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Reviewer A

The authors proposed the novel test to diagnose pulmonary cryptococcosis (PC) and distinguish it from other resembling pulmonary infections. They investigated the diagnostic accuracy of the serum sphingolipid (SPL) test related to PC, using lipid chromatography-mass spectrometry. As the results, the serum ceramide (Cer (d18:1/18:0)) was a specific diagnostic biomarker for PC. The manuscript was well written, but the following points need to be addressed for suitable publication.

・Could the diagnostic performance of the SPL tests differ in detecting different kinds of Cryptococcus species such as gattii and neoformans?

Reply: The admission criteria were patients with pulmonary cryptococcosis diagnosed by pathology or culture, but no clear distinction was made between gattii and neoformans. In southern China, most patients infected neoformans(Fang W, Fa Z, Liao W. Epidemiology of Cryptococcus and cryptococcosis in China. Fungal Genet Biol. 2015;78:7-15.). As to whether different kinds of Cryptococcus spp will lead to different SPL test results, further research is needed.

・In the "Introduction" section, the authors describes that "the sensitivity of serum CrAg detection is low in HIV-negative patients (<75%)". However, as far as I know, the sensitivity of cryptococcus antigen test in the diagnosis of PC is much higher in the previous cohort. (J Clin Microbiol. 2020;58:e01563-20.)

Reply: We have modified our text as advised "Nevertheless, it has been observed that both LFA and LA exhibit lower sensitivity in HIV-negative patients compared to HIV-positive individuals. Specifically, in cases of disseminated disease, the serum CrAg sensitivity for both LA and LFA in HIV-positive individuals exceeded 94%, whereas in HIV-negative patients, the sensitivity was only 78.1% and 82.6% for LA and LFA, respectively (14)." (see Page 4, line 93-97)

・If the patients with PC were complicated with cerebral nervous system involvement or dissemination, the accuracy of the test can change. What were the details of cryptococcal infections in the patients included in this study?

Reply: All the patients we included were patients with simple pulmonary cryptococcosis, and the patients had no neurological symptoms at the time of admission and follow-up. (see Page 5, line 142-144)

Reviewer B

This is a novel paper and has some interesting findings – but I think needs some fairly major amendments to tighten the findings. Please see below:

Introduction

• Line 62-63: “Cryptococcus is widely distributed in nature, but pigeon dung is considered the main source of infection with this fungus.”
o Note in many parts of the world, the main species causing infection HIV negative – and particularly patients who are apparently immunocompetent is Cryptococcus gattii, not Cryptoccous neoformans. The source of Cryptococcus gattii is trees such as the River Red Gum (Eucalyptus camldulensis) but also others. This may be worth mentioning given this paper is about Cryptococcus in non-HIV infected individuals – and a reference to species of Cryptococcus causing infection may be appropriate.

Reply: In southern China, most patients infected neoformans(Fang W, Fa Z, Liao W. Epidemiology of Cryptococcus and cryptococcosis in China. Fungal Genet Biol. 2015;78:7-15.). The admission criteria were patients with pulmonary cryptococcosis diagnosed by pathology or culture, but no clear distinction was made between gattii and neoformans. We have modified our text as advised “*Cryptococcus neoformans* and *Cryptococcus gattii* are the two primary etiological factors underlying cryptococcosis. Cryptococcosis attributable to *C. neoformans* predominantly manifests in HIV-positive individuals, whereas infections induced by *C. gattii* are more frequently observed in immunocompetent patients, including those who are HIV-negative.” (see Page 3, line 61-65)

• Line 75-78 is difficult to understand: “Most HIV-negative and even health PC patients present with only cough and expectoration due to the lack of specificity in the clinical manifestations, and the imaging manifestations are diverse.
o These lines are difficult to understand – I think the authors means the symptoms and imaging findings are similar to a number of other pathologies – both infective and non-infective.?

Reply: We have modified our text as advised and reorganized the paragraph to provide a clearer description “The majority of patients with PC typically present with non-specific symptoms such as cough and expectoration, accompanied by ambiguous imaging manifestations. Consequently, the disease can easily be confused with and misdiagnosed as other pulmonary conditions, including pulmonary aspergillosis (PA), tuberculosis (TB), lung cancer, and bacterial pneumonia. For instance, it has been reported that approximately 51.9% of South African miners tested for PC were mistakenly diagnosed with TB. Such misdiagnosis can lead to severe consequences as PC necessitates distinct treatment strategies compared to diseases with similar symptomatology. Therefore, it becomes imperative to explore novel diagnostic biomarkers that can facilitate early detection of PC, prevent the dissemination of Cryptococcal infection, and reduce PC-related mortality.” (see Page 3-4, line 78-87)

• Line 84: “pathological biopsy” – is this biopsy for histology?

