Critical Reviews in Microbiology

ISSN: 1040-841X (Print) 1549-7828 (Online) Journal homepage: http://www.tandfonline.com/loi/imby20

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To cite this article: Michele W. Tang, Karl V. Clemons, David A. Katzenstein & David A. Stevens (2016) The cryptococcal antigen lateral flow assay: A point-of-care diagnostic at an opportune time, Critical Reviews in Microbiology, 42:4, 634-642, DOI: 10.3109/1040841X.2014.982509

To link to this article: http://dx.doi.org/10.3109/1040841X.2014.982509

Critical Reviews in Microbiology

http://informahealthcare.com/mby
ISSN: 1040-841X (print), 1549-7828 (electronic)

Crit Rev Microbiol, 2016; 42(4): 634–642
 DOI: 10.3109/1040841X.2014.982509

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REVIEW ARTICLE

The cryptococcal antigen lateral flow assay: A point-of-care diagnostic at an opportune time

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Abstract

Cryptococcal meningitis is a devastating HIV-related opportunistic infection, affecting nearly 1 million individuals and causing over 500 000 deaths each year. The burden of disease is greatest in sub-Saharan Africa and Southeast Asia, where cryptococcal disease is the most common cause of meningitis. Rapid, accurate and affordable diagnosis of cryptococcal disease has been lacking in many of the most heavily affected areas. Here, we review a point-of-care assay for cryptococcal disease, the dipstick-formatted cryptococcal antigen lateral flow assay (LFA) (IMMY, Norman, OK). In comparison to culture, the assay is 99.5% sensitive and 98% specific. In comparison to other commercially available tests for cryptococcal antigen, the LFA has equal or superior sensitivity and specificity in CSF, plasma and serum samples. We discuss potential applications for the use of the assay in resource-limited settings, including what is likely to be an important role of the LFA in screening for early cryptococcal infection before clinical disease and in evaluating pre-emptive treatment.

Keywords

Cryptococcosis, cryptococcal antigen, cryptococcal meningitis, fungal diagnosis, lateral flow assay

History

Received 13 July 2014
Revised 6 October 2014
Accepted 6 October 2014
Published online 23 January 2015

Epidemiology

Cryptococcal disease is a life-threatening fungal infection caused by species of the basidiomycetous fungus, *Cryptococcus*. The organism is found worldwide, and the primary pathogenic species are distinguished by capsular antigen serotypes; *Cryptococcus neoformans* var. *grubii* and var. *neoformans* (serotypes A and D, respectively), *C. gattii* (serotypes B and C), and a hybrid AD type. Globally, disease due to *C. neoformans* var. *grubii* is the most common cause of cryptococcosis, primarily among individuals with impaired cell-mediated immunity. Worldwide, patients with AIDS have a disproportionate burden of disease, though the incidence of cryptococcal disease has been rising in organ transplant patients and other cohorts in developed countries (Pyrgos et al., 2013). *Cryptococcus gattii* primarily affects immunocompetent individuals, but does cause infections in immunosuppressed patients. It is endemic within Australia and considered an emerging infectious disease in British Columbia, Canada and the Pacific Northwest region of the USA (Galanis et al., 2010; Harris et al., 2011).

Cryptococcal meningitis is a devastating HIV-related opportunistic infection, estimated to affect nearly a million individuals per year, with a disproportionate number of infections (ca. 720 000 individuals per year) in sub-Saharan Africa (Park et al., 2009). Cryptococcal meningitis has become the most common cause of meningitis in many countries where the prevalence of HIV is high. Although disease incidence and mortality have declined substantially with the advent of antiretroviral therapy (ART) in North America and Western Europe, the mortality rate is still high in many resource-limited settings. Mortality is estimated to range from 35% to 70% in sub-Saharan Africa, and has been as high as almost 90% in some studies (Lessells et al., 2011; Makadzange et al., 2010), compared to 12% mortality in the USA (Centers for Disease Control and Prevention, National Center for Zoonotic, 2010). Worldwide, it is estimated that 624 700 deaths occur from cryptococcal meningitis within 3 months of diagnosis, representing an early mortality greater than or equal to that caused by tuberculosis (Park et al., 2009). In resource-poor countries, cryptococcosis may account for 13–44% of deaths in AIDS patients (Kozel & Bauman, 2012).

Diagnosis of cryptococcal disease

A key strategy in reducing the morbidity and mortality from cryptococcal disease is early diagnosis and treatment. However, rapid, accurate and affordable methods for the diagnosis of cryptococcal disease are not widely available in many resource-limited settings. Culturing the organism is considered the gold-standard diagnostic method, but this requires a lumbar puncture, and even when performed, culture-based methods have poor sensitivity, require a large quantity of specimen, significant laboratory infrastructure and skilled technicians, and results often take several days to return. Direct observation by microscopy of the organism in

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CSF, the principal method in resource-poor countries, is a rapid diagnostic approach but also has poor sensitivity and relies on a skilled microscopist to identify the organism. Because of these difficulties in diagnosis, detection of the cryptococcal antigen (CrAg), a component of the organism's glucuronoxylomannan (GXM) polysaccharide capsule, in serum or CSF has become the mainstay of diagnosis. The two main methods of testing for CrAg have been by latex agglutination (LA) or enzyme immunoassay (EIA). Both assays are highly sensitive, specific and yield rapid results; LA is usually considered the gold standard (Babady et al., 2009).

