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Original Article

Microbiology Section

Evaluation of Lateral Flow Assay for the Diagnosis of Cryptococcal Meningitis and its Comparison with the Gold Standard and Other Laboratory Tests

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ABSTRACT

Introduction: Cryptococcosis is a potentially fatal fungal disease affecting both immunocompromised and immunocompetent individuals. Hence, a Point Of Care Test (POCT) is required with higher sensitivity and specificity (100%), for the rapid detection of cryptococcosis which will be life saving for the patient.

Aim: To evaluate the usefulness of Lateral Flow Assay (LFA) for rapid detection of Cryptococcal Antigen (CrAg) from Cerebrospinal Fluid (CSF) sample.

Materials and Methods: This diagnostic/pilot study was conducted in Mycology Laboratory, Sri Ramachandra Medical College and Research Institute, Chennai, Tamil Nadu, India, over a period of six months (June 2019-November 2019). The CSF samples (n=37) from patients with suspected meningitis were considered for the study. Direct microscopy with gram stain, negative stain and histopathological stains was performed for all the CSF samples. Culture was done on basal medium, enriched media and special media. *Cryptococcus* genus specific Polymerase Chain Reaction (PCR) was also performed to confirm the isolates grown on the culture. Serological tests like Latex Agglutination Test (LAT) and LFA were also performed on all the samples. The LFA results were compared with microscopy, culture and LAT.

Results: Among the 37 suspected meningitis patients, three grew *Cryptococcus* in culture which was considered as the gold standard in the diagnosis of Cryptococcal Meningitis (CM). Direct microscopic techniques had 100% specificity however their sensitivity was less i.e., 67%. LAT had a sensitivity of 100% and specificity of 94%. However, LFA in present study had a very good sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) (100%).

Conclusion: The LFA for the detection of CM is considered to be an effective method when compared to the other conventional and serological methods with higher sensitivity and specificity. Hence, in present study authors evaluated the use of LFA in diagnosis of CM and found that this assay when used as a POCT, can give the results in short duration. Considering this as a pilot study, further studies including higher number of samples are essential to confirm the effectiveness of the findings.

Keywords: Cryptococcal antigen, Immunochromatography, Point of care test, Sensitivity, Specificity

INTRODUCTION

Globally, the annual estimate of Cryptococcal disease-related deaths exceeds 180,000 [1]. This fungal infection is preventable and treatable yet, due to the delay in diagnosis it continues to be associated with extreme morbidity and mortality [2]. The infection caused by *Cryptococcus* spp. is collectively known as Cryptococcosis, which is a major global health problem since the advent of Human Immunodeficiency Virus (HIV) pandemic in 1980 [3]. *Cryptococcus* spp. primarily causes pulmonary cryptococcosis as the route of entry of the propagules is by inhalation. This mainly occurs in immunocompetent individuals. In immunocompromised individuals the infection gets disseminated to the brain causing CM. The genus *Cryptococcus* includes three main species among which *Cryptococcus neoformans* (*C. neoformans*) is most commonly involved in causing CM [4].

The laboratory diagnosis of cryptococcosis is generally based on direct microscopic demonstration, culture, serological tests for the Cryptococcal Antigen (CrAg) detection and molecular assays. Microscopic methods are highly specific, but may have low sensitivity and require expertise for its interpretation [5]. Culture though considered as the gold standard, may be time consuming. PCR offers an excellent alternative for the early diagnosis of cryptococcosis compared to conventional methods, and can detect low fungal loads [6]. But PCR can be performed only in laboratories with good molecular setup and requires expertise for performance and interpretation.

Serological tests are found to have more sensitivity and specificity when compared to other tests. LAT is more sensitive but suffers from the limitation of false positivity as well as high rates of false negativity. Also, performing LAT is a tedious process, it require a rapid immunochromatographic test to detect CM with a short turnover time. LFA is instrument free, simple and a rapid diagnostic test having higher sensitivity and specificity when compared to LAT [7,8]. Thus, in this study, the aim was to evaluate the performance of LFA for diagnosing CM, by comparing the results obtained by LFA with the gold standard (culture) and other tests (microscopy and serology).

