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Editorial Board Member of *World Journal of Clinical Cases*, Zaza Demetrashvili, FACS, FICS, MD, PhD, Professor, Department of Surgery, Tbilisi State Medical University, Tbilisi 0177, Georgia. zdemetr@yahoo.com

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The *WJCC* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Journal Citation Reports/Science Edition, Current Contents®/Clinical Medicine, PubMed, PubMed Central, Scopus, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 Edition of Journal Citation Reports® cites the 2021 impact factor (IF) for *WJCC* as 1.534; IF without journal self cites: 1.491; 5-year IF: 1.599; Journal Citation Indicator: 0.28; Ranking: 135 among 172 journals in medicine, general and internal; and Quartile category: Q4. The *WJCC*'s CiteScore for 2021 is 1.2 and Scopus CiteScore rank 2021: General Medicine is 443/826.

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CASE REPORT

False positive detection of serum cryptococcal antigens due to insufficient sample dilution: A case series

Wen-Yu Chen, Cheng Zhong, Jian-Ying Zhou, Hua Zhou

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Wen-Yu Chen, Cheng Zhong, Jian-Ying Zhou, Hua Zhou, Department of Respiratory, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 314000, Zhejiang Province, China

Wen-Yu Chen, Department of Respiratory, The Affiliated Hospital of Jiaxing University, Jiaxing 314000, Zhejiang Province, China

Corresponding author: Hua Zhou, PhD, Doctor, Department of Respiratory, The First Affiliated Hospital, Zhejiang University School of Medicine, No. 1882 Zhonghuan South Road, Jiaxing 314000, Zhejiang Province, China. zhouhua1@zju.edu.cn

Abstract

At present, with the development of technology, the detection of cryptococcal antigen (CRAG) plays an increasingly important role in the diagnosis of cryptococcosis. However, the three major CRAG detection technologies, latex agglutination test (LA), lateral flow assay (LFA) and Enzyme-linked Immunosorbent Assay, have certain limitations. Although these techniques do not often lead to false-positive results, once this result occurs in a particular group of patients (such as human immunodeficiency virus patients), it might lead to severe consequences.

Key Words: Cryptococcosis; Capsular antigen detection; False positive; Tissue; Case report

Core Tip: Cryptococcosis is a pulmonary or disseminated infectious disease caused by Cryptococcus, which mainly causes pneumonia and meningitis, but also skin, bone or internal organs infection. the three major cryptococcal antigen detection technologies, latex agglutination test, lateral flow assay and Enzyme-linked Immunosorbent Assay, have certain limitations. Although these techniques do not often lead to false-positive results, once this result occurs in a particular group of patients (such as human immunodeficiency virus patients), it might lead to severe consequences. Therefore, once the test results are inconsistent with the clinical symptoms, it is necessary to reexamine the samples carefully.

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INTRODUCTION

Cryptococcosis is a pulmonary or disseminated infectious disease caused by Cryptococcus, which mainly causes pneumonia and meningitis, but also skin, bone or internal organs infection. Clinically, combined with clinical manifestations and microscopic examination results, the diagnosis was made, and then confirmed by fungal culture or tissue staining. Infection is caused by inhalation of Cryptococcus in human respiratory tract, and the primary infection focus is mostly lung, which causes Pulmonary cryptococcosis (PC)[1-3]. However, immunocompetent people who are infected with cryptococcosis often have insidious onset, usually without typical clinical symptoms, mostly of which are found in physical examination, and the imaging manifestations are diverse which cause trouble in the diagnosis of PC in normal population[4-6]. At present, the diagnosis of PC mainly includes three methods: Pathogen detection, immunologic test and molecular biological detection. The detection of cryptococcal capsular polysaccharide antigen (CRAG) is considered to be the most valuable and rapid serological diagnosis methods in routine examination of cryptococcosis[7]. There are three major CRAG detection technologies for now, atex agglutination test (LA), lateral flow assay (LFA) and Enzyme-linked Immunosorbent Assay. Generally speaking, CRAG titer > 1:4 indicates cryptococcal infection, and the higher the titer, the greater the diagnostic value. Current researches suggest that CRAG test has higher sensitivity and diagnostic specificity than traditional immunodiagnostic methods and is widely applied in clinic[8-10].