Reply: Yes, this refers to biopsy for histology, specifically lung tissue biopsy. We have modified our text as advised “the clinical diagnostic techniques employed for PC

primarily encompass lung tissue biopsy" (see Page 4, line 88-89)

• Line 85: cryptococcal antigen detection – suggest specify site ie serum

Reply: We have modified our text as advised "Recently, the detection of cryptococcal antigen (CrAg) in serum using lateral flow assay (LFA) and latex agglutination (LA) has emerged as the most rapid and widely adopted diagnostic method for PC" (see Page 4, line 91-93)

• Line 87-88: "…but the sensitivity of serum CrAg detection is low in HIV-negative patients (<75%)."
o The sensitivity for CrAg in Cryptococcus infection in HIV-negative patients is quite a lot higher than 75%.
☐ Eg Chen et al, 2014.:
In clinical practice, the sensitivity of the test [CrAg] for C. gattii infection remains high, including for HIV-positive patients—90% for lung disease, with 87 to 100% sensitivity for CSF CRAG tests (29, 98, 130, 239).

Reply: Thank you for advice; however, the reference you provided is specific for CSF (cerebrospinal fluid) and, therefore, is not suitable for our study, which focuses on sensitivity in serum. Instead, we have searched another study that reported a higher sensitivity of serum CrAg detection. We have modified our text as advised "Nevertheless, it has been observed that both LFA and LA exhibit lower sensitivity in HIV-negative patients compared to HIV-positive individuals. Specifically, in cases of disseminated disease, the serum CrAg sensitivity for both LA and LFA in HIV-positive individuals exceeded 94%, whereas in HIV-negative patients, the sensitivity was only 78.1% and 82.6% for LA and LFA, respectively (14)." (see Page 4, line 93-97)

Methods
• Line 114-115 does not flow well – the first sentence is incomplete

Reply: We have modified our text as advised "The inclusion and exclusion criteria for patients diagnosed with PC adhered to the guidelines for the diagnosis and management of invasive fungal diseases (21). The inclusion criteria were as follows: 1) age ≥ 18 years; 2) confirmed negative HIV test; 3) first onset of the disease; 4) provision of signed informed consent; and 5) presence of clinical and/or imaging manifestations indicative of PC, supported by microbiological examination or histopathological examination results meeting any of the following conditions: (a) Identification of cryptococcus in or cryptococcal growth from samples of blood, pleural effusion (consistent with a clinically determined site of infection), or smears of diseased lung tissue specimens collected under sterile conditions. (b) Detection of cryptococcus in or cryptococcal growth from pus smears of the extrapulmonary infection site, collected under sterile conditions from patients with disseminated infection. (c) Identification of granulomatous lesions through histopathological examination of specimens collected from diseased tissue via bronchoscopy, lung puncture, or surgery, accompanied by corresponding tissue inflammation and confirmation of cryptococci through special

staining." (see Page 5, line 128-142)

• It is not clear to me how PC patients were selected. Was it all patients at the hospital diagnosed with PC consenting to participate or was it a selection (and if so how was it selected.)

Reply: All patients with pulmonary cryptococcosis diagnosed by pathology or culture were included

• Similarly for PA and TB patients – was this all patients with this diagnosis being cared for at the hospital or a selection?

Reply: Patients with PA and TB were included based on diagnostic criteria, and all inpatients who met the diagnostic criteria were included without strict screening

• Health controls are first mentioned in line 127.

Reply: We have modified our text as advised "Healthy controls were also recruited from the First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). Healthy controls were selected to match the age and sex of the patient groups and were free of documented pulmonary infectious, chronic or malignant diseases." (see Page 6, line 151-155)

• An overall description of study design, the groups of patients included and how patients were selected under study design and participants would be helpful and then an overall statement regarding how outcomes for each would be compared.

Reply: Thank you for your suggestions. We have improved the description of patients and healthy controls based on the modification suggestions. (see Page 6, line 151-155) And the overall description of the study design could refer to Figure1. (see Page18)

• Line 160-170 – CrAg in BAL fluid is not routine at this stage and not part of any guidelines I am aware of, though I realise it's being investigated with perhaps promising results - I think worth commenting on in the introduction.

Reply: Thank you for your suggestions. We have also conducted a search for relevant references and discovered that the results do not support the routine use of the test in BALF (Med Mycol. 2018;56(6):774-777). Therefore, we have deleted the contents about CrAg test in BALF and modified our text as "Quantitative detection of CrAg in serum samples was performed using LFA according to the manufacturer's instructions. In brief, 40 μL of the specimen was placed on the test strip, and the readings were obtained using an immune-quantitative analyser after a 10-min incubation. A reading of ≥ 8 indicated a positive result for cryptococcosis." (see Page 7, line 184-187).

