

Evaluation of a Newly Developed Lateral Flow Immunoassay for the Diagnosis of Cryptococcosis

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Background. Cryptococcosis is a common opportunistic infection of human immunodeficiency virus (HIV)–infected individuals mostly occurring in resource-limited countries. This study compares the performance of a recently developed lateral flow immunoassay (LFA) to blood culture and enzyme immunoassay (EIA) for the diagnosis of cryptococcosis.

Methods. Archived sera from 704 HIV-infected patients hospitalized for acute respiratory illness in Thailand were tested for cryptococcal antigenemia using EIA. All EIA-positive and a subset of EIA-negative sera were tested by LFA, with results recorded after 5 and 15 minutes incubation. Urine from patients with LFA- and EIA-positive sera was tested by LFA. Antigen results from patients with positive cryptococcal blood cultures were compared.

Results. Of 704 sera, 92 (13%) were positive by EIA; among the 91 EIA-positive sera tested by LFA, 82 (90%) and 87 (96%) were LFA positive when read after 5 and 15 minutes, respectively. Kappa agreement of EIA and LFA for sera was 0.923 after 5 minutes and 0.959 after 15 minutes, respectively. Two of 373 EIA-negative sera were LFA positive at both time points. Of 74 urine specimens from EIA-positive patients, 52 (70.3%) were LFA positive. EIA was positive in 16 of 17 sera from blood culture–positive patients (94% sensitivity), and all sera were positive by LFA (100% sensitivity).

Conclusions. A high level of agreement was shown between LFA and EIA testing of serum. The LFA is a rapid, easy-to-perform assay that does not require refrigeration, demonstrating its potential usefulness as a point-of-care assay for diagnosis of cryptococcosis in resource-limited countries.

Cryptococcosis, caused by *Cryptococcus* spp., is one of the most common opportunistic infections among human immunodeficiency virus (HIV)–infected individuals [1]. Globally, an estimated 1 million new cases of cryptococcal meningitis occur per year, with more than 600 000 deaths. An estimated 88% of global cases and more than

90% of deaths from cryptococcal meningitis occur in sub-Saharan Africa and Southeast Asia [2]. Primary respiratory illness due to *Cryptococcus*, while uncommon in the United States and Europe, is more common in other regions, such as Southeast Asia [2].

Currently, cryptococcal diagnostics include microscopy and/or culture-based methods, or detection of cryptococcal antigen (CrAg) in body fluids using either latex agglutination (LA) or enzyme immunoassay (EIA) methods. Although culturing of the organism is considered the gold standard diagnostic method, it has poor sensitivity, requires a large quantity of specimen, and requires laboratory infrastructure including electricity (for centrifugation). Antigen tests such as LA or EIA performed on cerebral spinal fluid or serum are highly sensitive and specific diagnostic options that are less labor- and time-intensive than culture [3, 4]. However,

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these methods require refrigeration, a cold chain for specimen transport, and technical expertise; therefore, they are often performed only in reference/diagnostic labs far removed from patients, potentially limiting their clinical utility. In addition, the costs of these tests are not affordable for many clinics. As a result, cryptococcosis often goes undiagnosed in resource-limited countries.

Point-of-care tests (POCTs) show promise for enabling diagnosis of infectious diseases in remote care centers in low-resource countries. POCTs are assays that can produce results quickly, are simple to perform and interpret by personnel with no or minimal laboratory training, and ideally can be used without cold chain or advanced laboratory equipment [5]. Today, reliable and affordable POCTs are available for the detection of hepatitis B infection [6], HIV infection [7], malaria [8, 9], syphilis [10], cholera [11], and some neglected tropical diseases [12–14]. Additional advantages of POCTs include reduction of patient anxiety and waiting time and decreases in patient loss to follow-up [15]. To serve the communities most in need, the World Health Organization (WHO) recommended that POCTs should be Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-free, and Delivered to those who need it (ASSURED) [16, 17].

In 2009, a lateral flow immunoassay (LFA) for the detection of cryptococcal antigen was developed by IMMY (Immuno-Mycologics) as a potential POCT for diagnosis of cryptococcal infection. The LFA is stable at room temperature, has a rapid turnaround time, requires very little technical skill, and can be performed with minimal laboratory infrastructure. In this study, the performance of the LFA for CrAg detection was evaluated by testing archived specimens from HIV-infected patients hospitalized with acute respiratory illness in Thailand, and comparing these results with culture and EIA.