MATERIALS AND METHODS

This diagnostic/pilot study was conducted in the Mycology Laboratory, Sri Ramachandra Medical College and Research Institute over a period of six months (June 2019-November 2019). The study was started after obtaining clearance from Institutional Ethics Committee (IEC) (REF: CSF/19/MAY/77/156).

Inclusion criteria: The CSF samples from all patients of suspected meningitis (based on clinical presentation and biochemical analysis of CSF) which was sent to the laboratory in six months were included in the study.

Exclusion criteria: Specimens with visible lipids or any other obvious signs of contamination were excluded from the study.

Study Procedure

Sample collection and preservation of the culture: A total of 37 CSF samples from patients with suspected meningitis were

considered for the study. Direct microscopy, histopathological examination, culture and serological tests were performed for all the samples. Culture positive isolates were maintained in Sabouraud Dextrose Agar (SDA) slopes and stored at room temperature.

Phenotypic characterisation: Microscopic examination of all the 37 samples was done by Gram stain, Negative stains (India Ink and Nigrosin) and Histopathological stains (Giemsa stain and Haematoxylin & Eosin). All the samples were inoculated into basal fungal culture media (SDA and Oat meal agar), special media (bird seed agar, cumin seed agar, coriander seed agar) and differential agar {*Cryptococcus* differential agar, Creatinine Dextrose Bromothymol blue Thymine agar (CDBT)} for culture identification. *Cryptococcus* spp. utilise the phenolic and polyphenolic compounds present in the special media, producing melanin which is absorbed by the yeast cell wall forming a tan to reddish-brown pigmented colonies. Differential agar helps in the identification of different species of *Cryptococcus*, as *C. neoformans* produce dry blue coloured colonies, *C. gattii* produces brown mucoid colonies and *C. laurentii* produces dry brown colonies. CDBT helps in the further identification of the serotypes of *Cryptococcus* spp. In CDBT agar, serotype D (*Cryptococcus neoformans* var. *neoformans*) produces orange coloured colonies, serotype A (*Cryptococcus neoformans* var. *grubii*) does not grow, whereas *C. gattii* produces blue green colonies.

Genotypic characterisation: The PCR was performed for the isolates which grew in culture, for the confirmation of the identification by phenotypic methods.

DNA extraction (Phenol-Chloroform method): A loopful of colony was mixed with 500 µL of lysis buffer Tris HCl EDTA Sodium chloride Sodium dodecyl sulphate (TESS buffer) in a microcentrifuge tube and kept in a water bath at 100°C for 1 minute. To this mixture 500 µL of Phenol: Chloroform (1:1) was added and vortexed. The tube was centrifuged at 10,000 rpm for 10 minutes. The aqueous layer was transferred to a new tube and equal volume of chloroform was added, vortexed and centrifuged at 10,000 rpm for 10 minutes. Equal volume of ice cold absolute Isopropyl Alcohol (IPA) was added and mixed gently and the tubes were spun at 10,000 rpm for 10 minutes. The aqueous layer was discarded and the resultant Deoxyribonucleic Acid (DNA) pellet was washed in 200 µL of 70% ethanol and centrifuged at 10,000 rpm for 10 minutes. The alcohol was discarded and the tubes were air dried. The DNA pellet was resuspended in 50 µL elution buffer/nuclease free water and stored at -20°C until use.

***Cryptococcus* genus specific Polymerase Chain Reaction (PCR):** The PCR was performed using the genus specific primers CN4 F (5'ATC ACC CTA CCA TTC ACA CATT-3') and CN5 R (5'GAA GGG CAT GCC AGA TGT TTG3') along with suitable positive and negative controls. These primers flanking ITS rDNA region will generate a fragment of 136 bp.

