Clinical Usefulness of Lateral Flow Antigen Detection Assay and PCR for Laboratory Diagnosis of Cryptococcal Infections

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ABSTRACT

Introduction: *Cryptococcus neoformans* is the most common opportunistic fungal pathogen that affects the central nervous system and meningitis is caused by *Cryptococcus* a leading cause of morbidity and mortality among people living with HIV. About 5% of meningeal infections are caused by *Cryptococcus neoformans*, which is tops among non-tuberculous causes. Being the most common opportunistic infection in AIDS patients, it has been reported to account for about 2-7% of all opportunistic infection. India ink examination for the demonstration of the capsule is the most widely used technique for rapid diagnosis, although it has less sensitivity. Microscopic methods and fungal culture though considered as specific tests they show the sensitivity of 50–80%, and fungal culture takes 2-3 days for the report.

Aims and Objective: The present study was carried out to assess the utility of Cryptococcal antigen detection by lateral flow assay (LFA) for the diagnosis of cryptococcal meningitis.

Methodology: Cerebrospinal fluid (CSF) sample sent for India ink examination for *Cryptococcus* collected from 116 patients suspected of cryptococcal meningitis over 1 year were included in the study. These samples were subjected to Gram stain, fungal culture, antigen detection by LFA and PCR.

Results: Of the 116 CSF samples tested, 20 (17.2%) samples were positive by LFA, among which 5 (4.31%) samples were also positive in India ink preparation, Gram's staining, fungal culture and PCR.

Conclusion: Antigen detection for Cryptococci must be prioritized over microscopy and culture for the diagnosis. Identification of Cryptococci by antigen detection and molecular methods are very much useful in the laboratory diagnosis of Cryptococcal infection.

Key Words: Lateral flow assay, *Cryptococcus*, India ink, CS, Antigen, PCR

INTRODUCTION

Opportunistic fungal pathogens are gaining more importance over the last decades due to the increase in the cases of AIDS. Cryptococcosis is now considered an AIDS-defining illness and also an important cause of Meningitis seen in immunocompetent and immunocompromised individuals.³

Cryptococcus neoformans causes opportunistic infection in immunocompromised patients with underlying conditions such as leukaemia, HIV and cancer, or seen in those patients taking corticosteroid medications and leads to pulmonary infection, meningitis or meningoencephalitis. Cryptococcal meningitis (CM) tops the list with a prevalence of around

5% per cent in India and Cryptococcosis is the second most common fungal infection after candidiasis in HIV patients. Worldwide, around 7 to 10 per cent of patients with AIDS are affected.¹

Cryptococcal meningitis has been reported in India as the most common opportunistic infection in AIDS patients and accounts for 2-7% of all opportunistic infections according to studies conducted in large cohorts across places like Delhi (3 to 7%), Mumbai (4 to 7%), and Chennai (2%).²

To reduce the morbidity and mortality associated with the infection, early diagnosis and the provision of specific antifungal therapy are necessary.⁴ Diagnosis depends on detailed

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clinical history, clinical examination, imaging studies and laboratory tests like India ink examination for capsule demonstration, fungal culture, antigen detection and molecular methods.

Microscopic methods and fungal culture though considered as specific tests; show lesser sensitivity of 70–90% for microscopy and 80–92% for fungal culture.⁵ Culture for *Cryptococcus* takes more time and large volumes of samples are needed to get growth. Recently, lateral flow assay for *Cryptococcus* (CrAg) detection has become the widely recognized test for diagnosis of *Cryptococcus* infection which detects the presence of cryptococcal antigen in blood or cerebrospinal fluid before the appearance of clinical manifestations are considered as a screening test for the HIV infected patients before initiating ART for adults and adolescents.⁶ Molecular method like PCR detects the *Cryptococcal* DNA and provide an accurate result. So, considering the need for early diagnosis and initiation of the antifungal treatment, this study was carried out to determine the usefulness of antigen detection and PCR for diagnosis of Cryptococcal infection in HIV and non-HIV infected patients in a tertiary care hospital.

Methodology:

The study was carried out in the Department of Microbiology at JSS hospital for the period one year from January 2019 to December 2019 after obtaining the institutional ethical committee clearance (Ethical clearance letter number: JSS/MC/PG/6227/2018-19).

