# Diagnostic Value of Bronchoalveolar Lavage Fluid Cryptococcal Antigen-Lateral Flow Immunochromatographic Assay for Pulmonary Cryptococcosis in non-HIV Patients

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# Abstract

**Background:** The present study aimed to investigate the diagnostic value of cryptococcal antigen-lateral flow immunochromatographic assay (CrAg-LFA) in bronchoalveolar lavage fluid (BALF) of patients with pulmonary cryptococcosis (PC).

**Methods:** The subjects collected from March 2015 to October 2018 in 4 hospitals were divided into the pulmonary cryptococcosis (PC) group (n=72) and the non-pulmonary cryptococcosis (non-PC) group (n=236). The patients in the PC group were diagnosed by lung biopsy.

**Results:** The sensitivity, specificity, positive and negative predicted values of CrAg-LFA in the serum were 75.0%, 99.6%, 98.2%, and 92.9%, respectively, while those in the BALF were 93.1%, 100.0%, 100.0%, and 97.9%, respectively. Hence, the sensitivity of the CrAg-LFA in BALF was significantly higher than that in the serum of the patients in the PC group ($P<0.05$).

**Conclusion:** The CrAg-LFA is a rapid, simple and safe experimental method. CrAg-LFA in BALF samples can offer higher diagnostic value for PC than that noted in the serum samples of PC subjects. Furthermore, the BALF positive results were equivalent to the microbiological culture positive results in terms of diagnostic value.


# Introduction

Pulmonary cryptococcosis (PC) is a subacute or chronic lung disease caused by *Cryptococcus neoformans* infection, which can spread to the whole body. *Cryptococcus* is a localized invasive infection that occurs in the lung tissue(1). Traditional ink staining is not suitable for viscous respiratory secretions, and the culture positive rate is also low. The diagnosis of pulmonary cryptococcosis is mainly based on pathological examination and culture of biopsy specimens obtained from sterile sites, all of which are invasive and difficult to acquire. The detection of cryptococcal capsular polysaccharide antigen in serum has important clinical significance in the diagnosis of PC. The new cryptococcal antigen-lateral flow assay (CrAg-LFA) was developed in recent years and has the advantage of rapid detection. The simultaneous detection of galactomannan antigen (GM) in the serum and bronchoalveolar lavage fluid (BALF) for the diagnosis of pulmonary aspergillosis can confirm that GM sensitivity of BALF is significantly higher than that of serum GM (2). Nonetheless, the effectiveness of cryptococcal capsular polysaccharide antigen in BALF, notably using CrAg-LFA, in the diagnosis of PC has not been previously reported. In the present study, CrAg-LFA was used to detect the cryptococcal antigen in BALF and serum samples. This evidence was combined with the clinical pathological outcomes, so as to explore its diagnostic value for PC.

# Subjects And Methods

Subjects

From March 2015 to October 2018, patients who were admitted to the departments of respiratory medicine and thoracic surgery were screened consecutively. These departments were based in the Zhongshan Hospital, Xiamen University, the Zhangzhou affiliated hospital of the Fujian Medical University, the Second affiliated Hospital of the Fujian Medical University, and the Quanzhou First Hospital affiliated to the Fujian Medical University. The patients who exhibited nodular lesions near the lungs and lung shadows and were suspected with PC following ineffective general antibacterial therapy were included into our study.. The present study gained approval from the Ethics committees of Zhongshan Hospital, Xiamen University (approved no. 2017030). All patients signed informed consent forms.

Instruments and reagents

Cryptococcus antigen detection kit (CrAg-LFA detection) was a product of Immuno Mycologics, USA. The fungal identification card YST and the automated blood culture system Bact/Alert3D were purchased from Biomerieux Ltd., France. The BACTEC FX automated blood culture system was purchased from Becton Dickinson, USA.

Methods

Pathological examination

Percutaneous lung biopsy was guided by CT or B-ultrasound and tissue biopsy was conducted by electronic bronchoscopy. Conventional tissue fixation that included embedding and sectioning were performed by researchers who were blinded to the origin of the samples. The samples were detected using H&E staining and PAS, as well as special staining, such as hexamine silver.