Composition of PCR reaction mix and reaction condition: The PCR master mix was prepared containing 10 µL of PCR mix (Takara, Japan), 0.5 µL of forward (CN4) and reverse primer (CN5), 7 µL of template DNA, and the volume made upto 20 µL with sterile nuclease-free water. The reaction mixtures were amplified in a thermal cycler (Veriti 96 well, Applied Biosystems, USA), with the following program: 95°C for 5 minutes, followed by 35 cycles consisting of 95°C for 30 seconds, 67°C for 30 seconds, and 72°C for 30 seconds, with a final extension period at 72°C for 7 minutes. After thermal cycling, 10 µL of the amplified product was run on a 1.5% (wt/vol) agarose gel, stained with ethidium bromide, and visualised with ultraviolet light.

Serological tests: Serological tests like LAT test and LFA were performed on all the samples.

Latex Agglutination Test (LAT): Samples were centrifuged and the supernatant was used for the latex test of *C. neoformans* antigen detection. The test was performed using the CALAS kit Meridian Bioscience (Lot no: 140100K308), according to the manufacturer's instructions. Boiling the CSF specimens and then cooling them to room temperature prior to testing will limit the non specific interference. The positive control should give a positive reaction with the detection latex and a negative reaction with the control latex. LAT CSF titres of 1:4 or less are presumptive evidence of central nervous system infection by *C. neoformans*, still additional follow-up and culture are strongly recommended. CSF titres of 1:8 or greater (significant titre) from patients with suspected meningitis strongly suggest infection by *C. neoformans*.

Lateral Flow Assay (LFA): The LFA was performed using the BIOSYNEX Crypto PS (Lot no:180220 & 180221), according to the manufacturer's instructions. It is a rapid immunochromatographic test for the semi-quantitative detection and titration of *Cryptococcus* spp. capsular antigens in CSF to guide the diagnosis of CM. The test does not require any pretreatment of the samples.

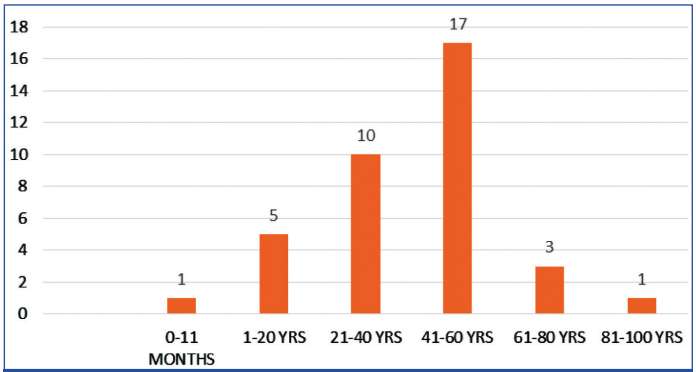
Interpretation: Two distinct coloured lines in the control (C) and test line (T1)-positive. Three distinct coloured lines in the control (C) and test lines (T1) and (T2)-Strong positive. A coloured line appearing in the control (C) and no lines on both the test lines-negative. The test line (T1) appears at 25 ng/mL of capsular antigen and the test line (T2) appears at 2.5 µg/mL of capsular antigen. No visible coloured line in the control line (C) but coloured lines present in both the tests (T1) and (T2) is considered invalid [9].

STATISTICAL ANALYSIS

The sensitivity, specificity, PPV and NPV were calculated using Statistical Package for the Social Sciences (SPSS), Version 17.0 software considering culture as gold standard.

RESULTS

Age and gender distribution: The age distribution of individuals suspected with meningitis ranged from 0 months-85 years. The age distribution is shown in [Table/Fig-1]. Among the 37 patients with suspected meningitis, 23 of them (62.16%) were male and 14 of them (37.84%) were female. Out of the three patients with proven CM, two were male and one was a female patient.

