Medical Mycology, 2022, 60, myac020
https://doi.org/10.1093/mmy/myac020
Advance Access Publication Date: 1 April 2022
Original Article

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Evaluation of a *Cryptococcus* capsular polysaccharide detection FungiXpert LFA (lateral flow assay) for the rapid diagnosis of *Cryptococcosis*

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Received 8 October 2021; Revised 22 February 2022; Editorial Decision 25 February 2022

Abstract

Cryptococcus is an opportunistic pathogenic fungus and is the major cause of fungal meningitis. The cryptococcal antigen (CrAg) lateral flow assay (LFA) is an immunochromatographic test system that has simplified diagnosis as a point-of-care test. In this study, we evaluated the diagnostic performance of Cryptococcal capsular polysaccharide detection FungiXpert (Genobio Pharmaceutical, Tianjin, China) using serum and cerebrospinal fluid (CSF) samples for the diagnosis of cryptococcosis and investigated the cross-reaction of the assays to pathogenic fungi and bacterium by comparing it to the U.S. Food and Drug Administration (US FDA)-approved IMMY CrAg LFA. Eighty CSF and 119 serum/plasma samples from 158 patients were retrospectively collected to test for qualitative or semi-quantitative detection of CrAg. Cross-reaction of the assays was tested using 28 fungi and 1 bacterium. Compared to IMMY CrAg LFA, the FungiXpert LFA demonstrated 99.1% sensitivity and 98.9% specificity in the qualitative test. In the 96 semi-quantitative CrAg assay results, 39 (40.6%) test titers of FungiXpert LFA were 1–2 dilutions higher than those of IMMY CrAg LFA. The Intraclass Correlation Coefficient of the Semi-quantitative results of CrAg titer tests via the two assays was 0.976. Similar to IMMY CrAg LFA, FungiXpert LFA showed cross-reactivity with *Trichosporon asahii*. Compared with the IMMY CrAg LFA, the FungiXpert LFA showed an equal, yet, excellent performance. However, it is important to note that these two assays have potential cross-reactivity to *T. asahii* when diagnosing patients. FungiXpert LFA is a rapid screening method for the effective and practical diagnosis and treatment of cryptococcosis.

Lay summary

The FungiXpert LFA was developed to diagnose fungal meningitis caused by *Cryptococcus* yeasts, by using serum or cerebrospinal fluid. It was compared to an existing lateral flow assay (LFA). The FungiXpert LFA performed well in qualitative and semi-quantitative tests.

Key words: *Cryptococcus*, *Cryptococcus* capsular antigen, lateral flow immunoassay, cross-reactivity, consistency.

Introduction

Cryptococcus is an opportunistic pathogenic fungus and the major cause of fungal meningitis in those immunosuppressed from HIV/AIDS, those receiving immunosuppressive therapies, and previously healthy individuals.[1–4] Some members of the *Cryptococcus gattii* species complex are also known to occasionally infect healthy individuals. In addition, cryptococcal disease is a spectrum ranging from pulmonary cryptococcosis to meningoencephalitis. Rapid and accurate laboratory tests are required to identify cryptococcal infections. Lateral flow assay (LFA) is an immunochromatographic test system for the qualitative or semi-quantitative detection of *Cryptococcus* capsular polysaccharide antigens in the serum, plasma, whole blood, and cerebrospinal fluid (CSF). Cryptococcal antigen (CrAg) LFA is an inexpensive point-of-care (POC) assay that has been extensively utilized with high sensitivity and specificity.[5,6] Furthermore, CrAg titers are strongly associated with mortality and can identify patients with the highest risk of death.[7–9] On the other hand, low CrAg LFA titer is crucial for the early diagnosis of cryptococcosis in HIV-negative patients, and lumbar puncture is recommended to be performed routinely for CSF testing when a positive low serum titer is reported.[10] Therefore, rapid semi-quantitative LFA reagent test results affect the clinical diagnosis and enable doctors to make titer-based treatment decisions at a lower cost at the bedside.[9]