Grouping

Human immunodeficiency virus (HIV) antibody was detected and the negative result was confirmed in all enrolled patients. All patients were divided into two groups: (1) The PC group consisted of patients who were diagnosed as PC and underwent serum and BALF CrAg-LFA as well as microbial culture prior to antifungal treatment. (2) The patients of the non-PC group provided blood samples, bronchoscopy results, and BALF samples for the assessment of the diagnostic procedure.

All PC patients were confirmed by (1) lung tissues pathological evidence through percutaneous lung biopsy or transbronchial lung biopsy: Non-caseating granulomas combined with devoured cryptococci cells and capsules in the cytoplasm of macrophages, (2) Positive culture of *Cryptococcus neoformans* from blood culture or biopsy specimen (3). The clinical data, pathogen detection, radiological imaging and the detection of the cryptococcal antigen in blood and BALF samples, as well as in their microbial culture of all included patients were collected.

Lumbar puncture was attempted to conduct in PC patients to rule out asymptomatic central nervous system involvement.

BALF specimen collection

BALF samples were collected from all included patients prior to antifungal treatment according to the standard operating procedure (SOP) by the use of the bronchoscope. Following the induction of local anesthesia with 2% lidocaine, nasal bronchoscopy was performed. The bronchoscope was inserted into the target sub-segment bronchus for HRCT positioning, and 30 ml of sterile saline was injected at 37℃. The injection was performed two times for bronchoalveolar lavage to the lesion site. Subsequently, 15 to 30 ml of BALF sample was recycled, placed in a silicone plastic bottle and immediately sent (at room temperature) for detection. The solution could be stored for 72 h at 4**℃**.

Microbial culture

The serum samples were collected from all included patients for blood culture. A total of 5 ml of fasting serum was collected prior to antifungal treatment from all patients, and the supernatant was stored in a refrigerator at -20°C. The alarm-positive culture flask was immediately transferred to the blood agar plate and the Shabao's plate to allow growth and separation, followed by identification using the YST card. The BALF or bronchoscopy brush was directly inoculated in the blood agar and/or in the Shabu's plates in order to allow growth and separation, followed by identification using the YST card.

CrAg-LFA

Using a blinded study design, 5 to 10 ml of the BALF and whole blood sterile samples were routinely collected, from which serum was isolated according to the instructions provided by the kit manufacturer. One drop of the sample dilution was placed into a small test tube, and mixed with 40 μl of the separated sample. Subsequently, the white end of a test strip was immersed into the solution, and was removed 10 min later for detection. A negative result was defined by the appearance of one quality control line, whereas a positive result was defined by a simultaneous appearance of a quality control line and a test line. A failed result was defined by the appearance of the test line only, which indicated that it required re-testing.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software. The data that followed non-normal distribution were analyzed using the Kruskal-Wallis test. The categorical data or grading data were analyzed by Chi-square test. A P value lower than 0.05 ($P<0.05$) was considered for significant differences.

# Results

Clinical characteristics and laboratory findings between PC and non-PC groups.

Among the 308 patients, 72 cases were definite diagnoses of PC, whereas 236 cases were diagnosed as non-PC. The diagnosis of PC was established in accordance with the standard criteria as described

previously (3) . More than half of PC patients were asymptomatic 40 (55.6%), and fever was more common to be seen when compared with non-PC group (31.9% vs. 17.3%, P=0.008). The underlying diseases among PC group included diabetes mellitus, old tuberculosis, lung cancer, malignancy, hematological disease, pulmonary vasculitis, however, most of PC patients were without underlying diseases. Regarding laboratory findings, only procalcitonin and galactomannan were higher in non-PC group than those in PC group, the others variables, including white blood cells, neutrophil, lymphocyte subsets, were not different between groups (Table 1). Bronchoscopic founding is no specific in PC patients.

