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Rapid diagnosis of cryptococcosis using an antigen detection immunochromatographic test

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Summary *Objectives:* Current methods for cryptococcal antigen detection have some limitations. This study aimed at evaluating a lateral flow assay (LFA) for the diagnosis of cryptococcosis in a French University medical center.
Methods: A retrospective study was performed on samples collected from patients with a definitive diagnosis of cryptococcosis (group I 66 samples; 28 patients) or with non-*Cryptococcus* invasive fungal infection (group II 18 samples; 17 patients). In addition, 274 samples from 205 consecutive patients, either suspected of cryptococcal infection or routinely

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screened during their follow-up, were prospectively tested (group III). Cryptococcal antigen was assayed using LFA and an EIA. A latex-based test was used for confirmation.
Results: Sensitivity calculated on group I and specificity on group II, were respectively at 100% and 90.0%. Two false positives were related to *Trichosporon* fungemia. Per-sample analysis on group III revealed sensitivity, specificity, positive and negative predictive values all at 100% for CSF, and at 100%, 98.9%, 75% and 100%, respectively for serum samples. LFA enabled the diagnosis of two cases of asymptomatic cryptococcosis.
Conclusion: The excellent diagnostic value and practicality (visual reading results in 15 min) of LFA make it fully appropriate for the diagnosis of cryptococcosis in this particular setting.

Introduction

Cryptococcosis is caused by basidiomycetous yeasts of the *Cryptococcus neoformans/Cryptococcus gattii* species complex. In most cases, cryptococcosis is responsible for life-threatening meningitis complicating the course of various states of immunosuppression such as HIV infection, unrevealed or uncontrolled by antiretroviral therapy, solid organ transplantation, chronic lymphoid leukemia, liver fibrosis, sarcoidosis, or any kind of affection requiring long-term corticosteroid therapy.[1] While the incidence of cryptococcosis has declined with the advent of Highly Active Anti-Retroviral Therapy (HAART), its outcome still remains poor, even in developed countries, with a mortality rate calculated in a large French survey at 17%, 3 months after the diagnosis.[2] As is the case for the majority of invasive fungal infections, the prognosis of cryptococcosis depends on an early diagnosis. The diagnosis of cryptococcosis combines direct diagnosis tests to visualize (Indian ink staining) and isolate (culture on appropriate medium) the pathogen from different fluids, and serodiagnostic assays (antigenic detection), mainly in serum and CSF. However, direct diagnosis can lack sensitivity in case of low fungal inoculum. Moreover, cultures supply a delayed result, since they usually take more than 72 h to be positive.[3] The detection of the capsular glucuronoxylomannan (GXM) antigen is thus an important complementary diagnostic tool. Antigen can be detected using immunoenzymatic (EIA) methods or agglutination of sensitized latex particles (LA) with good performances. However, there are some limitations of these tests that render them not fully suitable for rapid diagnosis, notably during night duty. Indeed, a preliminary 15-min centrifugation is mandatory for both EIA and LA tests. An additional time of 45 min (incubation of sample, enzyme conjugate, substrate and finally stop solution) is required to obtain results with EIA. For the latex-based test, an incubation step of the samples with pronase may be required in order to eliminate immune complexes, and reach an appropriate level of sensitivity.[4] Moreover, the test may be difficult to read, notably in the case of weak agglutination.

Recently, a new lateral flow assay (LFA) has been evaluated positively for the diagnosis of AIDS-related cryptococcal meningitis, mainly in resource-limited settings.[3,5,6] The purpose of this work was to evaluate this commercial test for the diagnosis of cryptococcosis in a French University hospital.

Material and methods

Samples

Samples were divided into three groups according to the patient's condition. Group I corresponded to 66 samples previously collected from 28 patients with a proven diagnosis of cryptococcosis: they either had positives cultures for *C. neoformans/C. gattii*, or two different, positive antigen detection assays, or a positive Indian ink staining plus detection of the antigen. All patients but two, were HIV-infected. There were 24 sera, 30 CSF, 7 urine samples and 5 BAL fluids. Group II gathered 18 samples (1 CSF and 17 sera) from 17 patients with proven or probable invasive fungal infection other than cryptococcosis. Those infections were due to *Trichosporon asahii* (2 patients; 2 sera, one CSF), *Histoplasma capsulatum* (2 patients; 2 sera), *Aspergillus fumigatus* (4 patients; 4 sera), *Pneumocystis jirovecii* (3 patients; 3 sera), *Candida albicans* (3 patients; 3 sera), *Rhodotorula* sp (3 patients; 3 sera). In addition, one serum and one BAL fluid, which results were considered as false positives, were included. They were collected from two patients for whom the diagnosis of cryptococcosis was finally ruled out since antigenic detection occurred only once and only with an Enzyme Immunoassay test.

