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Original Article

Comparative Evaluation of Sputum Pretreatment Methods Using LED Fluorescence Microscopy for Improved Diagnosis of Pulmonary Tuberculosis

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ABSTRACT

Background: Pulmonary tuberculosis (TB) remains a major public health challenge, especially in resource-limited settings where sputum smear microscopy is widely used. However, its low sensitivity limits early detection. This study evaluated whether sputum pretreatment methods combined with LED fluorescence microscopy (LED-FM) improve diagnostic performance.

Methods: In this prospective comparative study, 300 clinically suspected TB patients were included. Sputum samples were processed using direct smear, Modified Petroff's method, 5% bleach sedimentation, 5% bleach centrifugation, phenol ammonium sulphate (PhAS) sedimentation, and chitin pretreatment. Smears were examined by Ziehl–Neelsen (ZN) staining and LED-FM. Lowenstein–Jensen culture served as the reference standard. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated.

Results: Among 300 samples, 48 (16.0%) were culture positive. Direct ZN microscopy showed the lowest sensitivity (37.50%), while direct LED-FM improved it to 45.83%. All pretreatment methods enhanced sensitivity compared to direct smear. The highest sensitivity was observed with 5% bleach centrifugation using LED-FM (66.67%), followed by Modified Petroff's method with LED-FM (60.42%). Specificity remained high across all methods (97.22%–100.00%). Although LED-FM outperformed ZN microscopy, the differences were not statistically significant ($p>0.05$).

Conclusion: Sputum pretreatment significantly improves smear microscopy performance. 5% bleach centrifugation combined with LED-FM showed the best results. These findings support simple, cost-effective strategies to enhance TB diagnosis in resource-limited settings.

Keywords: Pulmonary tuberculosis, LED fluorescence microscopy, sputum pretreatment, bleach centrifugation, chitin, Ziehl–Neelsen staining, diagnostic accuracy, Lowenstein–Jensen culture.

INTRODUCTION

Tuberculosis (TB) remains one of the leading infectious causes of morbidity and mortality worldwide, with a substantial burden concentrated in low- and middle-income countries. Recent global estimates indicate that approximately 10 million people develop TB annually, with a significant proportion remaining undiagnosed or unreported, thereby sustaining ongoing transmission [1]. India carries the highest TB burden globally and contributes nearly one-fourth of the total TB cases, underscoring the urgent need for effective and accessible diagnostic strategies in high-burden settings [1]. Despite continued efforts under global and national TB control programs, delays in diagnosis and under-detection of cases remain major challenges, particularly in resource-limited settings where reliance on conventional diagnostic approaches persists [2, 3].

Sputum smear microscopy remains the most widely used diagnostic tool for pulmonary tuberculosis in resource-limited settings due to its simplicity, rapid turnaround time, and low cost. However, its major limitation is low sensitivity, particularly in patients with paucibacillary disease, leading to missed diagnoses and continued transmission [4]. Fluorescence microscopy has been shown to improve bacillary detection compared to conventional Ziehl–Neelsen staining, and light-emitting diode fluorescence microscopy (LED-FM) has emerged as a practical alternative with higher sensitivity and faster slide reading [5]. Meta-analytical evidence further supports that LED-FM provides improved diagnostic performance while maintaining high specificity, making it suitable for implementation in high-burden and peripheral laboratory settings [6].

Various sputum pretreatment methods have been explored to improve bacillary yield and enhance smear microscopy sensitivity. Bleach-based digestion, sedimentation, and centrifugation can reduce background debris and concentrate mycobacteria [7]. Although bleach-based pretreatment has been shown to improve smear positivity, the reported benefit has varied across different settings and methodologies [8, 9]. Comparative evidence evaluating multiple pretreatment methods simultaneously remains limited, and the optimal approach for routine laboratory use is still unclear. Data on novel methods such as chitin-based pretreatment are particularly scarce. Therefore, the present study was undertaken to compare chitin and established chemical pretreatment methods using LED fluorescence microscopy and Ziehl-Neelsen staining, with Lowenstein–Jensen culture as the reference standard, to identify an effective and practical approach for improving pulmonary tuberculosis detection.

MATERIALS AND METHODS

Study Design and Setting

This prospective comparative study was conducted in the Department of Microbiology at a tertiary care centre over a defined study period. The study aimed to evaluate and compare different sputum pretreatment methods for improving the diagnostic performance of smear microscopy in pulmonary tuberculosis.