Cryptococcal capsular polysaccharide detection K-set LFA (FungiXpert, Genobio Pharmaceutical, Tianjin, China) is a commercial assay produced in China and globally available. The objective of this study was to assess the diagnostic performance of FungiXpert LFA in testing serum and CSF samples and comparing it with the U.S. Food and Drug Administration (US FDA)-approved IMMY CrAg LFA (Immuno-Mycologics, Norman, OK, USA). Further, we investigated the cross-reaction of the assays to pathogenic fungi and a bacterium. According to the manufacturer's instructions, some cross-reactivity experiments have been performed, for example, high concentration (>0.1 mg/ml) antigens from *Paracoccidioides brasiliensis* exhibited some cross-reactivity with the IMMY CrAg LFA reagent. Infection with *Trichosporon* spp., *Schizophyllum commune, Coprinopsis cinerea, Alternaria alternata, Mucor circinelloides, Rhodotorula* spp., or *Capnocytophaga canimorsus* (gram negative bacterium) may lead to false-positive results at low titers. Rheumatoid factor, hydroxyethyl starch, soaps, disinfectants, contaminated sample transport vials, and insufficient treatment for proteins contained in samples have been reported to cause cross-reactivity.[11–17] However, the cross-reactivity of FungiXpert LFA has not been sufficiently explored across a wide range of contaminants. In the present study, we selected fungi and bacterium that were not analyzed in previous cross-reactivity experiments and pathogens mentioned in other cryptococcal antigen cross-reaction studies.[11] We evaluated the diagnostic performance of FungiXpert LFA as a point-of-care assay for the diagnosis of cryptococcosis.

Methods

Collection of samples and clinical information

We retrospectively collected a total of 199 samples (80 CSF and 119 serum/plasma) from 158 patients from West China Hospital of Sichuan University who were screened for cryptococcal antigens between October 26, 2018 and November 27, 2020. These samples were used to evaluate the performance of the FungiXpert LFA reagent. All samples were stored in a freezer at − 20°C. The gender and age of 158 patients were reviewed. At the same time, laboratory data and clinical diagnosis of CrAg positive patients were retrospectively reviewed.

Fungal and bacterial strains and sample preparation

To test for cross-reactivity of the two assays, 28 fungal strains belonging to 19 species and 1 bacterial species were cultured. The strains used included clinically isolated strains, ATCC standard strains, and the College of American Pathologists Proficiency Testing (CAP-PT) strains. The clinically isolated strains included *Cryptococcus neoformans, Rhodotorula mucilaginosa, Geotrichum candidum, Exophiala dermatitidis, Trichosporon asahii, Aureobasidium pullulans, Sporothrix schenckii* complex, *Acremonium, Alternaria alternata, Trichophyton tonsurans, Trichophyton mentagrophytes* complex, *Mucor racemosus, Fusarium oxysporum, Fusarium solani*, and *Capnocytophaga sputigena*. The ATCC standard strains included *Candida albicans* ATCC 90 028, *Candida parapsilosis* ATCC22019, and *Candida krusei* ATCC6258. The CAP-PT strains included *Candida tropicalis* CAP2013 F-1and *Aspergillus fumigatus* 2015CAP F-04. The clinically isolated strains were confirmed viaMatrix-Assisted Laser Desorption/Ionization–Time-of-Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Dalton-ics, Inc., Billerica, MA, USA). The Biotyper databases used were the main library DB5989 and the Filamentous Fungi Library 2.0, and internal transcribed spacer sequencing was performed (ITS1 and ITS4 were used). Ascomycota and Basidiomycota fungi were cultured at 35°C on Sabouraud dextrose agar (SDA). The other fungi were cultured at 28°C on potato dextrose agar. The bacterial strain was cultured at 35°C on a blood plate. After culturing, the strains were passaged once, incubated for 24 h and then tested. All the strains were prepared using the 0.5 McFarland standard fungus/bacterium suspension in 0.9% sterilized physiological saline. This procedure yielded a yeast stock suspension of 1×10^5 to 5×10^6 cells per ml.

Assay kit and procedure

The tests were performed using the cryptococcal capsular polysaccharide detection K-set LFA (FungiXpert, Genobio Pharmaceutical, Tianjin, China) and IMMY CrAg LFA (Immuno-Mycologics, Norman, OK, USA). All assays were performed in a

blinded manner by trained laboratory technicians, according to the manufacturer's instructions.