CT images of PC patients

Among the 72 PC patients, the lesions were localized in the peripheral lung fields, whereas in 66 cases the lesions were localized in both lungs and in 37 cases they were more commonly found in the lower lungs. In addition, 21 cases of single nodules (Figure 2a-c), and 32 cases of multiple nodules (Figure 2b), including 21 cases of right lung and 14 cases of left lung, were observed. A total of 8 cases with multiple plaques, 15 lump cases with multiple nodules and 7 cases of diffuse pulmonary parenchymal changes were diagnosed. A total of 9 cases had cavities or vacuoles in their lesions, 7 cases presented with enlargement of mediastinal lymph node, and 6 cases exhibited pleural effusion (Table 1).

Diagnostic efficiency of cryptococcal antigen and culture in serum and BALF for PC

The CrAg-LFA data and the blood culture results from serum and BALF of the PC and non-PC groups are shown in Tables 2 and 3, respectively. Only one PC patient (1.4%) had a positive serum cryptococcal culture. With regard to the serum CrAg-LFA data, the sensitivity, specificity, positive predictive value and negative predictive value were 75.0%, 99.6%, 98.2%, and 92.9%, respectively. Nine PC patients (12.5%) had positive cryptococcal culture in BALF. With regard to the BALF CrAg-LFA data, the sensitivity, specificity, positive predictive value and negative predictive value were 93.1%, 100.0%, 100.0%, and 97.9%, respectively. The PC group exhibited significantly higher sensitivity of BALF CrAg-LFA than that noted for the serum CrAg-LFA ($P<0.05$) (Table 4 and Table 5).

# Discussion

Due to non-specific clinical features, the diagnosis of PC depends mainly on pathological examination (4). The gold standard is lung tissue pathological biopsy, H&E staining, PAS and hexamine silver staining. The type of lesion is related to the immune status of the patients. Non-caseating granulomas are often formed in patients with normal immune function and consist of devoured cryptococci cells and capsules that are visible in the cytoplasm of macrophages. Granulomas are not likely to appear in patients with impaired immune function, although the alveolar cavity is filled with cryptococci spores. Specialized bacterial staining indicated PAS (+), AB (-), hexamine silver (+) and acid resistance (-). The majority of the etiological examinations aim to detect *Cryptococcus neoformans* by blood culture or punctured materials. The aforementioned examinations exhibit a long reporting time and a low positive rate, and the majority of them are risky and invasive, which renders them unsuitable to be implemented in critically ill

patients and patients with blood coagulation dysfunction or lesions. Moreover, these examinations exhibit limited value for early diagnosis of the disease. Clinicians expect from non-invasive examinations ideal specificity and sensitivity metrics in addition to the rapid analysis time. In recent years, the determination of cryptococcal polysaccharide capsular antigen in serum samples has provided etiological evidence for the diagnosis of PC. Currently, the methods for detecting the cryptococcal capsular antigen in serum include latex agglutination (LA), enzyme-linked immunosorbent assay (EIA) and lateral chromatography (CrAg-LFA) (5). A previous study has shown that CrAg-LFA exhibited higher accuracy than LA and EIA, resulting in a significant clinical value. This conclusion was derived due to its optimal stability at room temperature, the rapid detection procedure (10 min), the ease of operation and its limited high demand on laboratory equipment (6).

Since the sample was from the target lung lesion area, a large number of clinical studies have proven that the sensitivity, specificity and clinical application value of galactomannan (GM) detection in BALF samples are significantly higher than those noted in serum for the diagnosis of invasive pulmonary aspergillosis (7,8). Based on these results, we extracted bronchoalveolar lavage (BAL) samples at the lesion of PC with high-resolution CT positioning by lung biopsy of patients diagnosed with this disease in the present study. Furthermore, we compared the CrAg-LFA data derived from BALF with those derived from serum samples and examined the diagnostic criteria for PC. The results demonstrated that the sensitivity and specificity of CrAg-LFA on BALF samples were 93.1% and 100.00%, respectively, while those of serum samples were 75.0% and 99.6%, respectively. Therefore, the diagnostic value of CrAg-LFA with regard to PC infection in BALF samples was significantly higher than that noted in serum samples. The conclusion was consistent with previous report (9).