Testing serial dilutions of serum results in CrAg titers, and high titers have value in predicting immune reconstitution inflammatory syndrome (IRIS; Sungkanuparph et al., 2009), the presence of meningitis (Jarvis et al., 2012), and poor prognosis (Diamond & Bennett, 1974; Zuger et al., 1986). EIA is still used in many laboratories as it can be performed with higher throughput, yields objective results, and allows for automation, while performance of the LA is manual and subjective. Despite these improvements over culture and microscopy, CrAg testing is not widely available in many resource-limited settings. Both LA and EIA also require significant laboratory infrastructure and technical expertise, frequently limiting availability of these tests to reference laboratories. We have, in addition, noted false negative results, prozone (negative results with undiluted or low dilutions of high antigen titer specimens) effects, and unclear endpoints with the LA test on serum, unless pronase treatment of the sera is done routinely prior to testing (Hamilton et al., 1991).

In 2009, a point-of-care test (POCT) for the detection of cryptococcal antigen was developed by IMMY (Norman, Oklahoma). The CrAg lateral flow assay (LFA) is a rapid diagnostic dipstick test that is stable at ambient temperature, does not require a laboratory, electricity, or skilled technician, and provides results in 10 min. The test, using two monoclonal antibodies, has been shown to identify all four GXM serotypes (A–D) with high sensitivity (Gates-Hollingsworth & Kozel, 2013). One-drop (ca. 40 μL) of bodily fluid (plasma, serum, whole blood, CSF or urine) is placed in a reservoir in contact with the LFA test strip. The specimen is absorbed up the test strip via wicking. If GXM is present in the specimen, it will be captured on gold-conjugated, anti-GXM monoclonal antibodies, forming a visible line. A control line ensures proper flow and reagent reactivity, and assures validity of the test. Thus, a positive specimen will yield two lines, one each at the test and control lines, while a negative test result specimen has only a single positive control line. A helpful, simple figure demonstrating how the test is to be performed, and illustrating what the strip shows in test conditions, can be found in Kozel & Bauman (2012, Figure 3), and that is reprinted in Vijayan et al. (2013). Similar detailed illustrations are included with the kit. Positive and negative control reagents are included with the kit. Prozone effects are rare. Icteric, hemolyzed or lipemic specimens do not interfere with the test. Cross-reactions from other fungal diseases are extremely rare. The test can be performed as a qualitative test, or as a quantitative test to determine titers using serial dilutions of the sample. Individual tests can be done using an Eppendorf microcentrifuge tube as a reservoir, and multiple tests can be run using 96-well plates. Some reagents are preserved in azide, necessitating use of gloves with the kit, and such materials cannot be flushed down a drain, as contact with lead or copper plumbing could lead to explosive mixtures. For valid results, specimens for testing should not undergo multiple freeze–thaw cycles.

The CrAg LFA was approved by the USA Food and Drug Administration in July 2011, and has also received a CE mark of approval for marketing in Europe. The World Health Organization (WHO) has added the CrAg LFA to the latex agglutination test as a preferred method for diagnosis of cryptococcal disease (WHO, 2011). Upon evaluation, the LFA has been found to satisfy the WHO ASSURED (Affordable, Sensitive, Specific, User-Friendly, Rapid, Equipment-free and Delivered to those who need it) criteria for a POCT. Here, we review the test characteristics of the CrAg LFA in comparison with other diagnostic modalities for cryptococcal antigen, and discuss potential applications for its use.

CrAg lateral flow assay validation studies

The utility of the CrAg LFA has been validated in a number of studies. The validation studies we discuss below are summarized in Tables 1 and 2.

Binnicker et al. tested 634 serum and 51 CSF samples using four assays: the Cryptococcal Antigen Latex Agglutination System (relies on a polyclonal antibody; CALAS, Meridian Biosciences, Cincinnati, OH), Premier EIA (relies on a polyclonal antibody; Meridian Biosciences), Alpha CrAg EIA (uses the same antibodies as in LFA; IMMY) and CrAg LFA (IMMY; Binnicker et al., 2012). There was an excellent correlation ($\kappa \geq 0.9$) between the LA test, the Alpha CrAg EIA and CrAg LFA test on the serum samples. There was 100% agreement between all four assays on the 51 CSF samples. When testing serum samples using LA as the gold standard, Premier EIA was 55.6% sensitive and 100% specific, Alpha CrAg EIA was 100% sensitive and 99.7% specific, and CrAg LFA was 100% sensitive and 99.8% specific. The authors posited that the lower sensitivity of the Premier EIA observed with the serum samples is likely due to the different capture antibodies used by the Meridian versus the IMMY assays. The IMMY assays recognize all four GXM serotypes, while the Meridian EIA assay and the LA assay have reduced sensitivity for serotype C GXM (Gates-Hollingsworth & Kozel, 2013; Percival et al., 2011). Serotype C infection, it should be noted, does occur in AIDS patients in Africa (Karstaedt et al., 2002; Litvintseva et al., 2005). The authors also noted that the CrAg LFA is amenable to high-volume throughput, i.e. 20 samples could be tested in 17 min with CrAg LFA, compared to 50 min by Premier EIA, 60 min by Alpha CrAg EIA and 70 min by LA.