Finally, group III corresponded to 90 CSF and 184 sera included prospectively and collected from 205 consecutive patients, who were either suspected of cryptococcosis (neurological symptoms) or screened systematically (routine follow-up of HIV-positive patients) for cryptococcal antigen. In the case of clinical symptoms or positive antigen detection, these patients were investigated in depth, mostly using imaging, blood-cultures, lumbar puncture, and broncho-alveolar lavage (BAL) subjected to Indian ink staining and culture.

Antigen detection

Antigen detection was performed using the EIA Cryptococcal antigen test (Premier Cryptococcal Antigen, Meridian, Bioscience, France) and a latex assay (Crypto-Ag LA, Fumouze, France) according to the manufacturers' recommendations. Both tests use 50-μl specimens per reaction. For the EIA test, reading was done using a spectrometric dual wavelength (450/630 nm). Optical density $\geq$0.1 was considered as positive, $<$0.07 as negative and indeterminate between both. For the latex-based test, serum (not

Table 1 Positive cryptococcal antigen detection according to the diagnostic method and the group of patients.

	Cryptococcal antigen detection method		
	LFA	EIA	LA
Group I: Proven cryptococcosis (n = 28 patients)			
Serum (n = 24)	24	24	24
CSF (n = 30)	30	30	30
Urine (n = 7)	7	7	7
BAL (n = 5)	5	4	5
Group II: Other probable or proven fungal infections and false positive samples[a] (n = 19 patients)			
Serum (n = 18)	2[b]	5[c]	3[d]
CSF (n = 1)	0	0	0
BAL (n = 1)	0	1	0
Group III: Prospective study (n = 205 patients)[e]			
Serum (n = 184)	12	8	10
CSF (n = 90)	2	2	2

[a] One serum and one BAL fluid specimen which results were finally considered as falsely positive.
[b] Two sera from patients with *T. asahii* infection.
[c] Two and one sera from patients with *T. asahii* and *Rhodotorula* sp infection, respectively; one false positive sera in a patient without fungal infection.
[d] Two and one sera from patients with *T. asahii* and *Rhodotorula* sp infection, respectively.
[e] Definitive diagnosis of cryptococcosis for 6 patients.

CSF) were treated volume to volume with the pronase solution provided by the manufacturer for 15 min at 56 °C. Any kind of agglutination using the first dilution (1/2) of specimen was considered as significant of positivity.

CrAg Lateral Flow Assay (Immy, Bioscience, France) relies on the incubation of an immunocoated strip (no information available on the origin of the antibody used) with 40 μl of sample, previously mixed with a drop of diluent. After 10-min of incubation at room temperature, the test is read. The test includes a positive control that results in the appearance of a band on the upper part of the strip. The sample is considered positive if a second band forms below the former. All the positive samples in groups I and II were tested a second time. Technical sheet given by the manufacturer only describes the use of the LFA test on serum or CSF but as others,[7] we tested urine and even BAL fluids specimens.

All the samples from group I and II were tested for cryptococcal antigen using EIA, LFA and the LA test. Samples from group III were tested using both the LFA and the EIA test. In case of positivity of one of those tests, a latex-based assay was performed.

Results

All the samples from group I (proven cryptococcosis, retrospective study), whether they were serum, CSF, urine or BAL, tested positive with the LFA test, resulting in a 100% (n = 66 positive/66 samples) sensitivity (Table 1). There was a single case of discrepancy with other antigenic tests: a BAL fluid sample that was found negative with the EIA test, but positive with LFA and LA (dilution of ¼ for the latter).

Sixteen out of the 18 samples collected from patients with other invasive fungal infections (group II) were negative with the LFA test. Interestingly, a serum drawn from a patient with a disseminated *Rhodotorula* infection was found positive with the EIA and LA tests while negative with the LFA. The two LFA-positive sera came from two HIV-negative patients infected with *T. asahii*, and also gave positive results with the EIA and the LA kits. The two additional samples included in this group and considered as false positive returned negative results with the LFA and LA tests. Specificity of LFA test on this group was thus calculated at 90.0% (2 positive/20 samples) [IC95 76.8–100%].