However, recently, our team found 3 false positive detection cases of serum CRAG in immunocompetent patients with pulmonary lesions. One of the patients was diagnosed as PC, but the anti-cryptococcosis treatment was ineffective, and finally the patient was found to be misdiagnosed by tissue culture after lung puncture biopsy. The other two patients were detected by lung puncture and continuous re-examination of CRAG to exclude diagnosis of PC with negative results. In order to summarize the diagnosis and treatment experience of these three cases and enhance the diagnosis and treatment level of PC, the clinical data are shared as follows.

CASE PRESENTATION

Chief complaints

Case 1: A 53-year-old man, half a month ago, the chest computed tomography (CT) suggested a thick-walled cavity shadow in inferior lobe of left lung.

Case 2: A 67-year-old woman developed a cough without obvious inducement, accompanied by chest muffling and shortness of breath. These symptoms lasted for 2 wk.

Case 3: A 67-year-old male patient, was found to have new solid nodules in the middle lobe of the right lung during routine review.

History of present illness

Case 1: The patient, was found to have nodules in inferior lobe of left lung during physical examination one year ago. Half a month ago, the chest CT review of the man suggested a thick-walled cavity shadow in inferior lobe of left lung. The patient came to our hospital for further diagnosis.

Case 2: The patient developed a cough without obvious inducement, presenting paroxysmal coughing without sputum and accompanied by chest muffling and shortness of breath. These symptoms lasted for 2 wk, during which the patient had received antimicrobial therapy with oral administration of Moxifloxacin 0.5 g once a day for 5 d in the outer court, but the symptoms were still not relieved, and even aggravated. On December 19, 2018, the patient checked the lung CT examination and found that there were nodules in inferior lobe of left lung and multiple enlarged lymph nodes in mediastinum. The patient with left lung infection was further diagnosed in our hospital.

Case 3: The patient with liver transplantation two years before and had been treated with oral tacrolimus anti-rejection after liver transplantation. The patient was found to have new solid nodules in the middle lobe of the right lung during routine review and had no chief complaint. The patient came to our hospital for further diagnosis.

History of past illness

Case 1: A history of nodules in inferior lobe of left lung during physical examination one year ago, general health good.

Case 2: A history of asthma.

Case 3: A history of liver transplantation two years before and had been treated with oral tacrolimus anti-rejection after liver transplantation.

Physical examination

Case 1: The patient underwent a preliminary examination with the results of a body temperature of 36.2 ℃, blood pressure of 137/86 mmHg, pulse rate of 91 beats/min, respiratory rate of 20 beats/min and oxygen saturation of 98%. Lung auscultation suggested clear respiratory sounds in both lungs, no rales were heard, and other examinations showed no obvious abnormality.

Case 2: The patient underwent a preliminary examination with the results of a body temperature of 37.2 ℃, blood pressure of 119/73 mmHg, pulse rate of 100 beats/min, respiratory rate of 21 beats/min and oxygen saturation of 96% with two nasal cathedrals of 2L/ min. Further physical examination revealed that an enlarged lymph node about 1 cm × 2 cm in size could be touched in the left neck, which was soft with moderately activity. Lung auscultation suggested heavy respiratory sounds in both lungs, but no rales were heard, and other examinations showed no obvious abnormality.

Case 3: The patient underwent a preliminary examination with the results of a body temperature of 36.7 ℃, blood pressure of 126/75 mmHg, pulse rate of 83 beats/min, respiratory rate of 18 beats/min and oxygen saturation of 99%. Lung auscultation suggested clear respiratory sounds in both lungs, no rales were heard, and other examinations showed no obvious abnormality.

Laboratory examinations

Case 1: After admission, the CRAG detection was positive, and no other major were found in other items. The results of cerebrospinal fluid (CSF) were all negative. After 19 days of treatment, the detection of CRAG was negative.

Case 2: After admission, in routine hematology and biochemical laboratory examinations, tests for CRAG and T-cell detection of tuberculosis infection were positive, and no other major findings were found in other items.

Case 3: After admission, routine hematology and biochemical laboratory tests showed that the detection of CRAG was positive with no other major findings were found in other items. Before the treatment, the local hospital rechecked the test of CRAG and the result was negative. Three months later, the detection of serum CRAG showed a negative result.