Hansen et al. (2013) also compared the CrAg LFA with the Meridian Premier EIA and IMMY EIA, using 589 serum and 411 CSF samples. Qualitative agreement across all three assays was 97.7%. Twenty-three samples had discordant results; all 23 were negative by Meridian Premier EIA, 20 were positive by IMMY EIA or CrAg LFA, 2 were CrAg LFA positive only and 1 was IMMY EIA positive only. Ten of the

Table 1. Comparison of IMMY CrAg LFA to culture or composite reference standard.

References	Location	*N*	Specimen type	Test evaluated	Sensitivity (%)	Specificity (%)	Compared to	Comments
McMullan et al. (2012)	Sydney, Australia	106	Serum	IMMY LFA	100% (56/56)[a]	100% (50/50)	Culture	168 samples from 92 patients, 25 with cryptococcosis.
				Meridian LA	91.1% (51/56)	100% (50/50)		*Characteristics of 25 patients with confirmed cryptococcosis*:
		42	CSF	IMMY LFA	100% (9/9)	100% (31/31)		12/25 (48%) Immunocompetent.
				Meridian LA	100% (9/9)	100% (31/31)		4/25 (16%) HIV infected.
								9/25 (36%) Other immunocompromised.
		20	Urine	IMMY LFA	94.4% (17/18)	100% (2/2)		*Cryptococcus Disease Sites*:
								14/25 (56%) CNS.
								6/25 (24%) Pulmonary.
								5/25(20%) Other (fungemia, osteomyelitis, laryngeal disease).
								4/20 (16%) culture confirmed samples were *C. gattii*.
Lindsley et al. (2011)	Thailand	704	Serum	IMMY LFA	100% (17/17)	NA	Blood culture	HIV-infected patients in Thailand enrolled in pneumonia study
				Meridian EIA	94% (16/17)	NA		
		13	Urine	IMMY LFA	92% (12/13)	NA		
Boulware et al. (2014)	Uganda, South Africa	832	CSF	CSF Culture (100 µL)	94.2%	100%	Tested CSF culture, India Ink, CrAg LFA and CrAg LA. Used composite reference standard of CSF culture positive, or culture negative with ≥2 tests positive	HIV-infected patients with meningitis
				India Ink	86.1%	97.3%		
				Meridian LA/IMMY LA	97.8%/97%	85.9%/100%		
				IMMY LFA	99.3%	99.1%		
			Serum	IMMY and Meridian LA	98.3% (114/116)	N/A		
				IMMY LFA	99.6% (239/240)	92% (98/106)		
			Urine	IMMY LFA	97% (151/156)	85% (68/80)		
Kabanda et al. (2014)	Uganda	112	CSF	IMMY LFA	100%	100%	Tested CSF culture, India Ink, CrAg LFA and CrAg LA. Used composite reference standard of CSF culture positive, or culture negative with ≥2 tests positive	HIV-infected patients with meningitis
Jarvis et al. (2011b)	South Africa	62	Plasma	IMMY LFA	100% (62/62)	NA	Laboratory confirmed cryptococcal disease (CSF India Ink, Meridian CrAg EIA + with titers >1:1024, CSF culture +)	Paired blood and urine from HIV positive patients diagnosed with cryptococcal meningitis within the preceding 2 years.
		62	Serum	IMMY LFA	100% (62/62)	NA		
		62	Urine	IMMY LFA	98% (61/62)	NA		

NA = not available.
[a]Fraction on which the percentage is based. Sometimes not all specimens could be evaluated, because (e.g.) insufficient volume to perform all tests.

Table 2. Comparison of IMMY CrAg LFA to other CrAg tests.