In the present study, the colloidal gold method was used to test the cryptococcal capsular polysaccharide antigen. The colloidal gold test strip comprises a simple and rapid detection reagent that can detect all four (type A-D) serotypes of *Cryptococcus neoformans*. During the test, the sample is subjected to capillary action and is chromatographed by binding to a monoclonal antibody against the *Cryptococcus* antigen, which is considered a gold standard detection method. In the presence of cryptococcal antigen in the sample, binding to the gold standard anti-*Cryptococcus* antibody will occur. This gold standard antibody-antigen complex continues to be chromatographed on the membrane by capillary action and reacts with the test strip containing immobilized anti-*Cryptococcus* monoclonal antibody. The cryptococcal capsular polysaccharide antigen in the test sample can react with the colloidal gold-labeled antibody, and the antigen-antibody complex migrates on the surface of the test paper under capillary action, and ultimately binds to the monoclonal antibody immobilized on the nitrocellulose membrane. This binding produces a red line that is considered the test line (10). The test strip itself carries a quality control line. A positive result denotes simultaneous appearance of the quality control line and the test line, and a negative result denotes appearance of the quality control line alone. No negative control is required, and this method is considered more reliable.

In the present study, 13 of the 18 pathologically confirmed PC patients who had negative serum CrAg-LFA had positive BALF CrAg-LFA. In certain patients, no clinical symptoms appeared, although small lesions

were found at the edge of the lung and even near to the pleura of the lung as determined by CT (Figures 1a-d). In one rare case with bronchial *Cryptococcus*, the lesion was found in the lateral bronchus of the right middle lobe. The serum CrAg-LFA test for this case was negative, while the bronchial flushing fluid CrAg-LFA test was positive. It has been shown that the false negative results of the capsular polysaccharide antigen detection occur mostly during the early stages of infection (11). The higher positive rate and sensitivity noted in the BALF samples as opposed to the serum samples may be due to the infiltration of cryptococci colonies to the lung, resulting in a slow release of the capsular polysaccharide antigen to the blood. These events occur in the early stage of infection, notably in patients without systemic symptoms. In addition, the infection is associated with a high local cryptococcal load noted in the lung lesions. These speculations need further confirmation. In the present study, both the serum and BALF results of 5 confirmed cases were negative and corresponded to patients with small lesions and no symptoms. No case was found to have positive serum CrAg-LFA results and negative BALF CrAg-LFA results.

Bronchoalveolar lavage (BAL) is known as liquid lung biopsy. It is a simple, safe and convenient compared to the histopathological biopsy that can be implemented in primary hospitals. It can be used for cryptococcal antigen detection in case of biopsy unavailability. However, the process of collecting BALF and the requirements of the operators need to be further standardized. These procedures require a high demand of operational skills and accuracy to reach the sub-segment where the lesion is located for lavage, notably for small lesions. In the present study, the location of BALF collection was accurately positioned by lung CT, and only 60 ml of liquid was perfused to avoid the dilution of specimens caused by excessive perfusion. This resulted in avoiding result bias, which was a significant addition from the requirements stated by previous studies. Since less than 1 ml of fluid is required for CrAg-LFA, this method can increase the positive rate by collection of the centrifuged precipitation of the lavage fluid. The CrAg-LFA test exhibits significantly higher sensitivity, specificity and application value in BALF than in serum samples in a similar manner with the GM. However, GM indicates false positive results in testing both serum and BALF samples, and can be influenced by several factors (12,13). It has been reported that both latex agglutination and colloidal gold methods can occasionally lead to false positive results with regard to the detection of serum cryptococcal capsular polysaccharide antigen. In the present study, one out of 236 cases of non-pulmonary cryptococcosis also appeared weakly positive with regard to the serum CrAg-LFA test, which suggested that potential cross-reactivity was caused by specific factors in case of a low serum cryptococcal antigen titer. Therefore, semi-quantitative determination is advised in such cases. Finally, this case was pathologically diagnosed as rheumatoid lung disease. Whether such rare diseases cross-react with serum cryptococcal antigen to a certain extent requires confirmation with large sample clinical trials. In the present study, no false positive results were found in BALF samples from the four centers tested by CrAg-LFA, and the test exhibited a specificity of 100.00%. Therefore, we suggest that the diagnostic value of the CrAg-LFA test in BALF samples exhibits equivalent positive performance in detecting cryptococcal antigen to that of the microbiological culture, and that it is only inferior to the clinical value of the histopathological biopsy. Furthermore, lung puncture and other traumatic examinations are required only in cases with negative results.