Imaging examinations

Case 1: Half a month ago, the chest CT review of the man suggested a thick-walled cavity shadow in inferior lobe of left lung. After admission, high resolution CT (HRCT) examination of the patient indicated multiple nodules in inferior lobe of left lung, one with a thick-walled cavity and one with nodules, suggesting granulomatous inflammation and the possibility of cryptococcus (Figure 1A). After 19 d of treatment, chest CT reexamination, which revealed the formation of nodules with cavities in the left lower lobe. The lesions were enlarged, and internal cavity was narrowed compared to old CT photos (Figure 1B). The patient had three times of chest CT reexamination later, all of which indicated that the infection lesions in inferior lobe of left lung were shrinking.

Case 2: On December 19, 2018, the lung CT examination found that there were nodules in inferior lobe of left lung and multiple enlarged lymph nodes in mediastinum. After admission, the chest HRCT of the patient indicated left inferior lobe lung cancer, multiple mediastinal lymph node metastasis, and pleural effusion with a small amount of pericardial effusion (Figure 2). Attachment: cysts in pancreatic body. B-ultrasound examination presented that there were multiple TI-RADS2 types of nodules in right thyroid, multiple lymph nodes enlargement in the IV region of bilateral neck, fatty liver, no obvious abnormality in bilateral adrenal scanning and retroperitoneal scanning. After the treatment, two chest CT reexaminations later, both indicated that the left lower pulmonary lesions were continuously shrinking.

Case 3: After admission, the chest HRCT reexamination of the patient suggested nodules in the right middle lobe and bilateral lower lobe and proliferative lesions were considered (Figure 3A). Three months later, chest CT examination of the patient in local hospital revealed the absorption of lesions in right lung.

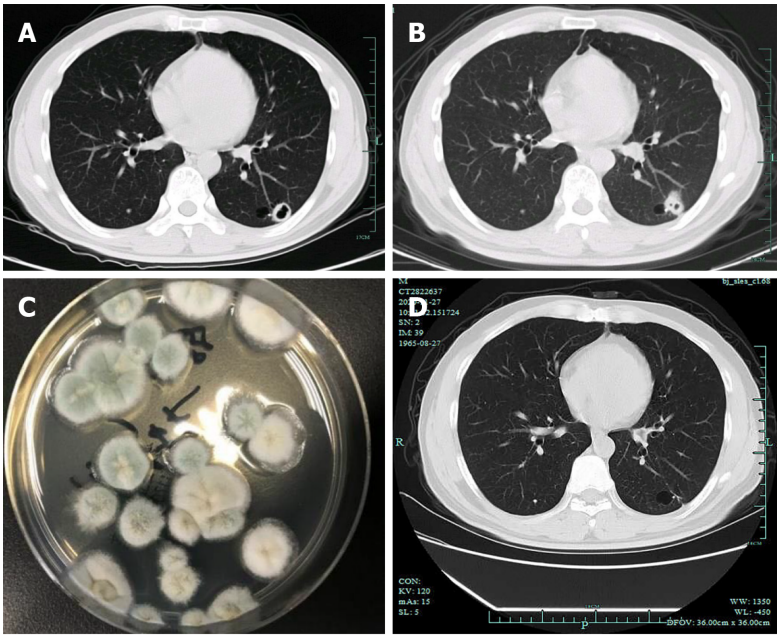

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Figure 1 High resolution computed tomography examination. A: High resolution computed tomography (CT) examination of the patient indicated multiple nodules in inferior lobe of left lung, one with a thick-walled cavity and one with nodules, suggesting granulomatous inflammation and the possibility of Cryptococcus; B: The lesions were enlarged, and internal cavity was narrowed compared to old CT photos; C: Tissue culture suggested Aspergillus spp.; D: The three times of chest CT reexamination after the treatment, all of which indicated that the infection lesions in inferior lobe of left lung were shrinking, suggesting that the treatment was effective.

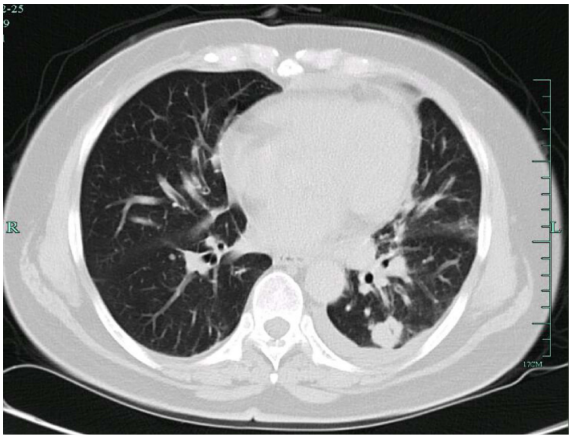

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Figure 2 Multiple mediastinal lymph node metastasis, and pleural effusion with a small amount of pericardial effusion.

