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Research Article

Rapid Detection of Bacteria in Cerebrospinal Fluid: A Comparison Between Fluorescent Microscopy and Conventional Gram Stain

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Abstract - *Background: Rapid diagnosis of bacterial meningitis is critical to reduce morbidity and mortality. However, the sensitivity of Gram staining is low, particularly in low inoculum and partially treated cases. Acridine Orange (AO) fluorescent microscopy has been suggested as a more sensitive alternative. Objectives: To evaluate the accuracy of Acridine Orange fluorescent microscopy versus Gram stain light microscopy for the diagnosis of bacterial pathogens in Cerebrospinal fluid (CSF). Methods: This prospective comparative study was conducted in the Department of Microbiology, UCMS and GTBH, Delhi, from November 2025 to February 2026. A total of 100 CSF samples from suspected bacterial meningitis cases were analyzed using Gram staining, Acridine Orange staining, and culture (gold standard). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy were determined. McNemar's test and Cohen's kappa were applied. Results: Of 100 samples, 30 were culture-positive. Acridine Orange demonstrated higher sensitivity (43.3%) compared to Gram stain (23.3%); however, the difference was not statistically significant (p = 0.10), indicating comparable performance in this sample size. Gram stain showed higher specificity (82.9%) than AO (77.1%). The agreement with culture was poor for Gram stain (κ = 0.069) and fair for AO (κ = 0.207), which indicates poor agreement of both tests with the gold standard. Conclusion: Acridine Orange staining shows improved sensitivity but limited agreement with culture. While it may serve as a useful screening tool, Gram staining remains essential for specificity and morphological identification. A combined diagnostic approach is recommended.*

Keywords - *Acridine Orange, Gram Stain, CSF, Meningitis, Fluorescent Microscopy.*

I. INTRODUCTION

Bacterial meningitis is a life-threatening condition requiring urgent diagnosis and management to prevent long lasting complication and death (1,2). Timely diagnosis and initiation of appropriate antibiotic treatment are essential to enhance the chances of favourable outcome (3,4). The diagnosis is made by examination of cerebrospinal fluid (CSF). Gram staining is used as a quick diagnostic stain because of its ease to perform and because it provides morphological information (1,5-7). However, its sensitivity is often limited, especially in cases with low bacterial load or prior antibiotic exposure (8). Acridine Orange (AO) is a fluorescent dye that intercalates with nucleic acids and facilitates visualization of bacteria under fluorescent microscopy, even at low concentrations (2). This characteristic suggests its potential use as an adjunct or alternative test. The aim of this study was to evaluate the diagnostic efficiency of fluorescent microscopy with Acridine Orange compared with Gram staining for rapid diagnosis of CSF samples.

II. MATERIALS AND METHODS

A. Study Design and Setting

A prospective, comparative laboratory-based study conducted in the Department of Microbiology, UCMS and GTBH, Delhi.

B. Study Duration

November 2025 to February 2026

C. Sample Size

A total of 100 consecutive CSF samples from patients with suspected bacterial meningitis were included.

D.Inclusion Criteria

CSF samples from clinically suspected cases of bacterial meningitis.

E. Exclusion Criteria

None

F. Laboratory Procedures

a. Gram Staining

CSF smears were prepared, heat-fixed, and Gram staining performed as per microbiological standards. Slides were examined and reported as routine practice (1).

b. Acridine Orange Staining

Smears were methanol-fixed and stained with Acridine Orange for 2 minutes and examined under luorescence microscopy using FITC filter (2). Acridine orange is pH dependent. At acidic pH (~4.0) → DNA fluoresces green, RNA orange-red. (Fig 1)

Figure 1. Reagents and Procedure for Acridine Orange Staining

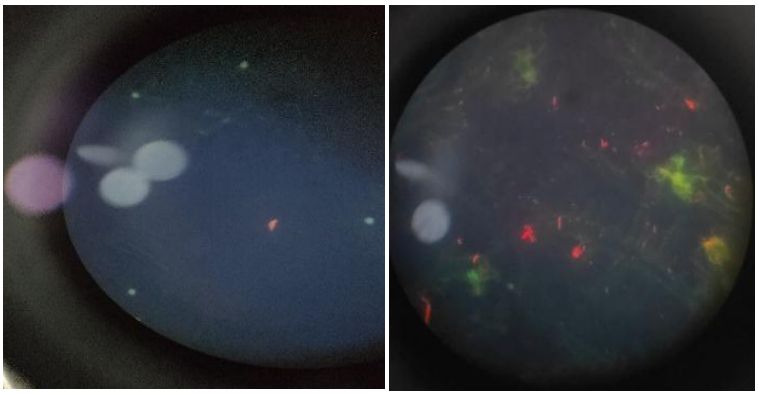

Figure 2. Acridine Orange Staining (1000× Magnification) Showing Bacteria in Palisade Arrangement

c. Culture

All samples were cultured using standard microbiological techniques and considered as the gold standard (5).

G. Statistical Analysis

Sensitivity, specificity, PPV, NPV, and diagnostic accuracy were calculated. McNemar's test was used to compare paired proportions, and the Chi-square test was applied for categorical variables. Agreement between tests and culture was assessed using Cohen's kappa coefficient.