Several limitations should be mentioned in the present study. First, we did not compare the diagnostic efficiency between LFA and conventional methods, such as LA or EIA. Second, the BALF CrAg-LFA value after treatment was not detected, therefore, we failed to evaluate the effectiveness of anti-cryptococcal therapy.

In summary, the CrAg-LFA test in BALF samples is of great significance for the early diagnosis of PC with small lesions, in which histopathological biopsy is not appropriate, due to its rapid, simple and safe method of analysis.

# Declarations

**(I) Conception and design:** Hui-Qing Zeng, Xue-Ying Cai, Xiao-Bin Zhang

**(II) Administrative support:** Hui-Qing Zeng

**(III) Provision of study materials or patients:** Hui-Qing Zeng, Xue-Ying Cai

**(IV) Collection and assembly of data:** Hui-Ying Zeng, Xiao-Bin Zhang, Dong-Yong Yang, Li Lin

**(V) Data analysis and interpretation:** Hui-Qing Zeng, Xue-Ying Cai, Xiao-Bin Zhang, Dong-Yong Yang, Li Lin, Mei-Jun Chen, Wei-Feng Guo

**(VI) Manuscript writing:** All authors

**(VII) Final approval of manuscript:** All authors


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### Conflict of interests

All authors declare that they have no any conflict of interests.

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# Tables

Table 1 Baseline characteristics between PC and non-PC patients

| Variable | PC group (n=72) | Non-PC group (n=236) | P value |
|---|---|---|---|
| Male gender, n (%) | 39 (54.2) | 146 (61.9) | 0.541 |
| Age, years | 48.4 (35-64) | 52.3 (38-70) | 0.862 |
| Symptoms | | | |
| Cough, n (%) | 32 (44.4) | 125 (60.0) | 0.205 |
| Expectoration, n (%) | 8 (11.1) | 75 (31.8) | 0.001 |
| Fever, n (%)[1] | 23 (31.9) | 41 (17.3) | 0.008 |
| Dyspnea, n (%) | 8 (11.1) | 59 (25.0) | 0.012 |
| Hemoptysis, n (%) | 8 (11.1) | 66 (30.0) | 0.003 |
| Asymptomatic, n (%) | 40 (55.6) | 64 (27.1) | 0.000 |
| Underlying diseases | | | |
| Diabetes mellitus, n (%) | 6 (8.3) | 34 (14.4) | 0.180 |
| Old tuberculosis, n (%) | 5 (6.9) | 15 (6.3) | 0.859 |
| Lung cancer, n (%) | 5 (6.9) | 40 (16.9) | 0.035 |
| Malignancy except lung cancer, n (%) | 3 (4.2) | 6 (2.5) | 0.474 |
| Hematological disease, n (%) | 1 (1.4) | 15 (6.4) | 0.131 |
| Connective tissue disease, n (%) | 0 (0.0) | 9 (3.8) | 0.123 |
| Pulmonary vasculitis, n (%) | 1 (1.4) | 7 (3.0) | 0.686 |
| Corticosteroids usage, n (%) | 6 (8.3) | 16 (6.8) | 0.654 |
| Immunosuppressive agent, n (%) | 4 (5.6) | 13 (5.5) | 0.988 |
| Laboratory findings | | | |
| White blood cells, ×10^9/L | 8.85 (4.21-12.3) | 6.87 (3.25-10.58) | 0.068 |
| Neutrophil, % | 78.6 (54.3-87.6) | 82.5 (65.9-91.3) | 0.742 |
| Procalcitonin, μg/L | 0.13 (0.04-0.32) | 0.21 (0.12-0.45) | 0.038 |