Pathological examinations

Case 1: Half a month ago, no significant abnormality was observed in bronchoscopy. After 19 days of treatment, the patient underwent CT-guided puncture biopsy of the left pulmonary lesions. Tissue culture suggested Aspergillus spp. (Figure 1C).

Case 2: On December 25, 2018, tracheoscopy suggested mucosal swelling of the left upper lobe bronchus. Intraoperative EBUS detected lymph node enlargement in the seventh and eleventh groups, and EBUS-TBA was performed in the seventh group. At the same time, the patient also underwent CT-guided lung puncture and left supraclavicular lymph node puncture biopsy. Pathological prompts of three above examinations were as follows, EBUS-TBNA: adenocarcinoma CK7 (+), TTF-1 (+), NapsinA (+), CK5/6 (-), P63 (-), CgA (-), ALK-Lung (-); left lung puncture: adenocarcinoma CK7 (+), TTF-1 (+), NapsinA (+), CK5/6 (-), ALK-Lung (-), P63 (-), Ki-67 (25%); left supraclavicular lymph node puncture: metastasis of lung adenocarcinoma CK(pan) (+), CK7 (+), TTF-1 (+), NapsinA (+), CDX2 (-), GATA-3 (-), CK20 (+). Further genetic testing suggested a mutation in L858R.

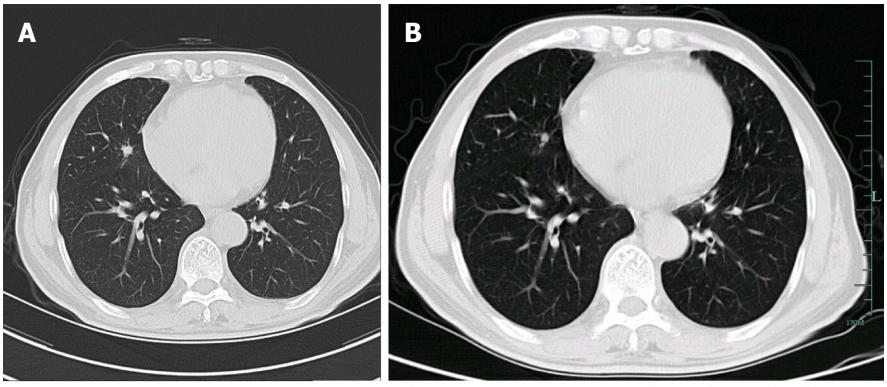

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Figure 3 High resolution computed tomography reexamination. A: The chest high resolution computed tomography reexamination of the patient suggested nodules in the right middle lobe and bilateral lower lobe and proliferative lesions were considered; B: the detection of serum cryptococcal antigen showed a negative result, suggesting that the treatment was effective.

Case 3: No evidence.

FINAL DIAGNOSIS

Depending on laboratory, radiological and pathological findings, Patient 1 was diagnosed as pulmonary aspergillosis.

According to laboratory, imaging and pathological examination, Patient 2 was diagnosed with lung adenocarcinoma in stage IV (cT2aN3M1c), and basically ruling out the possibility of cryptococcus infection.

Following the radiographic, clinical and laboratory examination, Patient 3 was diagnosed as community-acquired pneumonia.

TREATMENT

Case 1

The treatment regimen was Diflucan at a dosage of 400 mg intravenously once a day, and three d later the patient switched to oral administration and was discharged with medication. After 19 d oral administration of Diflucan (400 mg orally once a day), the lesions were still progressing after oral administration of Diflucan with the negative result of CRAG detection, the diagnosis of PC should be further verified and the treatment program was modified to Voriconazole 200mg orally twice a day, and the patient was discharged with medication.

Case 2

According to the genetic testing results, we developed an oral Conmana 125mg tid targeted therapy regimen.