Qualitative procedure

The procedure for the IMMY CrAg LFA is as follows: Pipette 40 μl of specimen diluent to a labeled disposable microcentrifuge tube. Add 40 μl of the specimen to the reservoir and mix by pipetting. Submerge the white end of a CrAg test strip into the specimen, observe, and record the results after 10 min. The procedure for the FungiXpert LFA is similar: Pipette 50 μl of the specimen diluent into a disposable microcentrifuge tube. Add 50 μl of the specimen to the reservoir and mix by pipetting. Pipette the mixed sample (50 μl) and add to the sample hole of the marked test card. Record the results after 10 min.

Semi-quantitative titration procedure

The procedure for IMMY CrAg LFA is as follows: Place 10 microcentrifuge tubes in a tube rack and label them 1–10 (1:5 to 1:2 560). Prepare dilutions starting with an initial dilution of 1:5. Pipette 160 μl of the specimen diluent to tube #1. Pipette 80 μl of titration diluent to each of the tubes labeled 2–10. Add 40 μl of the specimen to tube #1 and mix well. Transfer 80 μl of the specimen from tube #1 to tube #2 and mix well. Continue this dilution procedure up to tube #10. Discard 80 μl from the tube #10 and 40 μl from the tube #1 for the final tube volumes of 80 μl. Submerge the white end of a CrAg test strip into the specimen in each of the 10 tubes. Record the results after 10 min. The procedure for the FungiXpert LFA reagent is similar. The dilution steps were the same as those for IMMY CrAg LFA. Then, 50 μl of the mixed sample was pipetted into the sample hole of the marked test card. The results were recorded after 10 min.

Cross-reaction method

In this procedure, sample dilution is not required. The 0.5 McFarland standard fungus/bacterium suspension can directly be used for the experiment. The rest of the steps are similar to that of the clinical sample preparations used during the qualitative and semi-quantitative procedures. To calculate the accurate titer, the 0.5 McFarland standard positive fungus/bacterium suspension was diluted with 0.9% sterile normal saline at a 10-fold ratio, from 1:10 to 1:10 000; 10 μl of the diluted fungus/bacterium suspension was inoculated on SDA/Blood Agar Plates (BAP) for colony count.

Ethical statement

Ethical approval was obtained from the West China Hospital Sichuan University Biomedical Ethics Committee (approval number: 53,2018).

Validation study analysis

First, we compared the consistency of the qualitative results for the two assays. Using the IMMY CrAg LFA as a reference standard, we calculated the sensitivity and specificity of the cryptococcal capsular polysaccharide detection FungiXpert LFA for the qualitative (positive/negative) CrAg detection. Results that are inconsistent with the reference method are defined as false positives or false negatives. At the same time, the different qualitative results obtained from the two tests were compared by reviewing the patients' cases.

Statistical analyses were performed using the Statistical Package for the Social Sciences version 22.0 software (SPSS Inc., Chicago, IL, USA) with categorical data analysis to assess confidence intervals (CI) of proportion, overall percentage agreement, and kappa coefficients. The semi-quantitative titration results of the two kits were converted to logarithm scale and then analyzed for the intraclass correlation coefficient (ICC).

Results

Of the 199 samples tested, 113 were CrAg-positive (49 CSF and 64 serum/plasma) according to the IMMY CrAg LFA. The demographic data of 158 participants showed that there were 55 women (34.8%) and 103 men (65.2%), with a mean age of 49 $\pm$ 15 years. Of the 76 CrAg positive patients, 69 (90.8%) patients were diagnosed with cryptococcal infection, of which 53 (69.7%) patients were diagnosed with cryptococcal meningitis. *C. gattii/neoformans* species complexes were isolated from the CSF of 33 patients, and the other 20 patients were clinically diagnosed with cryptococcal meningitis from ink staining, cryptococcal antigens, and clinical symptoms. Additionally, 14 (18.4%) patients were diagnosed with pulmonary cryptococcosis. 1 (1.3%) patient was diagnosed with disseminated cryptococcal infection. 1 (1.3%) patient was diagnosed with cryptococcal intraosseous infection. 1 (1.3%) patient was diagnosed with probable cryptococcal infection, and 6 (7.9%) patients were diagnosed with alternative, non-cryptococcal infection and undiagnosed cryptococcal infection (Figure 1). Compared with the IMMY CrAg LFA, the FungiXpert LFA demonstrated 99.1% sensitivity (95% CI, 0.94, 0.99) and 98.9% specificity (95% CI, 0.93, 0.99) in the qualitative test. The overall qualitative agreement was 98.9% (kappa = 0.98) (Table 1). One of the 113 IMMY CrAg LFA positive samples (with a titer of 1:2) showed a negative result on the FungiXpert LFA. According to the medical records, this CSF sample with reduced titer after treatment was submitted for inspection on November 9, 2020 and was classified as false negative. One of the 86 IMMY CrAg LFA negative samples was positive on the FungiXpert LFA test (with a titer of 1:2). According to the medical records, the patient was clinically diagnosed with right breast invasive carcinoma through pathological biopsy, and hence the sample was classified as false positive (Table 1).