References	Location	*N*	Specimen type	Test evaluated	% Positive agreement	% Negative agreement	K statistic	Compared to	Comments
Binnicker et al. (2012)	Minnesota, USA	633	Serum	IMMY LFA	100% (9/9)[a]	100% (624/624)	1.0	Consensus result (3/4 agreement with Meridian EIA, Meridian LA, IMMY EIA, IMMY LFA)	Used archived CSF and serum samples.
				Meridian EIA	55.6% (5/9)	100% (624/624)	0.71		
				IMMY EIA	100% (9/9)	99.8% (623/624)	0.95		
				Meridian LA	100% (9/9)	100% (624/624)	1.0		
		51	CSF	IMMY LFA	100% (18/18)	100% (31/31)	1.0	Consensus result (3/4 agreement with Meridian EIA, Meridian LA, IMMY EIA, IMMY LFA)	
				Meridian EIA	100% (18/18)	100% (31/31)	1.0		
				IMMY EIA	100% (18/18)	100% (31/31)	1.0		
				Meridian LA	100% (18/18)	100% (31/31)	1.0		
Hansen et al. (2013)	Utah, USA	589	Serum	IMMY LFA	100% (41/41)	96.2% (527/548)	0.78	Meridian EIA	(589 serum, 411 CSF). 23 specimens discordant; all Meridian EIA negative, 22 IMMY LFA positive
				IMMY EIA	100% (41/41)	96.5% (529/548)	0.80		
		411	CSF	IMMY LFA	100% (15/15)	99.7% (395/396)	0.97		
				IMMY EIA	100% (15/15)	99.5% (394/396)	0.94		
Escandon et al. (2013)	Colombia	421	Serum	CrAg LFA	100% (16/16)	96.9% (405/418)	0.70	CrAg LA	Tested the stored sera of HIV-infected patients; 13 samples were CrAg LFA positive, but were not latex agglutination positive.
Lindsley et al. (2011)	Thailand	704	Serum	CrAg LFA	95.6% (87/91)	99.5% (371/373)	0.923 (0.88–0.97)	Meridian EIA (serum)	All patients were HIV infected. Serum LFA read after 15 min, urine LFA results read after 5 min.
Magambo et al. (2014)	Tanzania	140	Urine	Serum CrAg LFA	100%	73.8%	0.28	IMMY LFA (old diluent)	HIV infected patients with CD4 <200 were screened for serum CrAg positivity by LFA. 7.1% (10/140) were positive, and considered ''gold standard'' for asymptomatic CrAg positivity. Urine LFAs were compared to serum LFAs.
			Urine	Serum CrAg LFA	80%	91.5%	0.51	IMMY LFA (new diluent)	

[a]Fraction on which the percentage is based. Sometimes not all specimens could be evaluated, because (e.g.) insufficient volume to perform all tests.

discordant samples had sufficient volume for capsular serotyping with a panel of anti-GXM monoclonal antibodies. Four of these samples were consistent with serotype C, while the remaining samples yielded indeterminate or inconclusive results. This again suggests a lower sensitivity of the Meridian Premier EIA for serotype C. Overall, this study estimated LFA to be seven times more sensitive than the Meridian EIA.

McMullan et al. (2012) tested 106 serum, 42 CSF and 20 urine samples from 92 patients with known or suspected cryptococcal disease from two university hospitals in Sydney, Australia and tested them by LA and LFA. Twenty-five of the 92 patients had confirmed cryptococcal disease, diagnosed by culture, histology or molecular methods. Of those who were diagnosed by culture, 80% had *C. neoformans* and 20% had *C. gattii* infection. On serum specimens, CrAg LFA was 100% sensitive compared to LA, which was 91.1% sensitive. Both tests were 92.9–100% specific. Both assays had 100% concordance on CSF samples, and 17 of 18 urine samples from patients with known cryptococcal disease were positive by CrAg LFA (94.4% sensitivity). CrAg LFA titers were generally higher than were LA titers, with a mean ratio of LFA:LA = 1.53, but confidence limits ranged from 0.13 to 18.1. This study was noteworthy because most patients studied were not HIV patients, and they included cases with more localized forms of cryptococcal disease and *C. gattii* cases, thus giving insight into LFA efficacy in other population of interest.

Validation studies from resource-limited settings show similarly promising results. Archived sera from 704 HIV-infected patients, hospitalized for acute respiratory illness in Thailand, were evaluated for infection with *Cryptococcus* (Lindsley et al., 2011). The LFA was tested at 5 and 15 min. Seventeen of these patients had blood cultures positive for *C. neoformans*. All 17 sera were positive by CrAg LFA (100% sensitivity), and 16 were positive by Meridian Premier EIA (94% sensitivity). The authors also screened all samples with the Meridian Premier EIA, and 92 (13.1%) were positive. Ninety-one of the EIA positive samples were available for further testing, and CrAg LFA was positive in 87 of 91 (95.6% sensitive), with a kappa agreement of 0.96 when the LFA was read after 15 min incubation time. Sensitivity and kappa agreement were lower at 90.1% and $\kappa = 0.923$ when 5 min incubation time was used. Urine LFA was 92% sensitive compared to blood cultures, and was 71% sensitive compared with EIA-positive serum samples and 81% compared with LFA-positive serum samples. After this study, the recommended incubation time for LFA testing was increased to 10 min, the recommended dilution was decreased and the amount of conjugate in the strip increased.

