Original Article

Utility of Sunflower Agar for Laboratory Detection of *Cryptococcus neoformans*

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Abstract

Background: Early diagnosis and management of cryptococcal meningitis is associated with good prognosis and long-term survival. Culture methods have been found to be promising and definitive in the diagnosis of many infectious diseases. We look at the sunflower agar (SFA) cultural method for detection *Cryptococcus neoformans*, the causative agent of cryptococcal meningitis. **Materials and Methods:** This is a descriptive cross-sectional study carried out at Jos University Teaching Hospital, Jos Nigeria, to find out the performance of SFA for the detection of *C. neoformans*. Cerebrospinal fluids (CSFs) of the study subjects were collected, subjected to Indian ink microscopy, inoculated on Sabouraud dextrose agar (SDA) and SFA for identification of *C. neoformans*. **Results:** A total of 90 CSF samples were analyzed for the identification of *C. neoformans.* SFA and SDA were able to confirm 8 (50%) and 7 (43%) of the 16 capsulated yeast cells detected by Indian ink microscopy as *C. neoformans*. Both media were found to have similar sensitivity (100%), specificity (91.3%), positive predictive value (80%), and negative predictive value (100%) in comparison with Indian ink microscopy. In terms of turnaround time, 6 isolates were identified within an average of 48 h ($P = 0.017$) by SFA, while SDA detects 2 isolates ($P = 0.111$) at the stipulated period. **Conclusion:** SFA can be a good routine conventional culture media for laboratory detection of *C. neoformans.*

Keywords: *Cryptococcus neoformans,* Indian ink, meningitis, meningoencephalitis, Sabouraud dextrose agar, sunflower agar

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Introduction

Cryptococcus neoformans (C. neoformans) is found in the environment; it is abundant in the soil growing as a saprophyte.[1] There was strong evidence that *C. neoformans* var. grubii evolved from Africa.[2] It is commonly associated with pigeon and captive birds' droppings that provide appropriate conditions for its growth.[2,3] *Cryptococcus* species were also isolated from vegetables and fruits, house dust, air conditioners, air, and sawdust.[2,3] The main route of transmission to human is inhalation from the environmental source and penetration through skin.[4] Reactivation of a latent cryptococcal infection is the most common cause of opportunistic fungal meningitis,[4] a life-threatening fungal infection in AIDS patients.[5,6]

Pulmonary infection is usually the first primary cryptococcal infection with possible pleural effusion,[7,8] from the lung it can spread to infect any organ.[9] In immunocompetent host, pulmonary cryptococcosis is usually asymptomatic or associated with unusual presentation. The common species implicated with infection in immunocompetent individual is *C. neoformans* var. gattii which can present in a disseminated high mortality form.[10] The most common presenting symptoms associated with pulmonary infections include cough, chest pain, sputum production, weight loss, fever, and hemoptysis. Rarely, it is associated with dyspnea, night sweats, and obstruction of the superior vena cava. Significant

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number of cases are associated with signs and symptoms of subacute meningitis or meningoencephalitis.[9] Cryptococcal meningoencephalitis is the most common manifestation of cryptococcosis in immunocompromised individuals.[6-9]

C. neoformans infections cause an estimated one million cases of cryptococcal meningitis per year among people with HIV/AIDS and account for 625,000 deaths globally.[11,12] The highest burden of disease was reported in sub-Saharan Africa with an estimated mortality of 50% to 70%.[11-13] This high mortality in Sub-saharan Africa is linked to delay in presentation and late diagnosis which make early treatment difficult. Any approach leading to early diagnosis to improve early treatment of cryptococcal disease and its complications can reduce the associated high mortality. Prognosis is usually good when treatment start at early stage of the disease.[14]

The laboratory detection methods for the identification of *Cryptococcus* species (spp.) include; microscopic detection of its polysaccharide capsular yeast cell in cerebrospinal fluid (CSF), isolation in culture, histopathology or by rapid biochemical identification systems or using rapid lateral flow assay for detection of cryptococcal antigen in serum and CSF.[14,15] India ink preparations, alcian blue, and mucicarmine stains are used for microscopic identification of the capsulated *Cryptococcus* spp.[15-17] The importance of using chromogenic like special media for the diagnosis of cryptococcal meningitis cannot be overemphasized. Various plants seed has been tried for that purpose,[18] we chose sunflower seed to evaluate its performance in comparison with conventional Sabouraud dextrose agar (SDA) because of its availability in our environment and the simplicity of its preparation.