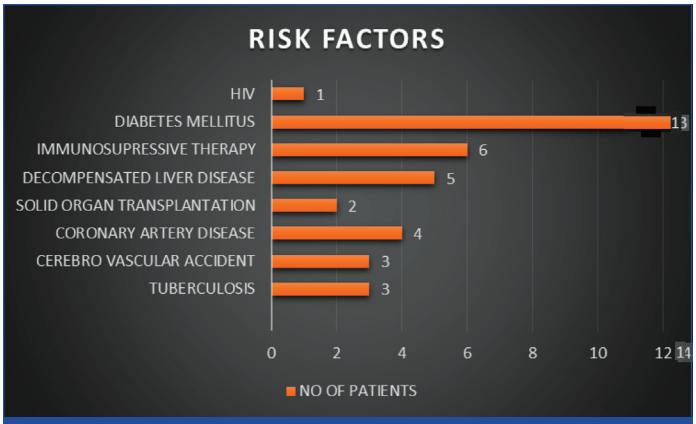


[Table/Fig-1]: Age distribution among patients with suspected meningitis.

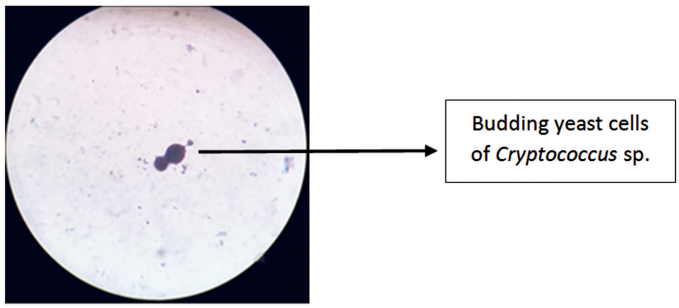
Co-morbid conditions and risk factors: Among the 37 patients with suspected meningitis diabetes mellitus (13/37), immunosuppressive therapy (6/37) and Decompensated Liver Disease (DCLD) (5/37) were the major risk factors/co-morbid conditions associated. The distribution of other risk factors/co-morbid conditions among the patients is shown in the [Table/Fig-2]. Out of the three patients with proven CM one patient had DCLD and one was on immunosuppressive therapy and the third patient was with uncontrolled diabetes mellitus.

Direct Microscopy

Gram stain: Among the 37 CSF samples, only two of them showed spherical gram positive yeast cells with narrow-necked budding [Table/Fig-3].

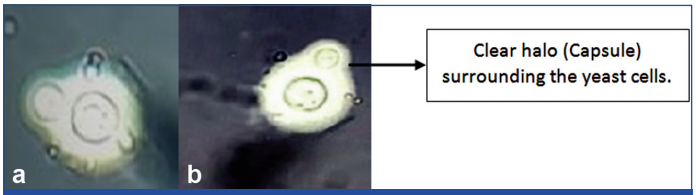


[Table/Fig-2]: Distribution of underlying risk factors/co-morbid conditions.



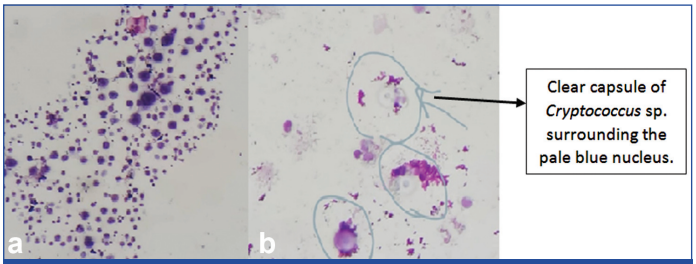
[Table/Fig-3]: Gram stain showing spherical gram positive budding yeast cells (100x).

Negative stains- India Ink and Nigrosin stain: Out of the 37 samples, only two samples showed spherical budding yeast cells with a clear halo around, which represents the presence of capsule in India Ink [Table/Fig-4a] and Nigrosin stain [Table/Fig-4b].



[Table/Fig-4]: Negative stain of CSF sample showing clear halo around the yeast cell- India Ink (a) and Nigrosin stain (b) (40x).