METHODS

The serum and urine specimens used in this study were collected as part of a pneumonia etiology study that included HIV-infected patients in Thailand, described elsewhere [18]. In brief, patients admitted to 1 of 8 hospitals in Sa Kaeo or 12 hospitals in Nakhon Phanom provinces in Thailand between 2004 and 2009 were enrolled if they displayed any sign or symptom of active infection (temperature $>38.2^{\circ}\text{C}$ or $<35.5^{\circ}\text{C}$ within 24 hours of admission; chills; abnormal total white blood cell count or differential), and had evidence of lower respiratory illness (abnormal breath sounds, tachypnea, cough, sputum production, or dyspnea). All patients provided informed consent for sample collection and use of samples for research purposes. Serum and urine specimens were collected soon after hospital admission and immediately after study consent. This study was approved by the Institutional Review Board of the Centers for Disease

Control and Prevention and the Ethical Review Committee of the Thailand Ministry of Health.

All testing was performed at the National Institute of Health, Nonthaburi, Thailand. The results of the LFA were compared with those obtained from 2 commonly used diagnostic tests for detecting *Cryptococcus* infection, blood culture and EIA. Blood cultures were obtained from a subset of study participants as a part of routine clinical care. Cultures were processed by automated BacTAlert blood culture system (bioMérieux), and pathogen identification of isolates from positive blood cultures was performed using standard microbiology methods (morphological and physiological tests).

Stored sera from all HIV-infected patients enrolled in the study from 2004 to 2009 were tested by EIA, using the Premier Cryptococcal Antigen enzyme immunoassay (Meridian Biosciences). LFA testing was performed on serum specimens from all patients with a positive serum EIA test and a random subset of patients with a negative serum EIA test. Additionally, urine from patients whose sera were positive by LFA and EIA were tested by LFA. No LFA testing was performed on urine of patients who had serum that tested EIA-negative.

The EIA was performed according to the manufacturer's protocol using 50 μL of specimen. For the EIA, wells with reactions of optical densities <0.07 units were considered negative; ≥ 0.07 to <0.100 was considered indeterminate; and ≥ 0.100 was considered positive. The LFA is a semiquantitative test system for the detection of capsular polysaccharide antigens of *Cryptococcus* species complex (*Cryptococcus neoformans* and *Cryptococcus gattii*). The LFA kit consists of immunochromatic test strips impregnated with monoclonal antibodies optimized to detect all 4 cryptococcal serotypes and a diluent. The LFA kit can be stored at room temperature for up to a year. To perform the assay, 20 μL of patient specimen was mixed with 2 drops of diluent in a 2-mL screw cap microtube (Sarstedt AG). The LFA strip was placed in the specimen and diluent cocktail and incubated at room temperature. Results were read after two incubation times: 5 minutes according to the manufacturer's instructions and a prolonged incubation time of 15 minutes. The presence of 2 bands (control band and test band) in the test zone of the LFA strip was interpreted as a positive result and a single band in the test zone (control band) was interpreted as a negative result.

A positive blood culture result for *C. neoformans* was used as the gold standard for confirmed *Cryptococcus* infection. Sensitivity was calculated among the group of patients with a positive blood culture result as: (number of patients with positive serum LFA) / (number of patients with positive blood culture) * 100. Specificity was not calculated in this group, as blood culture was not considered to be a method with sufficiently low false negative rates. Agreement between serum or urine LFA results and serum EIA results was calculated using the κ statistic.

RESULTS

A total of 704 HIV-infected study participants with available specimens were identified and were tested as outlined in Figure 1. Blood culture was performed on 325 patient specimens, with 18 of 325 yielding *C. neoformans*. Serum was not available from 1 of the 18 culture-positive patients for analyses in the study; thus the EIA testing was performed on 17 culture-positive sera. Of the 17 serum specimens tested, 16 were positive by EIA and 17 were positive by LFA with a 5-minute incubation time yielding a sensitivity of 94% and 100%, respectively. Thirteen of the 18 patients whose blood culture was positive for *Cryptococcus* also had a corresponding urine specimen, 12 of which were positive by LFA (92% sensitivity).