Case 3

Since the patient had been treated with oral tacrolimus anti-rejection after liver transplantation and had the basis of immune injury, the possibility of PC was considered as the preliminary diagnosis combined with the imaging examination and the positive detection of CRAG. The treatment of Diflucan by oral administration was recommended. The patient then returned to the local hospital for treatment, but before the treatment, the local hospital rechecked the test of CRAG and the result was negative. After contacting our hospital, a consensus was reached that anti-cryptococcal therapy should be replaced with antimicrobial therapy.

OUTCOME AND FOLLOW-UP

Case 1

The three times of chest CT reexamination after the treatment, all of which indicated that the infection

lesions in inferior lobe of left lung were shrinking, suggesting that the treatment was effective (Figure 1D).

Case 2

The two chest CT reexaminations after the treatment, both indicated that the left lower pulmonary lesions were continuously shrinking, suggesting effective treatment and basically ruling out the possibility of cryptococcus infection.

Case 3

Three months after the treatment, chest CT examination of the patient in local hospital revealed the absorption of lesions in right lung, and the detection of serum CRAG showed a negative result, suggesting that the treatment was effective (Figure 3B).

DISCUSSION

Since the above three cases all showed false positive detection of serum CRAG in the same period, our team attached great importance to it. We reviewed the examination results of the three patients and checked the possible factors one by one. Finally, we found that the false positive in this batch of specimens was caused by improper handling by technicians. Common methods for detection of CRAG includes LA, LFA and Enzyme-linked Immunosorbent Assay (ELISA). While we used LFA for detection, which is a simple and effective laboratory method for qualitative and semi-quantitative detection of the polysaccharide antigen in serum, plasma, whole blood and CSF by immunochromatography. The principle of LFA is essentially a "sandwich" immunochromatographic strip test, which requires adding the sample and sample dilution to a suitable container (such as a test tube), and the test strip is also placed in the container. During the test, the sample is chromatographed to the gold labelled anti-Cryptococcus antigen capture monoclonal antibody and the gold labelled control antibody located on the detection membrane. If there is a CRAG in the sample, it will bind to the gold labelled anti-cryptococcus antibody. The bound gold labelled antibody (GAB) antigen complex continues to be chromatographed on the membrane by capillarity and reacts with detection strips containing immobilized anticryptococcal monoclonal antibodies. The GAB antigen complex forms a "sandwich" structure at the detection strips and displays a visible detection strip. As long as there is normal chromatography and reagent reaction, the chromatography of any positive or negative samples will cause the gold labelled control antibody to move to the control strip, and the immobilized antibody will combine with the gold labelled control antibody to form a visible control strip. Positive test results will show two bands (test band and control band) and negative test results will show only one band (control strip). If no control strip appears, the test is invalid[8,11,12].

The kit used in our hospital is IMMY's CrAg Lateral Flow Assay (colloidal gold immunochromatography). First, add a drop of sample dilution to the microcentrifuge tube, then add 40 μL of sample. Next the white end of the CRAG test strip was immersed in the sample solution, and the result was read after 10 minutes. Diluting the sample is a critical step and usually about 50 μL of sample dilution is needed. Although this is not emphasized in the product specification, studies have shown that insufficient sample dilution is an important cause of false negative or false positives in test results[13,14]. According to the investigation, these three cases were caused by a newly employed technician who did not add enough sample diluent during the operation, which resulted in false positive in the test.

LITERATURE REVIEW

Although the detection of CRAG has high clinical value in the rapid diagnosis of cryptococcosis, we still cannot ignore the false positive results that may occur in the detection process. For once the above results are misjudged, it is easy to cause misdiagnosis. We searched on Pubmed with terms such as "cryptococcosis", "cryptococcal capsular antigen detection", "latex agglutination test", "colloidal gold immunochromatography", "enzyme-linked immunosorbent assay", and "false positive", and excluding the literature that simply discuss the "false-negative" of the above three techniques and the literature that study other methods for diagnosing cryptococcal. Only the reports on false positive results of CRAG detected by LA, LFA and ELISA were considered, including main clinical features and detection methods. A total of 4 cases were included (Table 1), as well as 8 other related studies. After reviewing the literature, we found that the three commonly used CRAG technologies (LA, LFA, and ELISA) in the market have the possibility of false positive results. Among the 4 included cases and 3 cases we reported, 4 cases adopted LA and 3 cases adopted LFA. A recently reported case of false positive adopted LA was from systemic lupus erythematosus (SLE) patient in active phase and complicated with Libman-Sacks endocarditis[15]. The patient developed onset of sudden disturbance of consciousness, recovered consciousness after 16 h, and the neurological examination was essentially normal, but the