Kappa interpretation:

- <0.20 = Poor agreement
- 0.21–0.40 = Fair agreement

A p-value <0.05 was considered statistically significant.

III. RESULTS

Out of 100 CSF samples:

- 30 were culture positive
- 70 were culture negative

Table 1. Diagnostic Performance

Parameter	Gram Stain	Acridine Orange
Sensitivity	23.3%	43.3%
Specificity	82.9%	77.1%
PPV	36.8%	44.8%
NPV	71.6%	76.1%
Accuracy	65%	67%

Acridine Orange demonstrated higher sensitivity; however, the difference was not statistically significant (p = 0.10), which suggests that this improvement might be incidental and requires further study with larger studies.

A. Agreement with Culture

- Gram stain: κ = 0.069 (poor agreement)
- Acridine Orange: κ = 0.207 (fair agreement)

Both tests showed limited agreement with culture, indicating that neither method alone is sufficiently reliable as a definitive diagnostic tool.

IV. DISCUSSION

Rapid and accurate diagnosis of bacterial meningitis is essential due to its high morbidity and mortality (3,4). The classic symptoms of meningitis are fever, neck stiffness and headaches, although these features are not present uniformly. Broadly, the most common causes of infectious meningitis are viral, bacterial, mycobacterial and fungal. Parasitic and non-infectious causes of meningitis also occur. Gram staining remains a cornerstone of laboratory diagnosis but has limitations in sensitivity, particularly in partially treated infections (1,8). Rapid identification of pathogens in normally sterile body fluid is essential for appropriate patient management, specifically antimicrobial therapy. Limited sensitivity and increased time to detection of traditional method prompted us to evaluate additional testing to contribute to the diagnosis of infection. Meningitis is diagnosed using Gram stain and culture of cerebrospinal fluid (CSF) collected by lumbar puncture. Because of the risk of rapid and permanent brain injury, intravenous antibiotic therapy is initiated immediately on clinical suspicion of bacterial meningitis, and CSF test results are subsequently used to modify the antibiotic spectrum.

Unfortunately, the sensitivity of CSF culture is low, partly because CSF is often collected after treatment, so many patients complete a course of empiric antibiotics without laboratory guidance to modify therapy. CSF Gram stain is performed urgently, 24 hours per day, on the basis of the assumption that the results are urgently useful in treatment decision making. However, the impact of CSF Gram stain is unknown, so this testing policy may not be appropriate. In the present study, Gram staining demonstrated low sensitivity (23.3%), consistent with previous reports (1). Acridine orange is a fluorescent dye that intercalates or binds with the nucleic acid (either DNA or RNA) present in organisms and fluoresces to emit various colors that help differentiate cellular organelles. This binding results from the electrostatic interactions of acridine molecules between the nucleic acid-base pairs. Due to its metachromatic properties, acridine orange (AO) is commonly used in fluorescence microscopy and flow cytometry analysis of cellular physiology and cell cycle status, including the fluorescent microscopic examination of microorganisms. Acridine Orange staining showed higher sensitivity (43.3%), likely due to its ability to detect nucleic acids and enhance visualization of bacteria even at low concentrations (2).

However, Acridine Orange showed slightly lower specificity, possibly due to non-specific fluorescence from cellular debris or extracellular nucleic acids (2). It allows differential fluorescent labeling of the cytoplasm/nucleus/lysosomes in living cells due to different accumulation in these particular subcellular domains. Similarly, it stains differentially the cytoplasm and the nucleus in fixed dead cells . Most puzzling, AO displays different fluorescent labeling behavior in regard to different cellular substrates. Regarding nucleic acids (NAs), AO has been widely and notably employed to differentiate simultaneously NAs strandedness, namely between DNA (orthochromatic green fluorescence) and RNA (metachromatic red fluorescence). Therefore, careful interpretation is required.(9)

Recent guidelines emphasize the importance of rapid diagnostic tools but highlight their limitations and the need for confirmatory testing (5,7). Our findings support the role of Acridine Orange as a screening tool, while Gram staining remains indispensable for morphological identification. Cerebrospinal fluid (CSF) microscopy analysis provides critical information in cases of suspected bacterial meningitis. However, rapid reporting of results is required for impact to be maximized and adverse outcomes to be minimized. Subsequently, CSF microscopy sensitivity and Turnaround time is an important metric for both patient care and, by extension, for laboratory accreditation. Optimization of CSF microscopy in busy laboratories can be achieved with staff education, checklists, and workflow changes without the need for expensive and complex instrumentation and automation.

V. CONCLUSION

Acridine Orange staining was found to be more sensitive but slightly less specific than Gram staining. The two methods are complementary and should be used together with culture to enhance the diagnosis of bacterial meningitis.

VI. LIMITATIONS

The present study has certain limitations. A small sample size could impact on the overall findings. Moreover, patient's history of antibiotic exposure was not assessed, which could have affected the microscopy and culture results, especially by reducing the bacterial density and false negative results. Furthermore, molecular diagnostic methods, such as polymerase chain reaction (PCR), were not included in this study, which could have provided higher sensitivity and served as a valuable comparative reference.

CONFLICT OF INTEREST

None.

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