Sera from all 704 patients were tested using EIA; 92 (13.1%) were positive. No sera tested in this study gave indeterminate results by EIA. Of 91 EIA-positive sera that were available for further analysis, 82 (90.1%) were positive by LFA with a 5-minute incubation time. Discordant results between EIA and LFA were most often observed in serum exhibiting lower EIA values (data not shown). A random subset of 373 serum samples was selected from the 612 EIA-negative sera and tested using LFA: 371 of 373 (99.5%) were LFA negative, and 2 (0.5%) were LFA positive. The resultant κ statistic describing agreement between LFA and EIA was 0.923 (95% CI, .877–.967).

Urine specimens were available from 74 patients whose sera were EIA positive. When these urine specimens were tested by

LFA using a 5-minute incubation, 52 (70.3%) were positive, and 22 (29.7%) were negative. Among urine specimens from 63 patients with LFA-positive sera, 51 (81.0%) were LFA positive, and 12 (19.1%) were LFA negative.

All 91 EIA-positive sera were also tested by LFA using an extended incubation time. When the results of the serum LFA were read after 15 minutes, 5 additional sera became positive ($n = 87$; 95.6%). Of these 5 sera, only 1 came from a patient with blood culture performed, which was negative for *Cryptococcus spp.* All EIA-negative specimens remained LFA negative after the extended incubation period, increasing the κ statistic to 0.959.

DISCUSSION

Resource-limited countries, especially those in sub-Saharan Africa and Southeast Asia, continue to experience a high incidence of cryptococcosis; managing this disease is a persistent public health challenge [2]. Although several CrAg tests are currently available for cryptococcal diagnosis, these tests are not readily accessible in resource-limited settings, resulting in no diagnosis or underdiagnosis of these often fatal infections. In the present study, a recently developed cryptococcal assay, the LFA, was evaluated for test sensitivity and agreement with other available diagnostic methods using archived serum and urine specimens from HIV-infected patients in Thailand. Results showed that, with serum specimens, the LFA was 100% sensitive when compared with the gold standard blood culture.

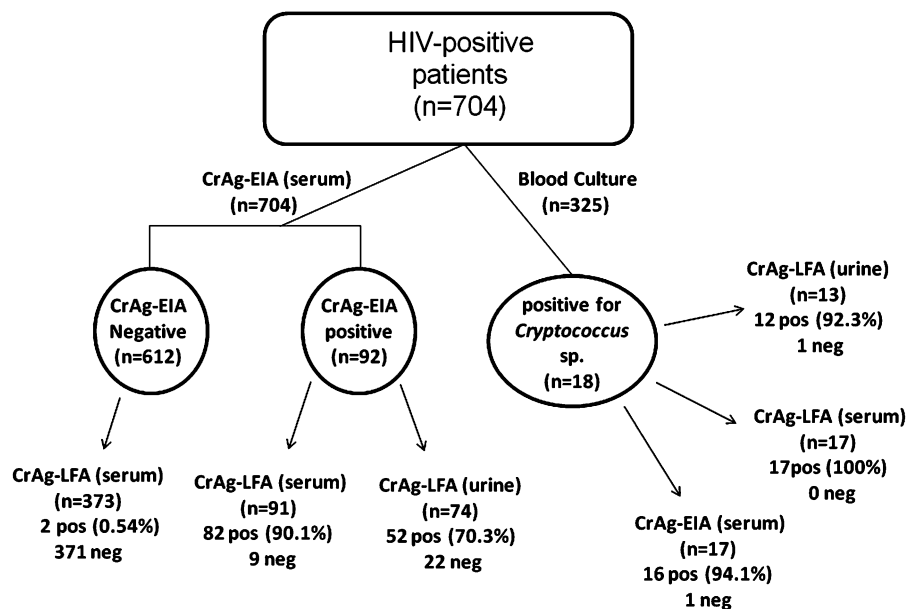


Figure 1. Flow chart of the specimen testing algorithm. A total of 704 sera were tested by cryptococcal antigen enzyme immunoassay (CrAg-EIA); 91 EIA-positive sera were tested by lateral flow immunoassay (LFA); a subset of CrAg-EIA–negative sera ($n = 373$) was further tested by LFA. Specimens from *Cryptococcus* blood culture–positive patients ($n = 17$) were tested by LFA and EIA. A total of 74 urine samples from serum CrAg-EIA–positive patients were tested by LFA. HIV, human immunodeficiency virus.