Additionally, we have commented the application od CrAg testing in BALF in the introduction as advised "Additionally, CrAg testing in lung aspirates has demonstrated its diagnostic value for PC (15), but concerns persist regarding its routine application in BALF (16)." (see Page 4, line 97-99)

• I note in the results there is mention of Evaluation of SPL biomarkers of the diagnosis

of PC and then refers to a validation cohort and a other cohorts – this is not explained in the methods. The process of testing a number of biomarkers then choosing one to explore further is not described in the methods.

Reply: Thank you for your suggestions. We have modified our text as advised. (see Page 7-8, line 226-259)

Results

• See comment in methods regarding need to explain different testing groups better.

Thank you for your suggestions. We have modified our text as advised.

• Line 229-231 could probably be said with more clarity.

Reply: We have modified our text as advised "The evaluation of specific diagnostic biomarkers for PC encompassed two key aspects: (1) verification of biomarker candidates in the validation cohort, comprising 20 controls and 20 PC patients, and (2) assessment of the sensitivity and specificity of the potential biomarkers using other pulmonary infection groups (Figure 1B)." (see Page 9, line 247-251)

• Line 270 – "In addition the level of Cer….may be related to the improvement of imaging"….

o This statement isn't supported with evidence. One patient is sited who had a Cer(d18….) level go down with treatment and also imaging improve. This isn't really enough to tie the findings together. Were there any patients whose radiology did not improver and Cer (d18..) level remain high?

Reply:

Indeed, we included only 4 patients during the follow-up period, and the lesions of these four patients improved after regular antifungal therapy, while the level of Cer (d18:1/18:0) decreased, but there was no statistical difference. This is also the limitation of our research. Therefore, in the future, we need to include more patients, especially those during the follow-up period, to study the correlation between Cer (d18:1/18:0) levels and patients' condition.

Discussion

• Line 315-318: …."our study found that Cer level gradually returned to normal after treatment and was positively correlated with prognosis of PC" – the results states the changes did not meet statistical significance over the course of treatment, that there is little change between level at 3-4 months and 6-12 months. There is no discussion of prognosis or clinical outcome at all.

Reply:

We have modified our text as advised "Therefore, in future studies, enrolling a larger cohort of PC patients from other medical institutions, including those who have undergone treatment and lung imaging, would be necessary to further evaluate the actual sensitivity and specificity of using Cer (d18:1/18:0) as a specific diagnostic biomarker for PC. Additionally, a larger cohort would allow for a better assessment of

changes in Cer (d18:1/18:0) levels during treatment and its implication for prognosis.."

(see Page 13, line 359-364)

Reviewer C

This article presents an original clinical sphingolipidomics approach to find and evaluate a new biomarker for the diagnosis of pulmonary cryptococcosis in non-HIV patients. The manuscript is clear, well-written, and interesting and the study design with a test and validation cohort to determine the performances of the new biomarker appears rigorous. I have a few minor comments:

1) The period of patient inclusion could be precise. Was the inclusion made prospectively or retrospectively? This last point should be specified in the methods section of the article.

Reply:

The inclusion was made prospectively. We have modified our text as advised "Patients are enrolled when they are diagnosed with pulmonary cryptococcosis after pathology or culture." (see Page 5, line 128-129)

2) It might be interesting to detail in a new table (which could be a supplementary one) some features about the 47 included patients with PC. For example, it is important to know whether pulmonary cryptococcosis (PC) is associated with meningitis or disseminated cryptococcosis as serum antigen tests are rarely positive, except in cases of disseminated cryptococcal infection (Gazzoni 2010). The diagnostic criteria (positive culture, direct examination, antigen, histopathology), the EORTC classification (Donnelly 2020) as possible, probable or proven cryptococcosis and the immune status of the included patients would be also interesting to know.

Reply:

In our study, we included pulmonary cryptococcosis patients diagnosed by pathology or culture, and they did not have neurological symptoms at the time of diagnosis and follow-up. Therefore, this study is mainly aimed at patients with simple pulmonary cryptococcosis. In previous studies, the CrAg test was up to 90% of patients with cryptococcosis(Chin Med J (Engl). 2018 Sep 20;131(18):2210-2215. Mycopathologia. 2021;186(5):717-728), while in our cohort, 74.07% of the patients tested positive for CrAg

3) Line 132: a reference to the guidelines for the diagnosis and treatment of TB in China could be added at the end of the sentence.

Reply:

Thank you for your suggestions. We have modified our text as advised. (see Page 6,

line 150)

4) Line 176: Was t test used to compare percentages ? Or is it a Chi-square test ?