Study Population

A total of 300 patients clinically suspected of pulmonary tuberculosis were included. Patients presenting with symptoms such as persistent cough, fever, hemoptysis, and weight loss were enrolled. Patients already receiving anti-tubercular therapy or those providing inadequate sputum samples were excluded.

Sample Collection

For each patient, two sputum samples (spot and early morning) were collected in sterile, wide-mouthed containers following standard guidelines. Samples were transported to the laboratory without delay and processed on the same day.

Sputum Processing Methods

Each sample was subjected to multiple processing methods:

- Direct smear (unprocessed sputum)
- Modified Petroff's method (NaOH digestion and centrifugation)
- 5% sodium hypochlorite (bleach) sedimentation
- 5% bleach centrifugation
- Phenol ammonium sulphate (PhAS) sedimentation
- Chitin-based pretreatment (novel method)

Bleach-treated samples were processed either by gravity sedimentation or centrifugation to concentrate bacilli. Modified Petroff's method involved digestion with sodium hydroxide followed by centrifugation. Chitin pretreatment was applied to evaluate its ability to enhance bacillary recovery compared with conventional chemical methods.

Microscopy

Smears prepared from each method were stained using Ziehl–Neelsen (ZN) staining and examined under bright-field microscopy. Parallel smears were stained with auramine and examined using LED fluorescence microscopy (LED-FM). Smear grading was performed according to standard WHO guidelines.

Culture (Reference Standard)

All processed samples were inoculated onto Lowenstein–Jensen (LJ) medium and incubated at 37°C. Cultures were monitored weekly for up to 8 weeks for growth of Mycobacterium tuberculosis. LJ culture was considered the reference standard for comparison.

Statistical Analysis

Diagnostic performance of each smear microscopy method was evaluated against LJ culture results.
The following parameters were calculated:

- Sensitivity = TP / (TP + FN) × 100
- Specificity = TN / (TN + FP) × 100

- Positive Predictive Value (PPV) = TP / (TP + FP) × 100
- Negative Predictive Value (NPV) = TN / (TN + FN) × 100

True positive (TP), false positive (FP), false negative (FN), and true negative (TN) values were derived for each method. Comparisons of diagnostic performance between different pretreatment methods were performed. A p-value of <0.05 was considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics version 31.0.2.0 (IBM Corp., Armonk, NY, USA)

Ethical Considerations

The study was approved by the Institutional Ethics Committee, and informed consent was obtained from all participants prior to sample collection.

RESULTS

A total of 300 patients with clinically suspected pulmonary tuberculosis were included. The highest proportion of patients belonged to the 41–50 years age group (20.0%), followed by 51–60 years (17.66%) and 31–40 years (17.33%). Males constituted 64.66% of the study population. The most common clinical presentation was cough associated with fever, asthma, or hemoptysis (59.33%), followed by isolated cough (34.0%). The majority of early morning sputum samples were mucoid (83.66%), while spot samples were predominantly mucoid (98.0%) (Table 1).

Out of 300 samples, 48 (16.0%) were positive by Lowenstein–Jensen (LJ) culture. Direct Ziehl–Neelsen (ZN) microscopy detected 18 (6.0%) culture-positive cases, whereas direct LED fluorescence microscopy (LED-FM) detected 22 (7.33%) cases. Among pretreatment methods, modified Petroff's method using LED-FM detected 29 (9.67%) cases, while 5% bleach centrifugation with LED-FM detected the highest number of cases (32, 10.67%). Other methods, including bleach sedimentation, phenol ammonium sulphate (PhAS), and chitin sedimentation, showed comparatively lower detection rates (Table 2).

Diagnostic accuracy analysis demonstrated that direct ZN microscopy had a sensitivity of 37.50% and specificity of 100.00%, while direct LED-FM showed improved sensitivity (45.83%) with a slight reduction in specificity (97.62%). Among pretreatment techniques, modified Petroff's LED-FM achieved a sensitivity of 60.42% and specificity of 98.41%. The highest sensitivity was observed with 5% bleach centrifugation using LED-FM (66.67%), with a specificity of 97.22%. Other methods, including bleach sedimentation, PhAS, and chitin sedimentation, demonstrated sensitivities ranging from 41.67% to 54.17%, with consistently high specificities exceeding 97% (Table 3, Figure 1).