Table 1 Case reports of false positive cryptococcal antigen

Ref.	Age (yr)/gender	Country	Underlying disease (including Immunosupressive disease or drug use)	Sample source/Detection method/Reagent company	Basis of diagnosis	Possible causes	Outcome
Matsumoto *et al*[24], 2019	58/F	United States	SLE with secondary immune thrombocytopenic purpura	CSF/LA/CALAS® Meridian Bioscience Inc., Cincinnati, Ohio	(1) Reexamination of serum LA suggested negative; (2) Both CSF ink staining and culture results were negative; and (3) Head MRI showed abnormal signals in the left superior frontal cortex, consistent with subacute ischemia; Cardiac hypertrophy suggested Libman-Sack endocarditis. The final diagnosis was "thromboembolic cerebrovascular transient ischemia"	Nonspecific interference with the autoantibodies of SLE circulation in patients	Survived
Augeret al [25], 2019	26/M	United States	None	CSF/LA	Blood and CSF culture identified Df-2 infection	Common antigenic surface components may exist in DF-2	
Volozhantsev *et al*[26], 2020	33/M	United States	Aplastic anemia; after bone marrow transplantation	Serum/LA/ IM Inc, American Microscan	The autopsy confirmed Trichosporon asahiti infection	Similar structures of polysaccharides may exist in Trichosporon asahiti	Died
Zhu *et al*[27], 2018	29/F	United States	Non-Hodgkin's lymphoma	CSF/LA/CALAS, Meridian Diagnostics, Cincinnati, Ohio, and CRYPTO-LA, International Biological Laboratories, Cranbury, New Jersey	CSF culture indicated Stomatococcus infection	Stomatococcus infection may cross-react with LA	Died
This case 1	53/M	China	None	Serum/LFA/ IMMY Immuno-Mycologics, Norman, Oklahoma, United States	(1) Anticryptococcal treatment failed; and (2) Lung puncture tissue culture suggested aspergillus	Insufficient sample dilution	Survived
Case 2	67/F	China	Bronchial asthma	Serum/LFA/ IMMY Immuno-Mycologics, Norman, Oklahoma, United States	Lung biopsy and left supraclavicular lymph node biopsy indicated lung adenocarcinoma	Insufficient sample dilution	Survived
Case 3	67/M	China	Post-orthotopic liver transplantation	Serum/LFA/ IMMY Immuno-Mycologics, Norman, Oklahoma, United States	(1) The local hospital reexamination of CRAG was negative; and (2) Antibacterial therapy was effective	Insufficient sample dilution	Survived

SLE: Systemic lupus erythematosus; CSF: Cerebrospinal fluid; LA: Latex agglutination; MRI: Magnetic resonance imaging; CRAG: Cryptococcal antigen.

initial CRAG of CSF was positive. Then the patient began to receive anti- cryptococcal therapy. CSF ink staining and culture results were both negative after 3 days, and the CRAG of CSF was negative after reexamination. The first positive result was considered unreliable, so the anti-cryptococcal treatment was suspended. The reason for this false positive result may be caused by the non-specific interference of circulating autoantibodies in active SLE patients, especially when the titer of serum anti-nuclear antibodies was high[15]. In addition, it has been reported that fungal infection caused by Trichosporon asahii or bacterial infection caused by Stomatococcus or Capnocytophaga can lead to false positive results in the detection of CRAG, and these positive results usually show low titer[16-18]. Besides, there are some rare cases of false positives adopted LA, including contamination of samples by substances in the BBL Port-A-cul specimen transport bottle and the inactivation of invertase vials of the test kits[19, 20].