Reply:

The percentages as categorical variables, therefore a Fisher's exact test was used to compare percentages. We have modified our text as advised and reorganized the paragraph to provide a clearer description "Measurement data were presented as the means with standard deviations (SD), while count data were expressed as frequencies or percentages. For continuous data that were normally distributed, the t test was used to compare the means of two groups, while the Mann–Whitney test was employed for non-normally distributed data. To assess the association between two categorical variables, such as sex and the presence of symptoms, the Fisher's exact test was applied." (see Page 7, line 189-195)

5) In table 2, the clinical characteristics of the pulmonary aspegillosis (PA) and tuberculosis (TB) groups have been compared with the PC reference group. There is a risk of increased alpha error if dedicated multiple comparisons are not performed.

Reply:

In this study, our main focus was on the identification of PC and PA, as well as PC and TB, so we did not conduct multiple comparisons

6) Among the differential diagnoses of PC, there are also bacterial pneumonia which have not been taken into account in this article. At least, they should be mentioned within the limits of the disussion, like lung cancer.
Indeed, our research has some limitations. For example, lung cancer patients are easily misdiagnosed as PC patients, but are not included in the study cohort because the patients initially included do not match the age of other patients in the cohort. However, in the follow-up study, we will include as many young lung cancer patients as possible, increase the sample size, and verify the diagnostic performance of Cer in PC and lung patients. (see Page 13, line 352-356)
In addition, bacterial pneumonia is clinically different from PC, bacterial pneumonia is often accompanied by fever, and the disease develops rapidly

7) The threshold of 18.00 nM was optimised to reduce false positives in the PA and TB cohort. Strictly speaking, it should be validated on a new cohort as was done for the 12.05 nM cut-off. At the very least, this should be mentioned in the limits of the discussion.

Reply: We have modified our text as advised "In addition, although adopting a cut-off value of 18.00 nM for Cer (d18:1/18:0) significantly reduces false positives for PA and TB patients, future studies should collect a new cohort of PA and TB patients to validate this finding." (see Page 13, line 356-358)

8) The comparison of the sensitivity of serum Cryptococcus antigen (CrAg) and Cer (d18:1/18:0) may be biased. The authors compared the performances of the two tests on the test cohort where the sensitivity of Cer (d18:1/18:0) is the best. It would be preferable to calculate the performance of the tests on the validation cohort, which may better reflect real life. Furthermore, no statistical test was used to compared the sensitivity of two tests. Specificity of the serum CrAg could be calculated on the pulmonary aspergillosis (PA), the tuberculosis (TB) and the control cohorts and compared to the Cer (d18:1/18:0) specificity. Finally, serum CrAg was used as a reference method whereas lung aspirates and Bronchoalveolar lavage fluids might be better than serum to diagnose PC (Liaw 1995, Senghor 2018).

Reply:

Indeed, there are some limitations to our research. We did not use statistical tests to compare the sensitivity of the two tests. In future studies, we will include more PA and TB patients and compare the specificity of serum CrAg and Cer (d18:1/18:0).

In a study conducted by Liaw in 1995, it was indicated that “direct measurement of CrAg in lung aspirate can be a rapid and useful test for diagnosis of PC.” However, a study by Senghor in 2018 concluded that “these results do not support the routine use of the test in BALF”. Therefore, we cannot agree that “serum CrAg was used as a reference method whereas lung aspirates and BALF might be better than serum to diagnose PC.”

In addition, as the acquisition of lung aspirates and bronchoalveolar lavage fluid requires bronchoscopy, this examination is invasive. Due to technical limitations and patients' refusal, the detection of serum CrAg is often used as a basis for diagnosis. As an equally non-invasive examination, we would like to compare the diagnostic efficacy of serum Cer (d18:1/18:0) with serum CrAg, especially for those patients with HIV-negative pulmonary cryptococcosis.

9) The authors should moderate their conclusion on the prognostic value of the Cer (d18:1/18:0) on the basis of their results. Indeed, the decrease was not statistically significant at 3-4 months and no decline was observed at 6-12 months. In addition, sera from only 4 patients were used to monitor changes in levels of Cer (d18:1/18:0). A larger cohort will be required to draw definitive conclusions.

Reply:
We have modified our text as advised “Therefore, in future studies, enrolling a larger cohort of PC patients from other medical institutions, including those who have undergone treatment and lung imaging, would be necessary to further evaluate the actual sensitivity and specificity of using Cer (d18:1/18:0) as a specific diagnostic biomarker for PC. Additionally, a larger cohort would allow for a better assessment of changes in Cer (d18:1/18:0) levels during treatment and its implication for prognosis..” (see Page 13, line 362-367)