcal infection, both in low-income areas and in the high-income settings. Infectious Diseases Society of America (IDSA) guidelines currently recommend 4 weeks of amphotericin for those with cryptococcal meningitis without HIV infection. This recommendation is based on no empiric data. Evaluation of how to shorten duration of therapy based on burden of infection (i.e. titer or quantitative culture) would be novel, and would spare patients exposure to toxic antifungal therapy.

In the last 20 years, cryptococcal testing has progressed from culture, which takes days for results, thereby clinically unhelpful with initial diagnosis, to India Ink, which is technically easy and quick, but with poor sensitivity, to a highly sensitive and specific point-of-care lateral flow assay that can be done at the patient bedside for rapid diagnosis. This evolution has greatly improved meningitis diagnosis and expedited initiation of effective treatment. Furthermore, the cost of $2.50 to $3.00 for the Immy CrAg LFA in resource-limited settings has made screening for cryptococcal infection a cost-effective strategy to prevent meningitis and death. (9, 31) CrAg titer predicts meningitis and death. Future areas of research include evaluation of customized therapy according to titer in persons with cryptococcal infection. The evolution of cryptococcal diagnostics highlights the enormous impact of point-of-care diagnostics in enhancing medical care and public health programs.

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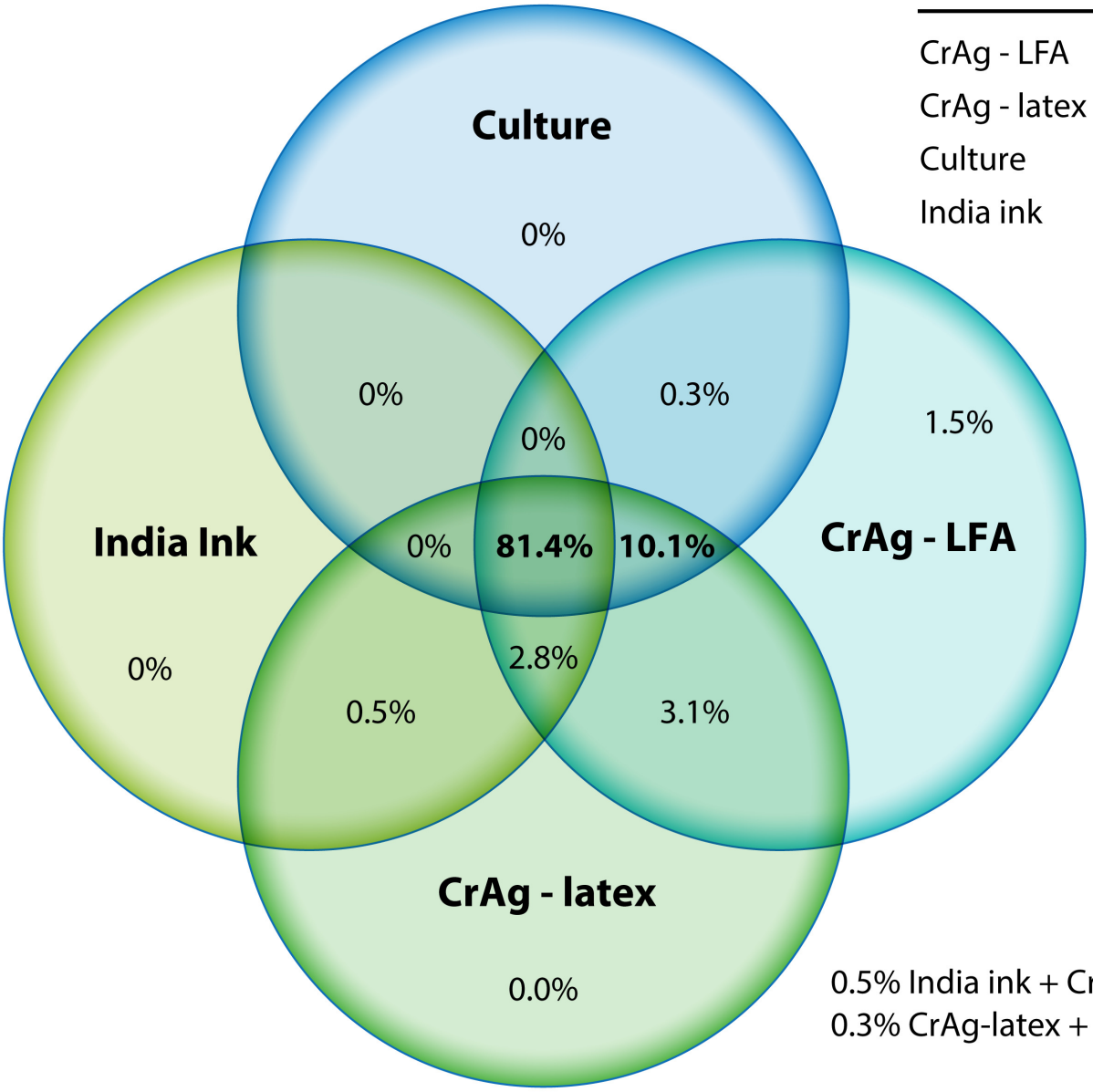
Figure 1: Venn Diagram of Distribution of CSF Diagnostic Testing in Uganda and South Africa during 2006-2012 (n=832).(8)