Additionally, the LFA had a high level of agreement with the cryptococcal EIA. When urine was evaluated, the LFA was found to be very sensitive (92%) when compared with blood culture, and moderately sensitive (70.7%) when compared with EIA-positive samples.

One of the limitations of this study was that the specimens were collected from patients hospitalized with acute respiratory illness for whom complete clinical details were not available, including whether they had meningitis. Patients with more invasive infections (ie, meningitis) may have a higher burden of circulating organisms and therefore antigen, and this may impact the performance of tests that measure CrAg. Accordingly, discrepant results between serum EIA and LFA were more often observed in serum with lower EIA optical density values, possibly reflecting the presence of low levels of circulating antigen.

This study was performed in a reference laboratory and therefore the performance of the LFA in a field or hospital setting is unclear at this time. However, the LFA was simple to perform and did not require any additional laboratory equipment. Incubation could be performed at room temperature and the assay itself could be accomplished in 3 easy steps since this method does not require any pretreatment of specimen (to remove rheumatoid factor). Finally, the LFA yielded results that were unambiguous. Thus, the LFA has many characteristics that may make it a valuable POCT in resource-limited areas. In addition, this study demonstrates that the CrAg LFA satisfies most of the WHO ASSURED criteria [16, 17]: specifically, the assay is sensitive, user-friendly (small specimen volume, simple to use), rapid (10–15 minutes to perform), and equipment-free (including no requirement for refrigeration). The rapid turnaround time will allow diagnoses to be potentially provided during patient visits, allowing treatment to begin immediately if warranted. Recently, Jarvis et al [19] strongly recommended the integration of CrAg screening into national antiretroviral treatment programs in sub-Saharan Africa to reduce the human and economic costs due to the disease [19]. Although not tested in this study, the LFA may also have utility as a screening tool for early diagnosis of cryptococcosis.

Extending the incubation time for sera from 5 to 15 minutes improved the sensitivity of the LFA when compared with EIA, thereby increasing agreement between the EIA and the LFA. Since the time of this study, the manufacturer of the LFA has modified the testing protocol, now recommending testing twice the volume of patient specimen, reducing the recommended specimen:diluent ratio from 1:5 to 1:2, increasing the amount of conjugate in the chromatographic strip, and increasing the recommended incubation time of the LFA to 10 minutes. Currently the LFA has CE marking (a mandatory conformance marking for the European Economic Area) for use in the European Union and in countries that use CE approval and has been

submitted to the United States Food and Drug Administration for approval (personal communication, Sean Bauman, IMMY).

For POCTs to have maximum value in remote settings, the use of minimally invasive, easily obtained, processing-free specimens is optimal. This study was performed with sera and urine as test specimens in a controlled laboratory setting (reference laboratory); however, in remote areas with insufficient technical expertise or specimen processing capabilities, sera may not be the ideal specimen type. The lower sensitivity of the LFA for urine compared with serum could be due to reduced excretion of CrAg into the urine, compared with the blood. HIV infection [20] and treatment of HIV with the antiretroviral drugs tenofovir and indinavir [21, 22] have been previously demonstrated to reduce the glomerular filtration rate and, potentially, reduce the amount of CrAg excreted in the urine. Urine has been used for antigen detection in other fungal [23–25] and nonfungal diseases [26, 27]. The kinetics of excretion of CrAg antigen in urine is unclear, and additional studies testing CrAg in urine need to be performed, including studies where urine is collected under controlled conditions.

Another minimally invasive specimen type is whole blood from a finger stick. Point-of-care assays for whole blood have been developed for viral and parasitic diseases [8, 9, 12, 13], all of which have been useful in resource-limited countries. Further studies evaluating the LFA using finger-stick blood would enhance the accessibility of this assay. Additionally the test's cost, ranging from \$1.25 to \$2.50 per test (depending on the country and volume of purchase) is based on the World Bank's list of economies, thereby ensuring affordability to the countries most in need.

In summary, this study demonstrates that the LFA is a sensitive test for *Cryptococcus* spp compared with the gold standard culture, and has a high level of agreement with EIA. Given the ease of use, temperature stability, minimal requirements for laboratory infrastructure, and potential low cost of this test, the LFA shows great promise as a POCT for diagnosis of cryptococcosis. The availability of this assay as a POCT for use in remote locations could have a meaningful impact on cryptococcal diagnosis.

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Potential conflicts of interest. All authors: No reported conflicts.

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