In a South African study, paired blood and urine samples were collected from 62 patients with active or recent cryptococcal meningitis (Jarvis et al., 2011b). All 62 patients had detectable GXM in serum, plasma and urine. However, GXM levels were 22-fold lower in urine than in serum or plasma. Serum or plasma could be used interchangeably, giving the same results. CrAg LFA was positive in 61 of 62 patients for serum, plasma and urine. One patient had plasma and serum samples that were indeterminate, and urine was negative. The authors determined that the sensitivity limit of the CrAg LFA was approximately 5 ng of GXM per mL. Even though urine concentrations of GXM were lower than serum concentrations, urine CrAg LFA was sensitive enough to diagnose almost all patients with cryptococcal meningitis. A very recent study (Magambo et al., 2014) also examined urine LFA for diagnosis, comparing the results to asymptomatic antigenemia, and compared a new diluent from the manufacturer for urine testing. They indicated the urine LFA had an unacceptably high rate of false positives, and the new diluent increased the specificity but decreased the sensitivity compared to the old.

Samples from 832 HIV-infected persons with suspected meningitis in Uganda and South Africa were evaluated with CSF culture, CrAg LA, India ink microscopy and CrAg LFA (Boulware et al., 2014). There was excellent concordance between LA and LFA results in both plasma and CSF samples. Using a composite reference standard of either CSF culture positivity, or >2 tests positive if CSF culture negative, CrAg LFA had the best performance with sensitivity 99.3% and specificity 99.1%. CSF culture sensitivity was 94.2% if 100 μL was cultured, but only 82.4% if 10 μL were cultured. CrAg latex agglutination was manufacturer-dependent, and sensitivity ranged from 97.0% to 97.8%, with specificity of 85.8–100%. India ink was the least sensitive, at 86%. CSF CrAg LFA titers were a median of 2.5-fold higher than CrAg LA titers, and 10-fold higher than LA in plasma. This result was noted to be more pronounced at the lower-titer range (<1:256). Others have estimated LFA to be ≥5-fold more sensitive than LA (Rajasingham et al., 2012).

A sub-study of the Boulware et al. study in Mbarara, Uganda evaluated the CSF of 112 subjects with suspected meningitis (Kabanda et al., 2014). Forty-seven (42%) had cryptococcal meningitis using a reference standard of ≥2 positive tests, performing CrAg LFA, CrAg LA, CSF culture and India Ink test. CrAg LFA was 100% sensitive and specific. In contrast, LA was 100% sensitive and 98.5% specific, CSF culture 95.7 and 100% and India ink microscopy 93.6 and 100%, respectively. LFA titers correlated with $\log_{10}$ CFU by culture. Higher LFA titers were also correlated with increased fungal burden by culture, and increased mortality. Serial antigen titers did not seem to correlate with clinical course.

The combined sub-Saharan study found the sensitivity, specificity, positive and negative predictive value of LFA in CSF to be 99.3, 99.1, 99.5 and 98.7%, respectively; CSF culture (if the minimum 100 μL volume was used) was 94.2, 100, 100 and 91.2%, respectively; microscopy 86.1, 97.3, 98.2 and 80.2%, respectively; Meridian LA 97.8, 85.9, 92.6 and 95.5%, respectively; and IMMY LA 97, 100, 100 and 95.3%, respectively (Boulware et al., 2014). The ''gold standard'' for these comparisons was a composite reference standard, employing CSF culture, CSF India ink assay and LA on CSF and serum. The LFA was particularly useful in patients studied earlier in their disease. The sensitivity of serum testing was 98.3% with LA and 99.6% with LFA. LFA titers were higher than LA titers by median 2.5-fold in CSF and 3.3-fold in serum. The urine LFA was 97% sensitive and 85% specific. A retrospective study in Colombia also suggested LFA to be more sensitive than their LA, with higher titers by LFA in more than half the samples (Escandon et al., 2013).

Pre-emptive screening and treatment for cryptococcal disease

Recent research and programmatic efforts have focused on the early detection and treatment of cryptococcal infection in HIV patients who have severe immunosuppression. The CrAg LFA plays an important role in the implementation of screening and treatment programs for subclinical cryptococcal disease because of its excellent test performance, ease-of-use and cost-effectiveness in comparison to earlier methods used to detect CrAg.

A study done in Uganda showed that cryptococcal antigenemia is detectable a median of 22 days before the onset of symptoms. Interestingly, it perhaps appears that in 11% of patients, CrAg may be detectable 100 days before development of symptomatic cryptococcal disease (French et al., 2002). Other evidence of long-term latent infection with *Cryptococcus* includes the high proportion of individuals with antibodies against the organism (Dromer et al., 1988), and a French study which found that the cryptococcal strains recovered from nine African immigrants were more similar to strains found in Africa than those found in Europe. The immigrants had been in France a median of 110 months, and had not been to Africa for as long as 13 years (Garcia-Hermoso et al., 1999). Although many patients with cryptococcal meningitis present while they are ART naïve, 20–30% present within the first 3 months after initiating ART (Jarvis et al., 2009; Lawn et al., 2006). Taken together, these data suggest an opportunity to screen for CrAg and to treat for cryptococcal disease in a high-risk population, prior to the development of symptoms and thus prevent or mitigate disease.