Helianthus annuus (sunflower) grows best in fertile, moist, well-drained soil with heavy mulch.[19] It contains caffeic acid, a substrate for the production of melanin that is utilize by *Cryptococcus spp*. It is commercially being used as ornamental plants, production of butter (sunflower butter), food for birds, cooking oil (polyunsaturated oil), and medical ointments and dyes.

Materials and Methods

Study area

The study was conducted at the Jos University Teaching Hospital (JUTH). CSF samples collected from patients with suspected meningitis were used in this research. The laboratory procedures were conducted at the microbiology laboratory of JUTH, Jos, Plateau State, Nigeria.

Study design

This is a descriptive cross-sectional study.

Study population

Ninety CSF samples from suspected cases of meningitis were collected based on the following.

Subject selection criteria

Immunocompromised patients with symptoms suggestive of meningitis and immunocompetent CSF samples with a negative bacterial culture. CSF samples with established laboratory diagnosis of meningitis were excluded.

Preparation of simplified sunflower agar

Fifty grams of sunflower seed [Figure 1] was pulverized (crush to powder) in a domestic blender and boiled in 1 L of distilled water for 30 min. The seed extract obtained was allowed to be cooled and filtered through 5 layers of gauze and then 1 g glucose and 15 g agar powder (HIMEDIA-RM201) were added. Agar Powder purified (HIMEDIA RM201) is an extensively purified by exhaustively extracting Agar with water and organic solvents to remove all nitrogenous compounds, inorganic salts and vitamins. It has low calcium and magnesium levels and is compatible with all culture media. The pH was adjusted to 5.6 using hydrochloric acid and NaOH, and the volume made up to 1 L and autoclaved at 120°C for 15 min. The prepared medium was dispensed in sterile petri dishes and slant in tubes. The prepared media was stored at a refrigerator temperature of 4°C–8°C.

The media maintained its performance for up to 8 weeks at the refrigerator temperature.

Preparation of Sabouraud dextrose agar

Commercially dehydrated SDA was purchased (HIMEDIA) and prepared according to the manufacturer guidelines. The composition was formulated, adjusted, standardized to suit performance parameters. Ingredients were dextrose 40.000 g/l, mycological peptone 10.000 g/l, agar 15.000 g/l with final pH at 25°C of 5.6. Sixty-five grams of the dehydrated powder was suspended in 1000 ml of distilled water, heated to boiling until the medium dissolved completely. It was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. The prepared medium was dispensed in Sterile Bijou bottles in slants and store at refrigerator temperature.

Specimens processing

Indian Ink microscopy

Using sterile Pasteur pipette, a drop of Indian ink was placed at the center of clean, dried, grease-free, and microscopic glass slide. A drop of CSF was placed on the drop of the Indian ink. This was gently mixed using a small clean wooden

Figure 1: Sunflower seed

applicator stick and covered with new clean coverslip. The wet mount preparation was then viewed at × 10 and × 40 for the identification of capsular yeast cells that gives presumptive diagnosis of *C. neoformans* as the causative agent of the disease. This procedure was also repeated on the in-house generated *Cryptococcus* species as a positive control.

Culture of cerebrospinal fluid on Sabouraud dextrose agar and sunflower agar

The collected CSF was kept in an incubator for 2 h and was then carefully decanted leaving lowest portion of about 0.5 ml (sediment), 0.5 ml CSF sediment was inoculated into each of SDA and sunflower agar (SFA) slant. In-house generated *Cryptococcus* species were also inoculated as positive control. All were incubated at 37°C for 24–72 h. The positive growth showed cream-colored smooth, mucoid yeast-like colonies on SDA. Yeast growth with characteristics brown color effect (BCE) on SFA gives preliminary identification of *C. neoformans*.