Cerebrospinal fluid samples received from clinically suspected cryptococci meningitis were included. CSF samples showing bacteria or yielding bacterial growth in culture were excluded from the study. Detailed clinical history as regards age, sex, occupation, history of presenting illness, history, retroviral status and antifungal treatment history was obtained and recorded in proforma after taking consent from patients.

CSF samples received in the Microbiology laboratory were processed without delay and were maintained at 37°C in an incubator before performing the tests. The samples were centrifuged at 1000 rpm for 15min and the sediment was used for microscopy (Gram stain and India ink examination), fungal culture and PCR while the supernatant was used for antigen detection.

Microscopic examination:

India ink preparation: A loopful of centrifuged sediment of CSF sample was suspended along with India ink on a sterile glass slide covered with the coverslip and observed under the microscope for the oval to spherical capsulated yeasts. Grams staining was also performed on deposit of centrifuged CSF sample.

Loopful of centrifuged deposit of CSF was inoculated onto two Sabouraud's dextrose agar slants and were incubated at 37°C and 25°C separately for 7 days. Slants were observed for growth after 48–72 hours. Growth observed was subjected to wet mount, Gram's stain and urease test. Species were identified by the Vitek-2 system.

A lateral flow test for the detection of cryptococcal antigen was performed using BIOSYNEX® CryptoPS kit. The test was done following the manufacturer's instructions and interpreted accordingly.

Polymerase chain reaction (PCR) was performed on the CSF sample. Extraction of DNA was done by using SpinStar Total DNA kit 2.0. The procedure was carried out according to the kit manufacturer's instructions. Real-time PCR was performed by *Cryptococcus* Real-Time PCR kit (RUO) from TechServ Healthcare Pvt Ltd, following the instructions in the manual provided. This assay was performed based on the hot-start version of modified *Thermus brockianus* DNA polymerase using fluorescent SYBR Green I dye. The modified hot-start Tbr polymerase gets activated following the initial denaturation step. The SYBR green I which is specific for double-stranded DNA binds to the amplified double-stranded PCR product, thereby permitting the direct detection of amplified DNA without labelled probes. This real-time PCR assay, specifically amplified the Internal transcribed spacer sequence which is a spacer DNA situated between the small-subunit rRNA and large-subunit rRNA genes.

RESULTS

A total of 116 CSF samples was received from suspected cases during one year (January to December 2019) by the microbiology laboratory of JSS hospital.

The 116 suspected CSF samples were subjected to Grams staining, India ink preparation, lateral flow assay and fungal culture. Out of 116 samples, 5 (4.3%) samples were positive by Gram stain and 111(95%) samples were negative. India ink preparation showed capsulated *cryptococci* in 5 of 116 samples (4.3%). In fungal culture 5 of 116 samples (4.3%) samples yielded the growth of *Cryptococcus neoformans*. In contrast, the Lateral flow assay detected cryptococcal antigen in 20 of 116 samples (17.24%). Among these 116 samples, 10 samples (5 positive and 5 negatives from LFA) were randomly selected and subjected to PCR. The 5 samples positive by Grams stain, India ink and which showed Cryptococcal growth in culture were also positive by PCR and five lateral flow negative samples were also negative by PCR.

Among 20 antigen-positive patients, the majority were in the age group of 31 to 40 years (7 patients, 35%) and 6 (30%) were in the age group of 41 to 50 years. Males were infected

in 70% (14 out of 20) than females 30% (6 out of 20). The primary presentation of illness of these 20 positive patients (LFA positive) was a headache, altered sensorium and fever which were present in all 20 (100%) patients. Vomiting was observed in 5 cases. Other manifestations were seizures and blurring of vision. All the antigen-positive patients were retro positive patients. Among 20 positive patients, 5 patients received fluconazole antifungal treatment by injection or orally for 4 weeks, 13 patients received fluconazole of 400 mg/kg/day for 2 weeks and 2 patients received Amphotericin B of 0.7mg/kg/day for 2 weeks since they were found allergic to fluconazole. Amongst the 20 positive patients, 17 (85%) patients recovered without any complications and 3 (15%) patients expired.

DISCUSSION

Cryptococcal meningitis continues to cause a significant burden of death among HIV infected individuals. The diagnostic use of detection of cryptococcal capsular polysaccharide antigen (CrAg) in serum and cerebrospinal fluid by lateral flow assay is a promising test for rapid diagnosis of cryptococcal infections.