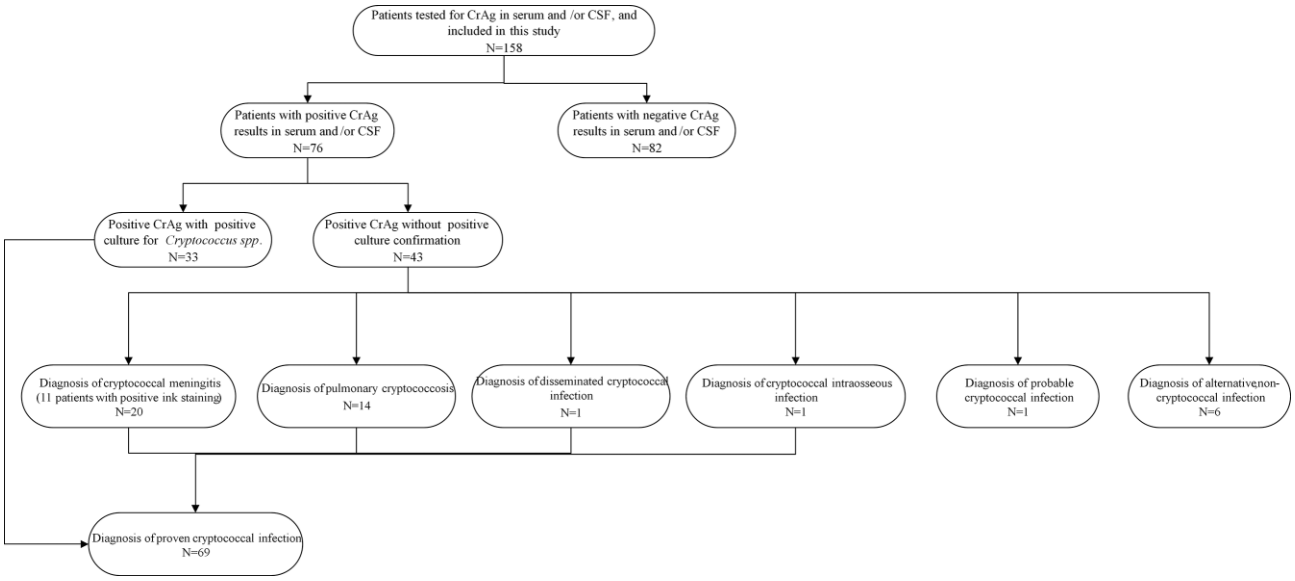

Figure 1. Summary of patients for whom serum and/or CSF samples were tested with the CrAg LFA included inthis study.

Table 1. Contingency tables comparing the results of the K-set LFA reagents and IMMY CrAg LFA using specimens (n = 199).

Assay and result		IMMY CrAg LFA Positive	IMMY CrAg LFA Negative	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Agreement (%)	Kappa
K-set	Positive	112	1	99.1(0.94,0.99)	98.8(0.93,0.99)	98.9	0.98
LFA	Negative	1	85				

LFA, lateral flow assay; CrAg LFA, cryptococcal antigen lateral flow assay; CI, confidence interval

Table 2. Intraclass correlation coefficient of the semi-quantitative results of CrAg titer tests by two assays.