Histopathological stains: Among the 37 samples processed, only two samples showed purple coloured spherical, budding yeast cells of *Cryptococcus* spp. in Giemsa stain [Table/Fig-5a] and a clear capsule surrounding the pale blue nucleus of *Cryptococcus* sp. in Haematoxylin and Eosin (H&E) stain [Table/Fig-5b].

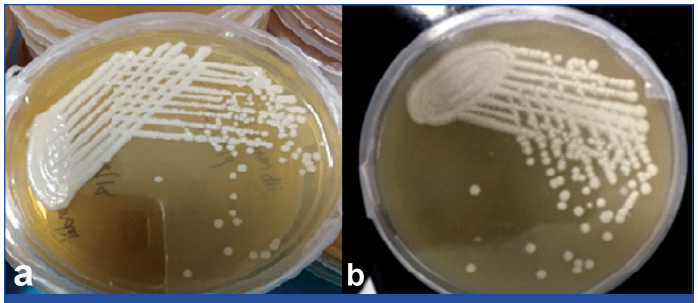


[Table/Fig-5]: Histopathological stains. Giemsa stain of CSF sample showing purple coloured yeast cells (100x) (a) and H&E stain of CSF sample showing clear capsule around pale blue nucleus (b) (100x).

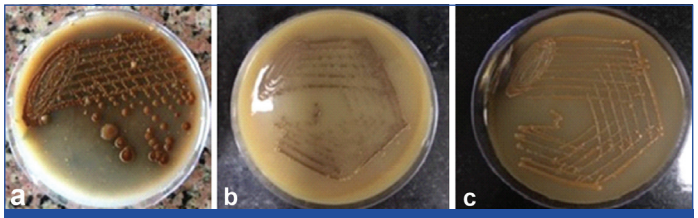
Phenotypic Identification

Basal media: Out of 37 samples, only three grew in basal culture media (SDA and Oat meal agar) producing creamy mucoid colonies after three days of incubation at 37°C [Table/Fig-6a,b]

Special media: Bird seed agar (BSA), Cumin agar and coriander agar: Among the 37 CSF samples, three which grew on basal media produced brown coloured colonies on BSA, Cumin agar and Coriander agar seen after 48 hrs of incubation at 37°c due to the utilisation of phenolic compounds present in the media [Table/Fig-7a-c].



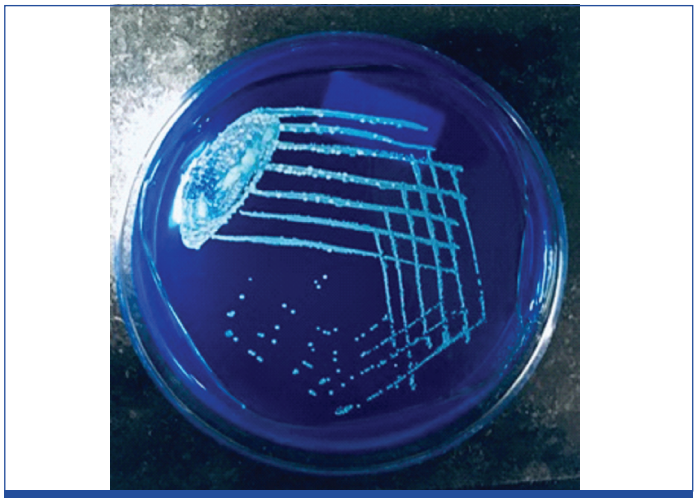
[Table/Fig-6]: Basal media creamy mucoid colonies- SDA (a) and Oat meal agar (b).



[Table/Fig-7]: Brown coloured colonies of *Cryptococcus* sp. on Bird seed agar (a), Cumin seed agar (b) and Coriander seed agar (c).