Among 116 CSF samples, India ink examination and Grams staining showed positive results in 5 (4.31%) samples which are in agreement with the similar study of Mahale K where 18 (7.43%) were positive out of 242 CSF samples.^{3,7} Since, India ink staining is the common diagnostic tool to identify *Cryptococci* in CSF, yet the sensitivity of India ink microscopy is only <86%.⁸ Fungal cultures in a similar kind of study showed 8.3% positivity (20 out of 242) while culture-positive patients were 4.31% (5 out of 116) in the present study.³ Low culture/microscopy positive rate in our study could be due to low CSF volume that was used for culture/microscopy or may be due to prior use of antifungal drugs for treatment or may be due to the presence of low count of yeasts during the early phase of the infection. The diagnostic value of culture can be improved by using a higher volume of CSF.⁹

The present study involved the detection of cryptococcal antigen among suspected patients by lateral flow assay. The seropositivity rate in our study was 17.24%. The study conducted by Lakshmi et al. showed the seroprevalence of 11.8% (25 out of 211) in their study, and they used the latex agglutination method (LA) for antigen detection.¹ Out of 20 samples tested in our study, five were also positive by India ink, Gram stain and culture. Fifteen more cases were detected by lateral flow assay which may be because lateral flow assay is more sensitive than fungal culture and India ink in early stages or may be due to persistence of antigen during treatment as observed in earlier studies conducted elsewhere.¹⁰

Among 116 CSF samples, 10 samples (5 antigen-positive and 5 antigen-negative samples) sent for Cryptococcal antigen detection were subjected to PCR. All the 5-antigen positive and 5 culture-positive samples were also positive by PCR. All 5-antigen negative were also negative by PCR showing PCR as a reliable technique in comparison to conventional techniques for the early diagnosis of *Cryptococcus* infection to reduce the mortality associated with it.²

Fever, headache and altered sensorium were present in all 20 patients positive by LFA findings which are analogous to the study by Vasanth Baradkar et al. where the headache was observed in (100%), altered sensorium (100%), fever (100%); neck stiffness in 90% of the patients.¹¹ A study conducted in south India observed headache in (92.31%), fever (79.49%), altered sensorium (71.79%) and neck stiffness in (66.67%) their patients.¹ Another study noted headache in 90%, fever in 85%, vomiting in 60% followed by altered sensorium in 40% of patients indicating varied clinical manifestations that can be seen in cryptococcosis patients.¹²

Among 20 positive patients' majority were in the age group of 31 to 40 years accounting for 7 (35%) and 41 to 50 years 6 (30%) findings which are keeping with earlier studies conducted by Susheelkumar et al. where 30 (65.2%) of the patients belonged to 25 to 49 years age group.¹³

Majority of infected patients were males 70% (14 out of 20) than female 30% (6 out of 20), showing a similar data of another study, where in 72 % (72 out of 100) were males and 28% (28 out of 100) were females which may be due to differences in exposure.^{11, 14}

Among 20 positive patients, 5 patients received fluconazole injection or orally for 4 weeks, 13 patients received fluconazole of 400 mg/kg/day for 2 weeks and 2 patients received Amphotericin B of 0.7mg/kg/day for 2 weeks since they were found allergic to Fluconazole. An improved response was seen in 17 patients irrespective of their HIV status to the treatment showing a recovery rate of 85% and 3 patients expired during the study period showing a mortality rate of 15%. The three patients had underlying risk factors like retro positive status, other clinical complications such as chronic alcoholic liver disease with pleural effusion, disseminated tuberculosis, intracranial arteriovenous malformation (AVMs) which could be the cause of death.

CONCLUSION

Although microscopy and fungal culture are considered as gold standard techniques for the identification of *Cryptococcus* infection, they may give false-negative results during the early stages of the disease. The Cryptococcal antigen can be present in the body despite negative culture and India ink tests that can be identified. Therefore, detection of Cryptococcal

antigen by lateral flow assay from the clinical samples such as CSF, serum or plasma would help in the early diagnosis of *Cryptococcosis* and screening the patients attending antiretroviral therapy (ART) clinics to treat the patients with antifungal drugs before the development and manifestation of *Cryptococcus*. PCR though expensive, is a good adjunct test for rapid diagnosis of cryptococcosis in seriously ill patients.

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