		95% Confidence interval		F test with true value 0			
	Intraclass correlation[b]	Lower bound	Upper bound	Value	df1	df2	Sig
Single measures	0.976[a]	0.964	0.984	80.729	95	95	0.000
Average measures	0.988[c]	0.981	0.992	80.729	95	95	0.000

[a]The estimator is the same, whether the interaction effect is present or not.
[b]Type C intraclass correlation coefficients using a consistency definition-the between-measure variance is excluded from the denominator variance.
[c]This estimate is computed assuming the interaction effect is absent, because it is not estimable otherwise.

CrAg titers were measured in 96 positive specimens (46 CSF and 50 serum/plasma). The minimum titer was 1:2 and the maximum titer was > 1:2 560. According to the manufacturer's instructions, the dilution > 1:2 560 was not further diluted. In the semi-quantitative CrAg assay results, 39 (40.6%) tests of FungiXpert LFA were higher than those of the IMMY CrAg LFA reagent. Among them, 29 cases (30.2%) of FungiXpert LFA results were one dilution higher than those of IMMY CrAg LFA and 11 cases (10%) were two-fold higher than those of IMMY CrAg LFA (Supplementary Data). The ICC of the semi-quantitative results of CrAg titer tests via the two assays was 0.976 (<0.4, weak correlation, >0.75, strong correlation) (Table 2).

Trichosporon asahii showed cross-reactivity with both assays (Table 3). Cross-reactivity to *Trichosporon asahii* was observed with the FungiXpert LFA and IMMY CrAg LFA with titers of 1:320 (3.1×10^2 colony forming units [CFU]/ml) and 1:160 (6.3×10^2 CFU/ml), respectively (Figure 2). Furthermore, strain variations within a single species did not affect the results of this study. Other fungi and bacteria did not show cross-reactivity (Table 3).

Discussion

The latex agglutination (LA) test has been considered the gold standard method in past studies.[18,19] The sensitivity of the IMMY CrAg LFA is superior to that of the LA test and enzyme

Table 3. Fungal and bacterium strains used in this study and CrAg LFA results.

		CrAg LFA result (CFU/ml)	
Tested fungi	Strain number	K-set	IMMY
Cryptococcus neoformans	2 002 281 141	2×10^1	4×10^1
Rhodotorula mucilaginosa	1 811 291 311	-	-
	1 812 091 171	-	-
	1 812 151 237	-	-
	1 812 021 164	-	-
Geotrichum candidum	1 811 251 155	-	-
	2 009 092 046	-	-
Exophiala dermatitidis	1 810 102 037	-	-
	2 008 102 024	-	-
Trichosporon asahii	1 903 221 157	3.1×10^2	6.3×10^2
	1 907 181 261	3.1×10^2	6.3×10^2
Aureobasidium pullulans	1 902 071 112	-	-
	2 006 091 186	-	-
	2 101 072 067	-	-
Sporothrix schenckii complex	2 005 132 019	-	-
	2 004 292 039	-	-
Acremonium	1 903 132 048	-	-
Alternaria alternata	2 012 312 072	-	-
Trichophyton tonsurans	1 810 171 245	-	-
Trichophyton mentagrophytes complex	2 008 272 025	-	-
Mucor racemosus	1 905 082 039	-	-
Fusarium oxysporum	1 903 251 241	-	-
Fusarium solani	1 912 041 304	-	-
Candida albicans	ATCC90028	-	-
Candida parapsilosis	ATCC22019	-	-
Candida krusei	ATCC6258	-	-
Candida tropicalis	CAP2013 F-1	-	-
Aspergillus fumigatus	2015CAP F-04	-	-
Tested bacterium			
Capnocytophaga sputigena	2 012 233 021	-	-