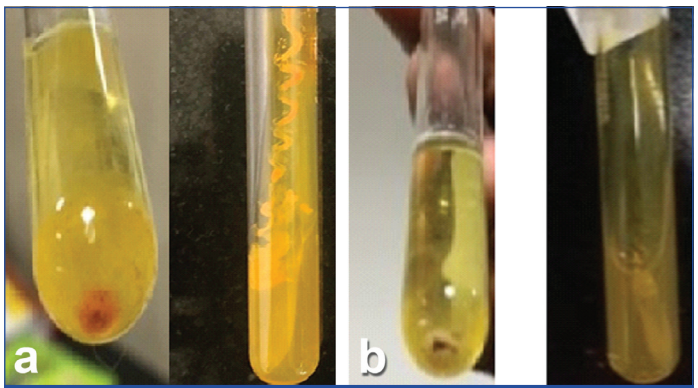
Differential Media

Cryptococcus **differential agar:** Out of 37 CSF samples, all the three culture positive isolates produced dry blue coloured colonies [Table/Fig-8]. Hence, identified as *Cryptococcus neoformans.*



[Table/Fig-8]: Dry blue colonies of *Cryptococcus* sp. on *Cryptococcus* differential agar.

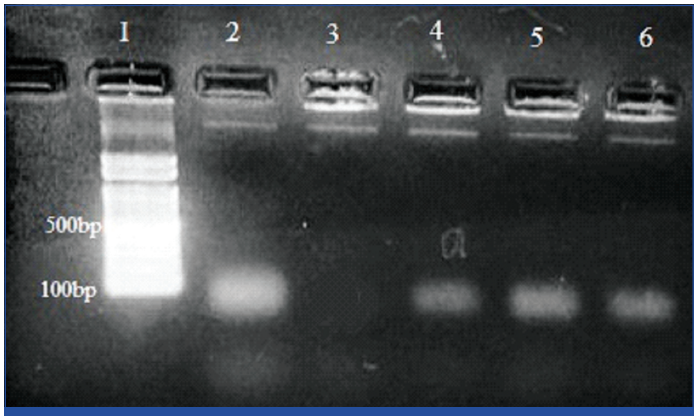
Creatinine Dextrose Bromothymol blue Thymine agar (CDBT): Among the 37 CSF samples, CDBT agar identified two as *Cryptococcus neoformans* var. *neoformans* (Orange colonies) [Table/Fig-9a] and one as *Cryptococcus neoformans* var. *grubii* (no growth) [Table/Fig-9b] in three culture positive isolates.



[Table/Fig-9]: Orange colonies of *Cryptococcus neoformans* var. *neoformans* (a) and No growth-*Cryptococcus neoformans* var. *grubii* (b) on Creatinine Dextrose Bromothymol blue Thymine agar (CDBT).

Genotypic characterisation: Genotypic characterisation was performed for the three culture positive isolates by PCR for confirmation.

***Cryptococcus* genus specific PCR:** *Cryptococcus* genus specific primers resulted in specific amplification of a single DNA fragment at ~136 bp for positive control (*C. neoformans* ATCC 14116) and all the three culture positive isolates. Negative control (*Candida albicans* ATCC 90028) did not yield any amplification [Table/Fig-10].