Among HIV+ individuals without central nervous system (CNS) signs or symptoms, and with CD4 $\leq$100 cells/mm^3, studies in South Africa, Kenya, Uganda, Cambodia and Thailand have identified detectable levels of CrAg in approximately 6–13% of patients (Jarvis et al., 2009; Liechty et al., 2007; Meyer et al., 2013; Micol et al., 2007; Pongsai et al., 2010). Most studies found low incidences of CrAg positivity (1–3.6%) of patients with CD4 cell-counts over 100 (Jarvis et al., 2009; Meya et al., 2010; Pongsai et al., 2010). However, a cross-sectional study of 369 HIV-infected, ART-treated and ART-naïve patients in Ethiopia found CrAg-positivity for 11% of patients with a CD4 cell count of <100 cells/mm^3, 8.9% of patients with CD4 between 100 and 150 cells/mm^3 and 5.7% with CD4 >150 cell/mm^3 (Alemu et al., 2013). None of the patients with a positive cryptococcal antigen test had a known history of cryptococcal disease. The increased prevalence of cryptococcal antigenemia in patients with higher CD4's may have been due to the inclusion of patients on ART. Eighty-four percent of those who were CrAg-positive were receiving ART, and likely had lower baseline CD4 cell-counts. Furthermore, the presence of increased numbers of patients with fever in the CrAg-positive group, Odds ratio (OR) 2.14 (95% CI 0.98–4.67), while not significant, suggests that some of the patients may have already been developing symptoms of cryptococcal immune reconstitution disease. The recent study by Magambo et al. also indicated that screening asymptomatic individuals initiating ART with CD4 counts between 100 and 200/mm^3 for antigenemia to be productive (6% positive), although there was a tendency for a single symptom such as headache or fever to be more common compared to the <100/mm^3 group, suggesting some may have been in early stages of cryptococcal meningitis.

Detection of cryptococcal antigenemia is predictive of developing symptomatic cryptococcal disease during the first year of antiretroviral therapy (ART), and an independent predictor of mortality (Liechty et al., 2007; Jarvis et al., 2009; Worodria et al., 2011). In South Africa, 6 of 21 (29%) patients who were CrAg-positive, and had no prior history of cryptococcal disease, developed symptomatic cryptococcal disease compared to none of the patients who were CrAg negative (Jarvis et al., 2009). Similarly, 4 of 12 (33%) patients with positive CrAg versus 1 of 119 (0.8%) with a negative CrAg, in a Thai study, developed meningitis within 1-year follow up (Pongsai et al., 2010). Development of symptomatic disease (particularly cryptococcal meningitis, CM) and mortality risk were highly correlated with CrAg titer in these studies.

A prospective study in Uganda analyzed CrAg positivity in a cohort of 609 individuals starting ART (Meya et al., 2010). Of 295 subjects with a CD4+ cell count $\leq$100 cells/mm^3 without a prior diagnosis of CM, 26 were CrAg positive (8.8%; 95% CI, 5.8–12.6%). Fluconazole therapy was provided to the patients at the discretion of the treating physician, with doses varying in the range of 200–400 mg for 2–4 weeks duration. Although ART alone may clear asymptomatic cryptococcal antigenemia in some patients (Jarvis et al., 2011a), five of these CrAg-positive patients received ART alone without fluconazole, and all died within 2 months of the initiation of ART (two patients died from CM). In a related study from Uganda, 10-week survival was 84% in patients with asymptomatic CrAg positivity when treatment with fluconazole was initiated, compared to 57% in patients treated for CM (Butler et al., 2012). The CrAg$^+$ asymptomatic cohort had an improved 5-year survival of 76% (95% CI: 59–93%) compared to 42% (95% CI: 35–50%) for the CM cohort ($p = 0.001$). Most mortality occurred early, with an excellent 5-year survival in patients who had survived 6 months (>88%).

Cost effectiveness of pre-emptive screening and treatment

A number of studies have examined the cost-effectiveness of pre-emptive serum CrAg screening and treatment, concluding that such strategies are likely to be cost-effective (Jarvis et al., 2011a; Meya et al., 2010; Micol et al., 2010). Primary prophylaxis with a prophylactic dose regimen of fluconazole without workup for CM has the risk of inadvertently starting inadequate therapy in asymptomatic CM, and many CM cases are asymptomatic in their early phase. Starting ART treatment without screening for subclinical cryptococcal infection and thus without use of fluconazole at that point in the infection appears to increase the risk of IRIS in those who then are developing CM.