Among the 205 patients prospectively screened or suspected of cryptococcosis, 199 were considered free of cryptococcal infection and had a negative LFA test. Three new cases of cryptococcosis with positive culture for *C. neoformans* were diagnosed during the study period. All had a positive LFA (3 serum and two CSF samples) with LA titration ranging from ½ to 1/256. Another patient had a previous history of cryptococcosis, and two of his sera were found positive with the LFA and the EIA test with a confirmation by the LA test (titration at 1/512 in both cases). Two HIV-positive patients without any clinical symptoms were tested positive for cryptococcal antigen in serum with the LFA. LA titration was at ½ and 1/32, respectively. For both patients, similar results were obtained from control sera drawn one week and one month later, in one case and 9 days later in the second one. EIA test was negative for the two sera with LA titration at ½. For both patients, despite in-depth investigation (lumbar puncture, blood and urine culture), infection with *C. neoformans* could not be demonstrated neither by direct examination (including Indian ink staining) nor by culture. Because of their severe immunodeficiency (CD4 cell count at 12 and 29/mm^3, respectively), the patients were treated with fluconazole. Finally, two patients tested positive on serum with the LFA while EIA and LA tests returned negative and mycological cultures remained negative. Both patients had a control serum with a negative LFA test, 5 and 7

days later, respectively. These two serum samples were considered as true false positives. Per-sample analysis gave sensitivity, specificity, positive and negative predictive values calculated on the 205 patients studied, were all at 100% (2 positive/2 samples) for CSF, and at 100% (10 positive/10 samples), 98.9% (2 positive/262 samples) IC95 [97.3—100%], 75% and 100%, respectively for serum samples.

Discussion

Despite its declining incidence, cryptococcosis remains a serious complication of undiagnosed HIV infection or other kinds of immunosuppressive conditions in western countries.[1,2] Appropriate therapy must be started as soon as possible since, most often, death occurs within 10 days following diagnosis, stressing the need for an anticipated diagnosis.[8] Current diagnostic procedures, whether direct or indirect, have limitations. These are particularly obvious during night duty since they require both a particular expertise to avoid false positive and false negative results (Indian ink staining) or a prolonged preparation time (centrifugation and/or pronase incubation) to reach adequate sensitivity.[4]

This latter limitation is overcome with the LFA that can be directly performed on the native sample. Thus, results can be obtained in less than 15 min without the need of any particular expertise. Lindsley et al. suggested increasing incubation time from 10 to 15 min in order to enhance the sensitivity.[5] In our experience, all strips were easily interpreted after a 10-min incubation, even though variation in the intensity of the specific band was observed. A semi-quantitative use of the test is possible with serial dilutions of the sample,[6] and quantification using laser thermal contrast has been reported.[3]

Our study performed in a French University Centre confirms the sensitivity of the test whether testing sera or CSF. However, one should note that most of the patients, who tested positive in our study, were HIV-positive, thus additional studies may be required to extend the results to other kinds of populations at risk of cryptococcosis. Nevertheless, it was interesting to note that, even in the case of a very low concentration of antigen as judged on the titration performed with the latex assay, the LFA returned a positive result. Indeed, the LFA was found positive while the EIA test remained negative in 2 sera from a patient with low antigen titration. This may correspond to subclinical forms whose diagnosis may be anticipated with the most sensitive test.[9] Indeed, while progressive meningitis remains the main clinical presentation, the routine use of systematic screening for antigenic detection in serum has shown that subclinical, such as isolated low-grade of fever or even asymptomatic, forms are common,[10] and must be diagnosed to prevent their evolution towards more severe forms.

Specificity was calculated in previous studies to be between 99.3[3] and 100%.[6] In a large study, Hansen et al. found an excellent concordance with the Meridian EIA test but without complete access to patient-level data, they were unable to fully conclude on the origin of discrepancies.[11] We found specificity at 100% on CSF prospectively tested (group III). However, two sera from this group were considered false positives. In the absence of positive culture for *C. neoformans*, this should prompt clinicians to perform another test as soon a possible, to confirm or infirm the diagnosis of cryptococcosis. The good predictive values obtained in our survey may vary according to the prevalence of cryptococcosis in the group investigated. We also found cross-reactions in two patients with *T. asahii* infections (group II). While of little concern in the setting of AIDS, these cross-reactions have already been described, using other tests of cryptococcal antigen detection, mainly in hematological or solid organ transplant patients infected with other basidiomycete pathogens such as *Trichosporon* sp or *Rhodotorula* sp, that may share common antigens with *C. neoformans*.[12] This should be kept in mind before reaching a definitive diagnostic conclusion. Nevertheless, one can note that the LFA remained negative in a case of *Rhodotorula* fungemia while EIA and LA tests were found positive.

In conclusion, the excellent negative predictive value of the CrAg Lateral Flow Assay, its ease of use (practicality and reading), as well as the rapidity of obtaining results (less than 15 min) are fully appropriate for diagnosis of cryptococcosis, notably during night duty when a patient with symptoms suggestive of neurologic cryptococcosis has to be tested in emergency in order to decide if an antifungal therapy has to be initiated. Nevertheless, the performances of the test (sensitivity and specificity) also support its use during the day, either to test patients with suspected cryptococcosis or asymptomatic patients at risk of cryptococcosis, mostly HIV-positive patients.

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