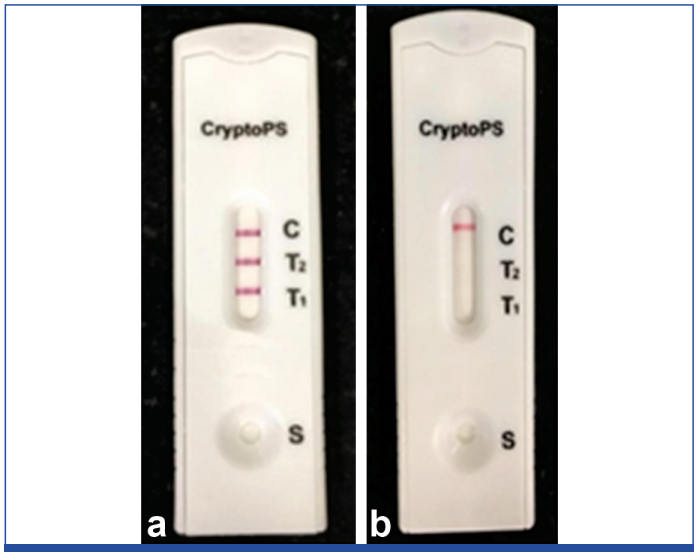


[Table/Fig-10]: *Cryptococcus* genus specific PCR targeting ITS rDNA region of *Cryptococcus* sp. Lane 1: DNA ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4: Isolate 1, Lane 5: Isolate 2, Lane 6: Isolate 3.

Serological Tests

Latex Agglutination Test (LAT): Out of the 37 CSF samples, five of them showed agglutination in LAT. Among the five samples, one sample showed a titre of >1:64, two of them showed a titre of 1:16 (Significant titre: 1:8). Two samples which showed a titre of 1:8 were direct microscopy and culture negative.

Lateral Flow Assay (LFA): Out of the 37 CSF samples screened by LFA, three samples (LAT titre >1:64 and 1:16) showed strong positive result by producing three distinct coloured lines (C, T1 and T2) [Table/Fig-11a] and the rest showed a negative result [Table/Fig-11b]. All the three samples were culture positive. The LFA results were compared with conventional and other serological tests [Table/Fig-12].



[Table/Fig-11]: Lateral Flow Assay (LFA)-positive test (a) and negative test (b).

Tests	Sensitivity (%)	Specificity (%)	*PPV (%)	*NPV (%)
Culture	100	100	100	100
Negative stain	67	100	100	97
Gram stain/Histopathological stains	67	100	100	97
Latex agglutination test	100	94	60	100
Lateral flow assay	100	100	100	100

[Table/Fig-12]: Sensitivity and specificity of different diagnostic tests in Cryptococcal Meningitis (CM) (n=37).
*PPV: Positive predictive value; *NPV: Negative predictive value

DISCUSSION

The CM is a life-threatening opportunistic infection. Management of CM still remains a challenge because of the delay in diagnosis. Starting the patient on oral fluconazole at an early stage can reduce risk of CM and its mortality [10,11]. Since for the sake of advantage, early screening and prompt initiation of pre-emptive fluconazole therapy, decreasing the time from first clinic visit to testing and afterwards treatment are crucial. Hence, in this study the performance of LFA which detects the cryptococcal capsular antigen was assessed, which can be a POCT and performed even by trained nurses in a hospital care setting.

Three patients with proven CM in present study were in the age group of 41-60 years. It has also been established in previous studies that elderly population was most commonly affected by CM [12]. Ageing is a process that negatively impacts the immune system and its capability to function. Also, associated disabilities and co-morbidities are common in the elderly [13]. These factors make the elderly individuals more vulnerable to infectious diseases [14]. However, the characteristic presentations of infectious diseases are not always noted in elderly patients, making it difficult to make an early diagnosis leading to a delay in treatment [15]. Hence, timely diagnosis and management of these age group patients acts as an important life saving measure.

Majority of the patients with suspected meningitis, and two out of the three patients with CM were men. Previous studies by Tay ST et al., and McClelland EE et al., also have reported an increased incidence of the disease among men (74/96 and 12/28 respectively) [16,17]. McClelland EE et al., in his study had also stated that, macrophages from females phagocytosed more cryptococcal cells than macrophages from males, also male macrophages had a higher fungal burden and showed increased killing by *Cryptococcus* spp. This may be due to an interaction of *Cryptococcus* with testosterone that results in increased Glucuronoxylomannan (GXM) release and *Cryptococcus*-mediated macrophage death [17]. The above data suggested that *Cryptococcus* strains are adapted to different hosts and the male individuals may be more prone to cryptococcal infection.