Meya et al. (2010) performed a cost-effectiveness analysis based upon a 609-person cohort in Uganda. In those patients with a CD4 cell-count <100 cells/mm^3, the number needed to

test was 11.3 (95% CI, 7.9–17.1) to detect one CrAg-positive individual. To prevent one death, 15.9 people (95% CI, 11.1–24.0 people) would need to be screened and treated to increase survival for one person for 130 months on ART. The authors estimated the CrAg (EIA or LA) test at a cost of $16.75 USD per test, which encompassed reagents ($5.43 per test), and laboratory and personnel costs. The LA costs include the costs of cold-chain shipping and refrigeration, which appear disproportionately high in Africa. The financial calculation translated to $190 USD to detect one CrAg-positive individual and $266 USD to save a life. Hospitalization costs for cryptococcal meningitis were not included in the estimate, as the analysis assumed rapid early mortality with cryptococcal meningitis. Assuming an average increase in life expectancy of 12.5 years with ART in Africa, the authors calculated that this equated to $21 per disability-adjusted life year (DALY) saved. In Cambodia, it was estimated that screening would cost $180/life year gained (Micol et al., 2010).

Using the CrAg LFA the cost-effectiveness of pre-emptive screening and treatment is dramatically improved. For resource-limited settings the CrAg LFA is priced at $2–4 USD per test. There are also significantly reduced laboratory personnel and infrastructure costs, for a total cost of $2.50–4.50 USD per test. Using the results from Meya et al., the cost of detecting one individual with asymptomatic CrAg-positivity would then decrease from $190 to as little as $28.37 USD, the cost of saving a life with screening and pre-emptive fluconazole would decrease from $266 to <$40 USD, and cost per DALY saved would decrease from $21 to $2–4 USD (Rajasingham et al., 2012). In Tanzania (Magambo et al., 2014), using an overall frequency of 7% antigenemia in asymptomatic (<2 symptoms suggestive of cryptococcal meningitis) ART candidates with <200 CD4 cells/mm^3, screening 67 such persons with LFA ($134–268) would correspond to one death from cryptococcal meningitis that potentially could be avoided. All these cost calculations meet WHO Commission on Macroeconomics and Health criteria for a very cost-effective intervention. For a resource-poor healthcare system, screening and pre-emptive fluconazole would appear cost effective if the prevalence of cryptococcal infection was $\geq$1%. In the US, where the prevalence of cryptococcal antigenemia is lower than sub-Saharan Africa, the costs of treating CM are also very much higher. This has led some to suggest that screening of patients with CD4 counts <100 would be cost-saving if the prevalence in the US is >0.1% (Rajasingham & Boulware, 2012). A recent multicenter study of 1872 stored serum samples from the US found an overall prevalence of 2.9% CrAg positivity from HIV-positive patients with CD4 <100 cells/mm^3, suggesting that screening may be warranted in the United States (McKenney et al., 2014).

Applications and future directions for CrAg LFA

In 2011, the WHO issued Rapid Advice on the Diagnosis, Prevention and Management of Cryptococcal Disease in HIV-Infected Adults, Adolescents and Children (WHO, 2011). In HIV-infected patients suspected of having possible cryptococcal meningitis, rapid diagnosis with either a serum or plasma CrAg LA or LFA is recommended, in addition to a lumbar puncture (if not contraindicated). A positive CrAg from serum or plasma also allows clinicians to immediately initiate therapy and risk-stratify those patients in need of a therapeutic high-volume lumbar puncture for management of increased intracranial pressure; increased intracranial pressure is one of the main drivers of mortality in CM patients (Denning et al., 1991).

Diagnosis of cryptococcal infection using urine or whole blood specimens is a true POCT, accessible in rural areas without access to a laboratory to analyze plasma or serum. Urine or whole blood show a great deal of promise, but are still undergoing evaluation and are not yet FDA-approved. Questions with urine include the best time of day for sampling, whether concentrating the urine can improve sensitivity, and whether false positivity can be reduced. A recent study indicated LFA testing on saliva is sensitive (88%) in patients who are symptomatic with suspected cryptococcal disease, but insensitive (27%, though 100% specific) in asymptomatic AIDS patients (diagnosed via blood cryptococcal antigen; Kwizera et al., 2014).

The WHO conditionally recommends routine serum or plasma CrAg screening in ART-naïve adults, where the CD4 cell count is <100 cells/mm^3 and where the population has a high prevalence of cryptococcal antigenemia (>3%; WHO, 2011). Both LA and LFA are recommended for screening, however, LFA has several advantages over LA in implementation of a screening program in both urban and rural areas. Multiple samples can be run simultaneously with LFA (such as in a reference laboratory), whereas this is more difficult with LA. CrAg EIA could also be used in a reference laboratory to run multiple specimens, but EIA was not included in the guidelines due to its higher cost and increased laboratory requirements. In rural or extremely resource-limited settings, CrAg LFA has, as mentioned, the advantage of not requiring electricity, laboratory infrastructure or highly skilled labor.