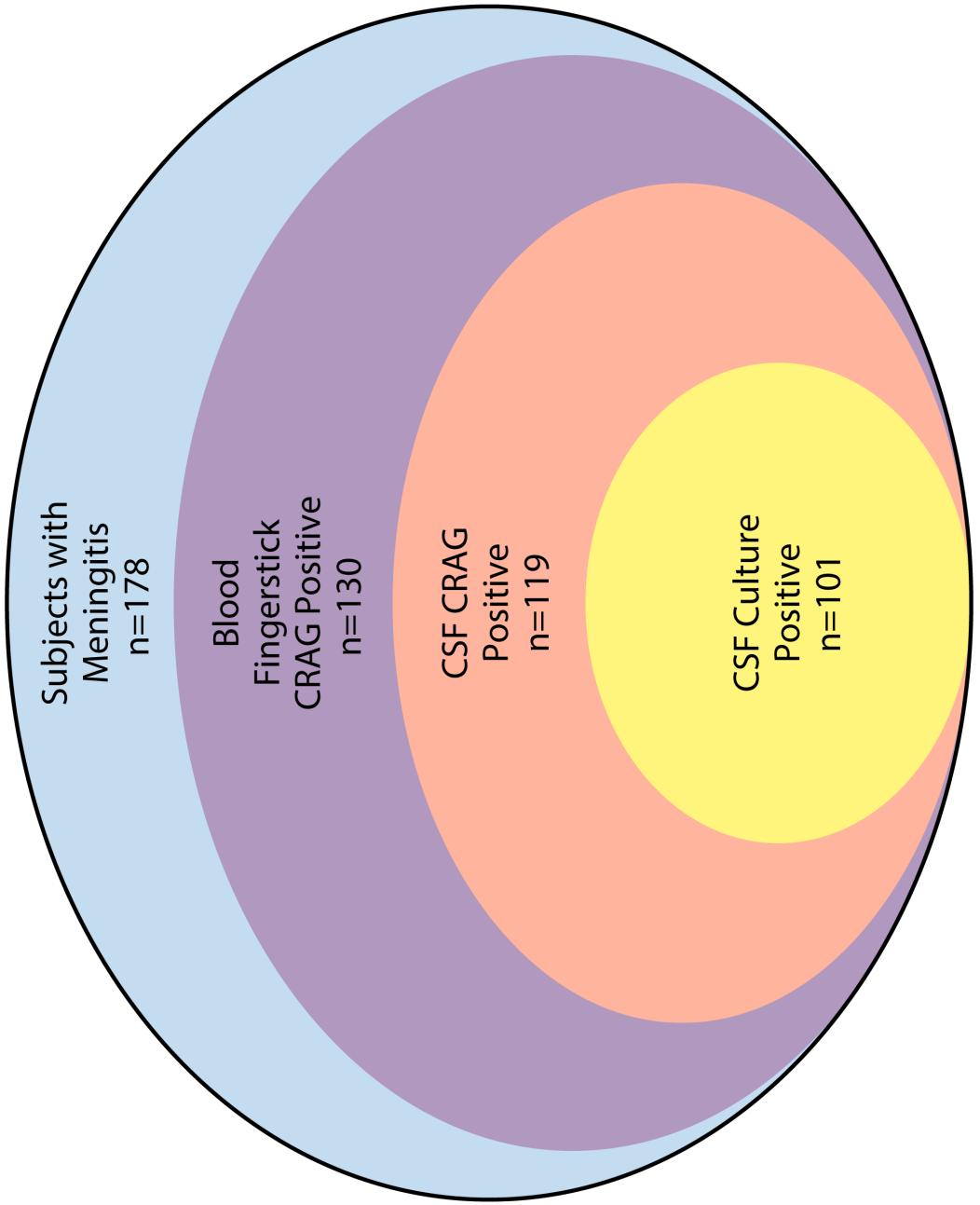
Figure 2. Venn Diagram of the Distribution of Positivity by Blood CrAg, CSF CrAg, and CSF Culture (16).