The DCLD was identified as one of the major risk factors in one of the patients with proven CM, in present study. Decompensated liver cirrhosis was identified as an independent risk factor in previous studies for the occurrence of invasive cryptococcosis [18-20]. The pathogenesis could involve multiple aspects. First, although *Cryptococcus* spp. typically enters the human body via inhalation through the respiratory system, people may become infected by ingesting contaminated food [19]. The presence of collateral circulation in decompensated liver cirrhosis allows the ingested *Cryptococcus* to bypass the liver scavenger system and directly enter the circulatory system, thereby causing cryptococcaemia and being further disseminated into the central nervous system. Other reported possible risk factors include diabetes mellitus, lymphoproliferative malignancy, haematological malignancy, cancer, autoimmune diseases, and lung diseases [21]. The second patient in present study with CM was on immunosuppressive therapy. Cell mediated immunity has also been shown to play a critical role in the host's defense against *C. neoformans* in animal models [22,23]. The third patient with proven CM was a female patient with uncontrolled diabetes mellitus. Diabetes mellitus was present in 8.5-33% of cryptococcosis cases in reported series [24,25]. Diabetes mellitus is a relative common condition in the age group of 41-60 years and uncontrolled diabetes mellitus leads to lowering of immunity and hence predispose to infections.

The laboratory diagnosis of cryptococcosis is generally based on direct demonstration, culture, and antigen detection by LAT.

In present study, direct microscopic methods like gram stain, negative stain (India ink and Nigrosin) and histopathological stains though were 100% specific, showed a low sensitivity (67%) when compared to culture, which is the gold standard test for the diagnosis of CM. This finding was similar to the finding of other studies which also stated that microscopic methods, though specific, showed a sensitivity of 50-80% [5,26,27]. This means that there will be many false negatives and few false positives, when CM is diagnosed with this test. In the present study, the NPV for the direct microscopy was found to be 97%, which means that there is always a 3% chance of people who are tested negative to have the disease. Also, microscopy requires laboratory infrastructure, it is dependent on fungal concentration and is highly operator dependent rather than the test performance [28].

In this study, all the samples which grew on culture were positive by PCR, thereby confirming its identification. Culture is considered as the gold standard for diagnosis of CM. But still culture takes time and requires more labour and large volumes of samples. Hence, both microscopy and culture cannot be used for the early diagnosis of CM.

In present study, LAT had 100% sensitivity and 94% specificity, but a low PPV of 60%. This means that there is only a 60% probability for the patient who is tested positive to have the disease. Hence, LAT may have lot of false positive reactions. Though earlier studies also had demonstrated a good sensitivity and specificity of >99% for LAT and is less labour intensive than culture, it also requires technical expertise and laboratory infrastructure [29,30]. Culture and LAT cannot be performed in resource limited settings, thus limiting their clinical utility [31]. Henceforth, a serological test which will have less false positive reactions and easy to perform without instruments would be ideal for rapid diagnosis of CM.

In the present study, LFA had higher sensitivity, specificity, PPV and NPV of 100%, when compared to the LAT. Blood, serum, or CSF can be used in LFA and is stable at room temperature, has rapid turn-around time of (<15 min), and user friendly [9]. The LFA meets the World Health Organisation's Affordable Sensitive Specific User friendly Robust and Rapid Equipment free Deliverable (ASSURED) to those who need them criteria for being affordable, sensitive, specific, rapid and robust, equipment free, user-friendly, and deliverable to end-users [32]. Moreover, the present paper suggest that LFA surpass the other standard diagnostic tests that are currently been used for the detection of CrAg.

Limitation(s)

Since present study was performed with low sample size, further studies including higher number of samples need to be performed to establish the accuracy of present study findings.

CONCLUSION(S)

The availability of this assay as a POCT not only in tertiary healthcare settings, but also in remote locations could have a meaningful impact on cryptococcal diagnosis, assisting in early treatment thereby reducing the morbidity and mortality rate. In view of the advantages of LFA over other tests, consideration should be given to establish the CrAg LFA as the current gold standard for the detection of cryptococcal infection.

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