There still remain many operational questions about optimal CrAg screening and treatment strategies. These include the optimal method of screening; for example, as a true POCT in the field, versus as a reflex laboratory test performed on all samples with CD4 cell counts <100 cells/mm^3. The development of point of care CD4 testing for HIV infected individuals in rural resource-limited settings presents the possibility of reflex testing of those with low CD4 counts. Appropriate diagnostic workup, and treatment for those who test CrAg-positive but do not have symptoms of cryptococcal meningitis is still not well defined. Fluconazole at 200–400 mg for 2–4 weeks decreased mortality in Uganda, but 6 of 21 (29%) patients receiving fluconazole (5 of 6 without ART) still died. Various fluconazole regimens have been proposed. A therapeutic regimen recommended by the WHO is fluconazole 800 mg/day for 2 weeks, followed by 400 mg/day for 8 weeks, then maintenance with fluconazole 200 mg/day. This recommendation is conditional, with a low quality of supportive evidence, and more studies are needed. Fluconazole treatment may present problems owing to drug–drug interactions with protease inhibitors used for ART and antituberculous therapy.

The importance of operational considerations is highlighted by a programmatic study in Kenya where screening

and treatment for early cryptococcal disease in patients initiating ART with CD4 <100 cells/mm^3 has been implemented since 2009 (Meyer et al., 2013). Patients found to be CrAg positive by LA in a reference laboratory were referred for treatment with fluconazole at 1200 mg/day for 2 weeks, followed by 800 mg/day for 8 weeks, followed by 200 mg/day as secondary prophylaxis. One thousand six hundred one (1601) HIV infected individuals with CD4 cell counts <100 cells/mm^3 were identified, and their outcomes compared to a historic control cohort. Overall mortality was 25% in both the intervention and the historic control cohort. Within the intervention group, individuals who were serum CrAg positive had significantly lower overall survival [HR 1.8 (95% CI: 1.2, 2.9); $p = 0.009$]. The authors point out several limitations, including a low (52%) overall uptake of the intervention. Only 66% of the individuals with CD4 cell counts <100 cells/mm^3 had a serum CrAg performed. Eleven percent were CrAg positive, and 86% of these individuals received fluconazole. The authors suggest that their results may be due to higher-than-expected mortality in the historical control group leading to an underpowered study, poor intervention uptake rates, or possibly poor efficacy rates of high dose fluconazole.

More data is sure to be published over the next few months and years, as a number of countries, including South Africa, are exploring operational issues and implementing widespread screening and treatment programs. The point-of-care CrAg LFA is likely to play a large role in implementation of such programs, particularly in settings where CrAg testing was previously unaffordable or in rural areas with little laboratory infrastructure. The availability of a test that does not require a laboratory, coupled with point of care CD4 testing, could significantly increase the fraction of patients at risk tested for CrAg.

Finally, the optimal timing of ART, and sequence of ART and antifungal therapy for cryptococcosis, has been studied, and different recommendations in that matter can be found. Concern has also been expressed that if the ''screen and treat asymptomatic positives with fluconazole'' strategy were adopted in the USA, where fluconazole monotherapy (although the standard in resource-poor countries) is not the recommended modality, as alluded to earlier, a number of patients with asymptomatic CM will be treated in what is regarded as a less than optimal manner. Requiring an LP in such asymptomatic antigenemic patients, before embarking on any therapy, would ameliorate that problem.

A recent development is the use of laser thermal contrast to accurately titer specimens (Boulware et al., 2014). The LFA positive test line, which contains gold, after laser radiation results in heat, and the intensity of the heat is measured. This approach would eliminate all subjectivity from the determination of the test endpoint and decrease the additional time and reagent costs associated with traditional titers, obtained by serial dilution.

Conclusion

The CrAg LFA has now been evaluated in multiple studies from resource-rich and resource limited settings. Sensitivity and specificity have consistently been excellent in comparison to other commercially available tests for cryptococcal antigen. In fact, the CrAg LFA has been shown in multiple studies to have superior sensitivity compared to the Premier EIA, LA, culture or microscopic examination. Its low cost ($2–4 USD per test for resource-limited countries), temperature stability and ease-of-use make it an ideal point-of-care test, meeting WHO ASSURED criteria. The CrAg LFA arrived at an opportune time, when clinics and hospitals in resource-limited settings are examining strategies, including lowered costs and accessible CD4 counts, for early diagnosis and treatment to reduce the unacceptable morbidity and mortality caused by cryptococcal disease.

Declaration of interest

Salary support was provided by the National Institutes of Health (NIH), USA (NIAID NRSA 5F32AI098584-02) to M. W. T. At the request of the investigators, IMMY has provided CrAg LFA kits to M. W. T. and D. A. K. for NIH-supported AIDS Clinical Trials Group studies, and to M. W. T., K. V. C. and D. A. S. for use in an experimental animal model of cryptococcal disease to study IRIS, funded by a Polin AIDS grant to the California Institute for Medical Research.

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