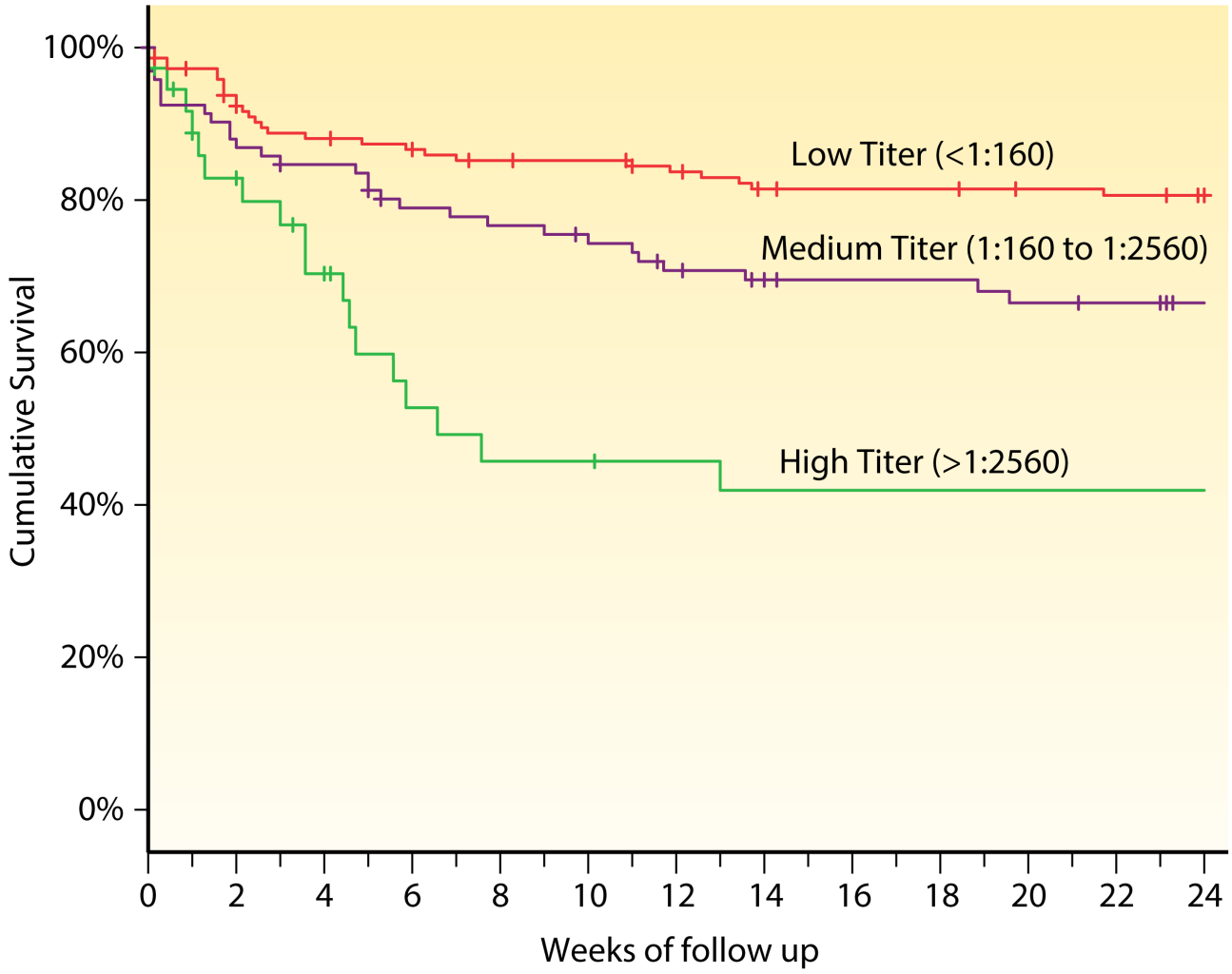
Figure 3. Survival by CrAg titer in 287 Asymptomatic HIV-infected Persons with Cryptococcal Antigenemia in four cohorts in Ethiopia, South Africa, Tanzania, and Uganda.(26, 27, 29, 32)



Sensitivity	
CrAg - LFA	99.3%
CrAg - latex	97.0%
Culture	94.2%
India ink	86.1%

0.5% India ink + CrAg LFA
0.3% CrAg-latex + Culture





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