LFA is considered to have better operability and stability than LA and the Chinese consensus believes that LFA method has a low probability of false positive, and has replaced the traditional screening method for cryptococcal infection, due to its simple operation, fast detection speed (< 15min), simple technology, less experimental instruments, and no need for refrigerated reagents[14-17]. Some studies

Table 2 Possible causes of false positive results in three cryptococcal antigen detection techniques

Detection method	LA	LFA	ELISA
Sample	Cerebrospinal fluid/Serum	Cerebrospinal fluid/Serum/Plasma /Whole blood	Cerebrospinal fluid/Serum
Possible causes of false positive	Serum of rheumatoid factor, agarose dehydration, hydroxyethyl starch, and containing Fe^{3+}/dL > 200 mg was present, Circular slides are not properly washed, the inactivation of Streptomyces protease in the kit and some nonspecific reactions in patients with HIV infection occur	LFA has antigenic cross-reaction with aspergillus which may lead to false positive results[1]; Sample dilution is insufficient[2]	Samples are Infected with other microbial infections, such as Trichosporon[1]; Reagents and samples to be tested are contaminated[2]

[1]Lateral flow assay (LFA) should not be used as a screening test for the general population, but only should be performed when clinical evidence suggests the possibility of cryptococcosis.
[2]LFA may also have potential interference factors, including samples pretreated with 2-mercaptoethanol or those containing vaginal ointment, caffeine, ascorbic acid, itraconazole, amphotericin B, acetaminophen or acetylsalicylic acid. However, the above factors have not been systematically evaluated.
LA: Latex agglutination; LFA: Lateral flow assay; ELISA: Enzyme linked immunosorbent assay; HIV: Human immunodeficiency virus.

have found that when the antigen titer in the sample is too high or the sample is not diluted enough, a post-zone phenomenon, also known as Prozone phenomenon (It is also called HOOK effect in the kit instruction), may occur which will interfere with the antigen-antibody reaction necessary for the display of positive test, resulting in false negative results[21-23]. But, accoeding to the investigation, it is puzzling that the three cases we reported had false positive results due to insufficient dilution of samples. This phenomenon is extremely rare, and only a few literatures have mentioned it, but its mechanism has never been discussed in depth. We proposed a relatively reasonable explanation. LFA detection of CRAG is achieved by capturing cryptococcal capsular polysaccharide components in serum or CSF samples with antibodies against cryptococcal capsular polysaccharide. This polysaccharide component is not unique to Cryptococcus, many microorganisms in nature secrete capsular polysaccharides (such as Streptococcus pneumoniae, Streptococcus group B, Streptococcus suis, *etc.*)[24-26]. Although LFA can detect CRAG of four major cryptococcus serotypes (type A and D are cryptococcus neoformans, type B and C are Cryptococcus Gattinii), capsular polysaccharides produced by other microorganisms are likely to be associated with CRAG in a cross-structure. False positive results may occur when the sample is not sufficiently diluted and there is a similar structure of CARG in the patient. As mentioned above, this may be the cause of false-positive capsular antigen results after certain fungal or bacterial infections.

In addition, through literature review, we found that with the progress and promotion of LFA and LA in recent years, ELISA was rarely used to detect CRAG in clinical diagnosis of cryptococcosis, but there were still reports on the comparison of three detection technologies[27-32]. Through these reports and some related works, we summarize the limitations of the three techniques in detecting CRAG (Table 2).

CONCLUSION

At present, with the development of technology, the detection of CRAG plays an increasingly important role in the diagnosis of cryptococcosis. However, the three major CRAG detection technologies have certain limitations. Although these techniques do not often lead to false positive results, once this result occurs in a special group of patients (such as human immunodeficiency virus patients), it might lead to serious consequences. Therefore, once the test results are inconsistent with the clinical symptoms, it is necessary to carefully reexamine the samples. Especially for LFA and LA, the samples can be fully diluted or segmented dilution to avoid false positive results. It is certainly that in the diagnosis, fluid and tissue culture should also be improved, combined with imaging, ink staining and other methods to further improve the accuracy of the diagnosis.

FOOTNOTES

Author contributions: Chen YW and Zhong C contributed equally to this work and should be considered as co-first authors; Chen YW conceptualized, drafted, and led the writing of the manuscript; Zhou H and Zhou JY provided overall conceptual guidance for the study; Chen YW closely worked with Zhong C to develop the article; all authors have contributed to the writing and reviewed and approved the final manuscript.

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ORCID number: Wen-Yu Chen 0000-0003-0412-2913; Cheng Zhong 0000-0002-1936-3448; Jian-Ying Zhou 0000-0002-8924-935X; Hua Zhou 0000-0001-6397-3203.

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