| | | | |
|---|---|---|---|
| CD4+ lymphocyte, counts/μl | 845.2 (515.6-1245.4) | 798.6 (496.8-1145.3) | 0.698 |
| CD8+ lymphocyte, counts/μl | 648.5 (415.9-1047.9) | 705.6 (523.5-1125.9) | 0.418 |
| Positive 1, 3-β-D glucan | 6.1 (3.9-8.4) | 7.3 (4.9-11.6) | 0.872 |
| Serum galactomannan | 0.31 (0.14-0.45) | 0.65 (0.41-1.35) | 0.021 |
| CT images | | | |
| Single nodule, n (%) | 21 (29.2) | 62 (26.3) | 0.628 |
| Multiple nodules, n (%) | 32 (44.4) | 84 (35.6) | 0.175 |
| Diffuse pulmonary parenchymal changes, n (%) | 7 (9.7) | 89 (37.7) | 0.000 |
| Two lungs involved, n (%) | 37 (51.4) | 158 (66.9) | 0.016 |
| Cavities or vacuoles | 9 (12.5) | 31 (13.1) | 0.888 |
| Mediastinal lymph node, n (%) | 7 (9.7) | 26 (11.0) | 0.756 |
| Pleural effusion, n (%) | 6 (8.3) | 38 (16.1) | 0.099 |
| Positive serum microbial culture[2] | 1 (1.4) | 0 (0.0) | 0.234 |
| Positive BALF microbial culture[2] | 9 (12.5) | 0 (0.0) | 0.000 |

Abbreviation: PC, pulmonary cryptococcosis, CT, computer tomography, BALF, bronchoalveolar lavage fluid.

[1]: Temperature was high than 37.3°C. [2]: *Cryptococcus. Neoformans*.

**Table 2.** Cryptococcal antigen and culture results in serum between PC and non-PC groups

| Group | Number of cases | Cryptococcal antigen | | Culture (Cryptococcus) | |
|---|---|---|---|---|---|
| | | Positive | Negative | Positive | Negative |
| PC | 72 | 54 (75.0) | 18 (25.0) | 1 (1.4) | 71 (98.6) |
| Non-PC | 236 | 1 (0.4) | 235 (99.6) | 0 (0.0) | 97 (100.0) |
| Sum | 308 | 55 | 253 | 1 | 168 |
| $x^2$ | | 209.177 | | | |
| P value | | <0.001 | | P=0.426* | |

*: Fisher exact probability method was used since the expected count of two cells is less than 5.

**Table 3.** Cryptococcal antigen and culture results in BALF between PC and non-PC groups

| Group | Number of cases | Cryptococcal antigen | | Culture (Cryptococcus) | |
|---|---|---|---|---|---|
| | | Positive | Negative | Positive | Negative |
| PC | 72 | 67 (93.1) | 5 (6.9) | 9 (12.5) | 63 (87.5) |
| Non-PC | 236 | 0 (0.0) | 236 (100.0) | 0 (0.0) | 121 (100.0) |
| Sum | 308 | 67 | 241 | 9 | 184 |
| $X^2$ | | 280.665 | | | |
| P value | | <0.000 | | <0.000* | |

*: Fisher exact probability method was used since the expected count of one cell is less than 5.

**Table 4.** Comparison of cryptococcal antigen detection from serum and BALF between PC and non-PC groups

| Group | Number of cases | Serum | | BALF | |
|---|---|---|---|---|---|
| | | Positive | Negative | Positive | Negative |
| PC | 72 | 54 (75.0) | 18 (25.0) | 67 (93.1) | 5 (6.9) |
| Non-PC | 236 | 1 (0.4) | 235 (99.6) | 0 (0.0) | 236 (100.0) |
| Sum | 308 | 55 | 253 | 67 | 241 |
| $X^2$ | | 209.177 | | 280.665 | |
| P value | | <0.001 | | <0.001 | |

Abbreviation: BALF, bronchoalvolar lavage fluid, PC: pulmonary cryptococcosis.

**Table 5.** Detection of cryptococcal antigen in serum and BALF samples in the PC group (%)

| Group | Serum group | BALF group | P value |
|---|---|---|---|
| Sensitivity | 75.0 | 93.1 | <0.05 |
| Specificity | 99.6 | 100.0 | >0.05 |
| Positive predictive value | 98.2 | 100.0 | >0.05 |
| Negative predictive value | 92.9 | 97.9 | <0.05 |

# Supplementary Files

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