CrAg LFA, cryptococcal antigen lateral flow assay; CFU, colony forming units

immunoassay (EIA) (serum and CSF); other advantages of this method over EIA and LA include lower costs and ease of use.[20–22] In this study, we evaluated the diagnostic performance of the semi-quantitative FungiXpert LFA versus that of the IMMY CrAg LFA. The FungiXpert LFA have the same advantages as the IMMY CrAg LFA. They are rapid, simple to use, and equipment-free; they require a small specimen volume and can be stored at room temperature. The FungiXpert LFA is comparatively cheaper as well. But the FungiXpert LFA would be more environmentally friendly if a strip was not in a large plastic cassette. Both FungiXpert LFA and IMMY CrAg LFA yielded unambiguous results. Compared with the IMMY CrAg LFA, the FungiXpert LFA showed an excellent performance, with 99.1% sensitivity and 98.9% specificity in qualitative testing. Notably, all false positives and negatives were obtained with low-titer samples (1:2) that probably had low Ag/organism burden. Thus, expected errors for borderline samples. We reviewed the cases of two patients with different qualitative results for the two assays. One of the IMMY CrAg LFA-positive samples was negative on FungiXpert LFA reagent testing. This CSF sample with reduced titer after treatment was submitted for inspection on November 9, 2020. The patient was treated in the neurology department of our hospital because of a headache and dizziness in June 2019. The India ink stain was positive and the CSF was cultured, revealing *Cryptococcus* species complex when tested by MALDI-TOF MS. The results of CrAg in the blood and CSF were both 1:2 560. The patient was diagnosed with cryptococcal meningitis and subsequently transferred to the department of infectious diseases for treatment. Amphotericin B, voriconazole, and flucytosine were administered, and symptomatic treatment to reduce intracranial pressure was provided. Subsequently, the patient was discharged when the symptoms were mitigated. The patient was admitted to the hospital for follow-up visits on April 20, June 20, August 20, and November 20. After the treatment, both ink staining and CSF culture were negative. The titers of CrAg in blood and CSF gradually decreased (the titers

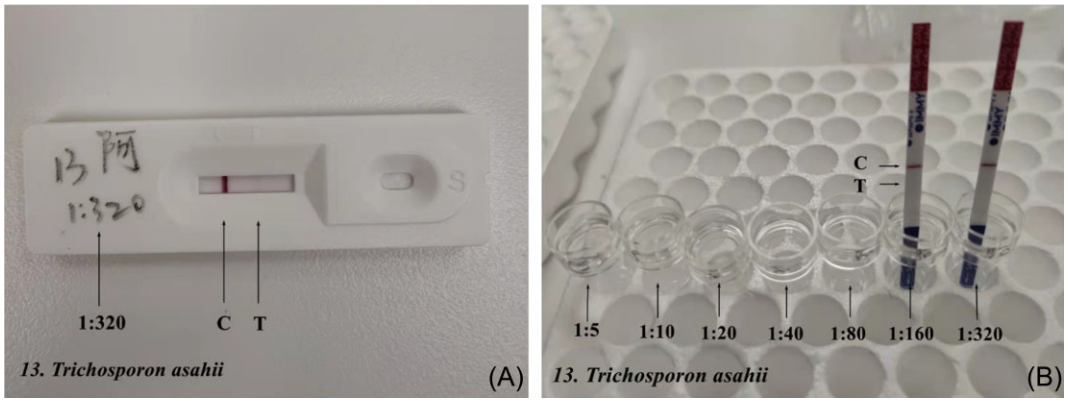

Figure 2. Cross-reactivities to Trichosporon asahii were observed with the two reagents. (A. FungiXpert LFA. B. IMMY CrAg LFA).

of CrAg decrease faster in CSF than in blood). Based on the medical records, we speculate that this inconsistency is related to the detection sensitivity of cryptococcal antigens of the two assays. Because the kinetics of antigen clearance is slow,[3,23,24] we speculated that the IMMY CrAg LFA demonstrated a higher sensitivity to detect lower concentrations of antigen.

Another inconsistent result was obtained where the IMMY CrAg LFA negative sample was positive on the FungiXpert LFA with a titer of 1:2. This patient was admitted to the hospital for one month because of cough and sputum production. All the infection-related tests were negative. It was suspected that the patient had right lung adenocarcinoma based on sputum shedding cytology. Subsequently, the patient was clinically diagnosed with right breast invasive carcinoma through pathological biopsy. We speculated that the false-positive results of the K-set reagent are caused by non-specific antibodies in the patient's tumor. According to Dubbels et al. 34% (13/38 cases) of all positive results were considered false positives, with semiquantitative titers ranging between 1:2 and 1:5. The study also mentioned that the false-positive cases were ultimately diagnosed as tumor-related diseases, such as large diffuse B-cell lymphoma and glioma.[25] Consequently, we recommend careful clinical correlation prior to establishing a diagnosis of cryptococcal infection in patients with first-time positive K-set reagent titers of 1:2.