Lactophenol cotton blue wet mount

All growth that show characteristics yeast colonial morphology on culture were subjected to lactophenol cotton blue (LPCB) wet mount. Yeast cell with characteristics capsule gives preliminary identification of *Cryptococcus* species.

Urease test

All growth that show characteristics yeast colonial grow pattern were inoculated on urea agar in small bijou bottle. This was incubated at 37°C for 48 h. The cultures that show characteristic pinkish color changes were considered urease positive.

Interpretation of results

The cultured specimen that show characteristic yeast colonial morphology, BCE on SFA, yeast cell morphology on LPCB wet mount, and urease positive were identified as *Cryptococcus species* in this study.

Data analysis

The data were analyzed using Epi Info^{tm7}, 2013 and ISO Wizard statistical software. In all, critical $P < 0.05$ was regarded as statistically significant. Data were presented in tables.

Ethical considerations

Ethical approval was obtained from Ethical Committee of the JUTH.

Results

A total of 90 CSF samples were analyzed using Indian ink microscopy, SFA, and SDA cultures. All the results were ready within 72 h. A characteristic dark yeast cells surrounded by halo [Figure 2] were observed in 16 (17.78%) of the 90 CSF samples analyzed by Indian ink microscopy. A characteristic whitish mucoid like growths [Figure 3] were observed in 11 (12.22%) on SDA. Growth with BCE [Figure 4] was observed in 8 (8.8.00%) of cultured samples on SFA. In terms of urea hydrolysis [Figure 5], 7.7.00% and 8.800% of SDA and SFA were positive, respectively. A yeast-like morphological appearance was observed in all growths on both SDA and SFA by LPCB wet mount analysis. The rate of detection *C. neoformans* before urea hydrolysis by Indian ink, SDA, and SFA was found to be 16 (17.78%), 11 (12.22%), and 10 (11.11%), respectively [Table 1]. Urea hydrolysis results [Table 2] showed that 7 (7.78%) were positive for SDA and 8 (8.89%) positive for SFA.

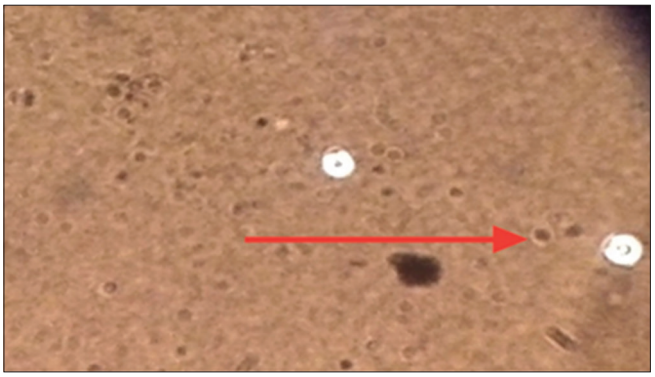

Figure 2: Indian ink wet mount showing capsulated yeast cell

Figure 3: Creamy mucoid growth on Sabouraud dextrose agar

Figure 4: Culture growth on sunflower agar (sunflower agar) (a): Growth with no brown colour effect. (b) Growth with brown colour effect

The turnaround time to the final presumptive identification of *C. neoformans* by SDA and SFA were also compared [Table 3]. After 24 h incubation, two results were ready for SFA and none for SDA, growth was also observed in 4 SFA and 3 SDA culture, respectively, after 48 hours' incubation. After 72 h, 1 culture was positive for SFA and 5 for SDA. In total, SFA and SDA were able to detect (confirmed) 6 and 1 isolates at 48 h, respectively.

In this study, growth on SFA with BCE and evidence of urea hydrolysis on SDA growth give presumptive identification of *C. neoformans* for the respective media. Indian ink microscopy, SDA, and SFA cultures were all 100% for both sensitivity and negative predictive value (NPV). In terms of specificity both SDA SFA was found to have a specificity of 91.3% with excellent positive predictive value (PPV) of 80% [Table 4].

Discussion

High burden of cryptococcal meningitis among HIV-infected individual warrant a need of simple laboratory procedure for rapid diagnosis with high degree of specificity and accuracy.

Figure 5: Urea utilization reaction; Arrow pointing positive pinkish urea hydrolysis reaction

Table 1: Rate of isolation of *Cryptococcus neoformans* on Indian ink microscopy Sabouraud dextrose agar and sunflower agar

Methods	*n*=90		*P*	95% CL
	Positive, *n* (%)	Negative, *n* (%)		
Indian ink	16 (17.78)	74 (82.22)	0.14	5.90-42.12
SDA culture	11 (12.22)	79 (87.78)	0.82	33.95-42.84
SFA culture	10 (11.11)	80 (88.89)	0.55	22.30-41.87

SDA: Sabouraud dextrose agar, SFA: Sunflower agar, CL: Confident interval

Indian ink microscopy is the common laboratory procedure employed for the laboratory diagnosis of cryptococcal meningitis.[14] It is associated with high false positivity due to presence of leukocytes, myelin globules, fat droplets, tissue cells in the CSF, deteriorated reagent and when few organisms $<10^3$ colony-forming unit.[14] In terms of culture, SDA culture method is the common cultural methods employed, but it requires further biochemical analysis for presumptive identification.[18] Melanin production has been associated with virulence of *C. neoformans* in a number of studies.[20] Media containing substrate (caffeic acid) for the production of melanin were recently being a focus of research for the laboratory isolation *C. neoformans* var. neoformans.[20] Various plant seed known to contain caffeic acid were tried, these includes sunflower seed, mustard seed, tobacco seed, birdseed,[20] and henna seed.[21] Sunflower seed agar was found to be a simple and inexpensive tool for the presumptive identification of *C. neoformans* in clinical microbiology laboratories.[22] SFA is simple to prepare as shown in this study, using readily available sunflower seed and commercially available agar powder. We obtained the seed at no cost from bushes around where the sunflower grow. The prepared media were stored at refrigerator temperature, and the performance was maintained for up to 8 weeks. It also has an excellent capacity for the isolation of *C. neoformans.* Its sensitivity, specificity, PPV, NPV, and degree of accuracy is as good as that of SDA as observed in this research which agreed with similar study conducted by Ruchi *et al*. in India.[18] Its earlier statistically significant turnaround time ($P < 0.017$) as compared to that of SDA ($P < 0.111$) helps in earlier diagnosis and warrant possibility early initiation of treatment. In this study, SFA was found to be associated with a high degree of accuracy (91.3%) in establishing the diagnosis of cryptococcal meningitis; its high specificity limits the possibility of having high false-positive cases.

Conclusion

Readily available sunflower seed and simplicity in preparing the medium can make SFA a good chromogenic culture method for the laboratory diagnosis of cryptococcal infections in a poor resource setting.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

Table 2: Rate of urea hydrolysis from growth on Sabouraud dextrose agar and sunflower agar

Methods	*n*=90		Urea hydrolysis, *n* (%)	*P*	95% CL
	Positive growth, *n* (%)	Negative growth, *n* (%)			
SDA	11 (12.22)	79 (87.77)	7 (7.78)	0.64	30.15-40.82
SFA	10 (11.11)	80 (88.89)	8 (8.89)	0.77	18.28-27.23

SDA: Sabouraud dextrose agar, SFA: Sunflower agar, CL: Confident interval

Table 3: Comparison of turnaround time between Sabouraud dextrose agar and sunflower agar to presumptive identification of *Cryptococcus neoformans* (*n*=8)

Period of incubation (h)	Number of isolates identified by SDA	Number of isolates identified by SFA
24	0	2
48	3	4
72	5	2
P	0.111	0.017

SDA: Sabouraud dextrose agar, SFA: Sunflower agar

Table 4: Comparison of performance between Sabouraud dextrose agar and sunflower agar

Procedure	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Indian ink	100.0	67.7	53.3	100.0
SDA culture	100.0	91.3	80.0	100.0
SFA culture	100.0	91.3	80.0	100.0
Mean	100.0	89.65	75	100.0
SD	0	2.33	7.07	0
Variance	0	5.45	50.0	0

SDA: Sabouraud dextrose agar, SFA: Sunflower agar, PPV: Positive predictive value, NPV: Negative predictive value, SD: Standard deviation

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