The primary constituent of the capsule of *Cryptococcus* yeasts is glucuronoxylomannan (GXM), which is a polysaccharide antigen target for immunoassays in the diagnosis of cryptococcosis.[26] The two assays in our study utilized antibodies that are highly sensitive to GXM by lateral flow immunoassay. However, the clarity of the color band of the reaction results is different because of the different production processes. We observed a disparity between the FungiXpert LFA and IMMY CrAg LFA in the endpoint titers. The ICC of the semi-quantitative results of CrAg titer tests via the two assays was 0.976, indicating that the semi-quantitative results of the two kits were in good agreement. Meanwhile, the endpoint titers of the FungiXpert LFA were higher than those of the IMMY CrAg LFA in 39 of 96 positive patients. Considering that the endpoint titers are related to different detection assays, clinicians and laboratories should be aware that the same semi-quantitative CrAg assay should be used to risk-stratify the probability of central nervous system disease at diagnosis or to monitor the effectiveness of patient treatment.

With the introduction of molecular techniques, the taxonomy of the *C. neoformans* and *C. gattii* species complexes was revised. The species within *C. gattii/neoformans* species complexes include: *C. neoformans s.s., C. deneoformans, C. gattii s.s., C. bacillisporus, C. deuterogattii, C. tetragattii and C. decagattii*.[27] At present, some rapid semi-quantitative LFAs have been developed.[6,28] There are at least 4 commercial Conformite Europeenne for In Vitro Diagnostic (CE-IVD) CrAg LFAs available, but not all detect the 7 members of the *C. gattii/neoformans* species complexes.[29] *Cryptococcus bacillisporus* and two *Cryptococcus tetragattii* strains could not be detected with the Dynamiker LFA. The Biosynex LFA could not detect one *C. bacillisporus* and one *C. deuterogattii* strain and detected none of the six *C. tetragattii* strains. However, all seven pathogenic *Cryptococcus* species were detected by the IMMY diagnostics and FungiXpert LFAs(29).

In this study, we investigated the cross-reactivity of FungiXpert LFA and IMMY CrAg LFA with a fungus/bacterium suspension density of 0.5 McFarland. *Trichosporon asahii* showed cross-reactivity in both assays with titers of 1:320 (3.1×10^2 CFU/ml, FungiXpert LFA) and 1:160 (6.3×10^2 CFU/ml, IMMY CrAg LFA). Members of the genus *Trichosporon* express GXM in their cell walls, similar to *Cryptococcus* species.[30] Although there are relatively few cases of infection caused by *T. asahii*, clinicians should consider the possibility of *Trichosporon* infection when the CrAg test is positive and the treatment for cryptococcosis is ineffective. The test results for *Capnocytophaga sputigena* and *Alternaria alternata* that yielded false positive results in other studies,[11] were negative in this study. The results of cross-reactivity of other pathogens in this study were also negative, showing that the two assays had an excellent specificity.

There are some limitations to our study. First, the number of samples used for comparison was low, especially for samples with a titer below 1:10. Second, this was a basic study using pathological suspensions of preserved strains and not clinical specimens. Therefore, further studies using clinical samples such as blood or CSF from patients with infectious diseases caused by *T. asahii* are required to be performed. Since molecular typing methods are not carried out in routine clinical work, we were uncertain about the samples were used for comparison, especially with which *Cryptococcus* species caused the infection. In our study, only *Cryptococcus neoformans* was used for cross-reactivity experiments.

As a domestically produced reagent that has obtained CE certification, the commercial reagent Cryptococcal Capsular Polysaccharide Detection FungiXpert LFA is cheaper than the imported products; thus, it is a good choice for many domestic laboratories. In conclusion, FungiXpert LFA tested on serum and CSF in individuals had high sensitivity and specificity compared to the IMMY CrAg LFA test. Part of the endpoint of the FungiXpert LFA was higher than that of the IMMY CrAg LFA. However, the same semi-quantitative CrAg assay should be used to monitor the effectiveness of patient treatment. Clinicians and laboratories should be aware that assays have potential cross-reactivity with *T. asahii* when diagnosing the patients. The FungiXpert LFA is a rapid screening method that can provide an effective and practical method to diagnose cryptococcosis and monitor the effectiveness of patient treatment.

Authorship contributions

The authors alone are responsible for the content and writing of this paper.

Funding

Received no funding

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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