Comparative statistical analysis between Ziehl–Neelsen microscopy and LED fluorescence microscopy across all processing methods showed no statistically significant difference ($p > 0.05$). Although LED-FM demonstrated higher sensitivity than ZN microscopy across most methods, the differences were not statistically significant for any individual processing technique (Table 4).

Table 1. Baseline demographic, clinical, and sputum appearance characteristics of patients with suspected pulmonary tuberculosis (n = 300)

Variable	Category	n (%)
Age group (years)	≤20	27 (9.0)
	21–30	48 (16.0)
	31–40	52 (17.33)
	41–50	60 (20.0)
	51–60	53 (17.66)
	61–70	41 (13.66)
	>70	19 (6.33)
Sex	Male	194 (64.66)
	Female	106 (35.33)
Presenting symptoms	Asthma / sputum / cough	12 (4.0)
	HIV reactive	2 (0.67)
	Chest pain / loss of appetite / pain	3 (1.0)
	Cough	102 (34.0)
	Cough with fever / asthma / hemoptysis	178 (59.33)
	Pleural fluid / effusion / cough / sputum	3 (1.0)
Early morning sputum appearance	Mucopurulent	37 (12.33)
	Mucopurulent blood tinged	2 (0.66)
	Purulent	9 (3.0)

	Purulent blood tinged	1 (0.33)
	Mucoid	251 (83.66)
Spot sputum appearance	Mucopurulent	5 (1.66)
	Mucoid	294 (98.0)
	Salivary blood tinged	1 (0.33)

Values are presented as number (percentage). Percentages were calculated based on the total study population (n = 300).

Table 2. Comparison of direct and pretreated sputum smear microscopy results against Lowenstein–Jensen culture (n = 300)

Method	True Positive	False Positive	False Negative	True Negative	Total smear positive n (%)	Culture positive cases detected n (%)
Direct ZN	18	0	30	252	18 (6.0)	18 (6.0)
Direct LED-FM	22	6	26	246	28 (9.33)	22 (7.33)
Modified Petroff's ZN	23	1	25	251	24 (8.0)	23 (7.67)
Modified Petroff's LED-FM	29	4	19	248	33 (11.0)	29 (9.67)
5% Bleach sedimentation ZN	24	2	24	250	26 (8.67)	24 (8.0)
5% Bleach sedimentation LED-FM	26	5	22	247	31 (10.33)	26 (8.67)
5% Bleach centrifugation ZN	25	2	23	250	27 (9.0)	25 (8.33)
5% Bleach centrifugation LED-FM	32	7	16	245	39 (13.0)	32 (10.67)
PhAS sedimentation ZN	22	5	26	247	27 (9.0)	22 (7.33)
PhAS sedimentation LED-FM	24	5	24	247	29 (9.67)	24 (8.0)
Chitin sedimentation ZN	20	7	28	245	27 (9.0)	20 (6.67)
Chitin sedimentation LED-FM	21	7	27	245	28 (9.33)	21 (7.0)

TP: true positive; FP: false positive; FN: false negative; TN: true negative. Culture on Lowenstein–Jensen (LJ) medium was considered the reference standard for diagnosis of pulmonary tuberculosis.

Table 3. Diagnostic accuracy of direct and pretreated sputum smear microscopy methods against Lowenstein–Jensen culture (n = 300)

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Direct ZN	37.50	100.00	100.00	89.36
Direct LED-FM	45.83	97.62	78.57	90.44
Modified Petroff's ZN	47.92	99.60	95.83	90.94
Modified Petroff's LED-FM	60.42	98.41	87.88	92.88
5% Bleach sedimentation ZN	50.00	99.21	92.31	91.24
5% Bleach sedimentation LED-FM	54.17	98.02	83.87	91.82

5% Bleach centrifugation ZN	52.08	99.21	92.59	91.58
5% Bleach centrifugation LED-FM	66.67	97.22	82.05	93.87
PhAS sedimentation ZN	45.83	98.02	81.48	90.48
PhAS sedimentation LED-FM	50.00	98.02	82.76	91.14
Chitin sedimentation ZN	41.67	97.22	74.07	89.74
Chitin sedimentation LED-FM	43.75	97.22	75.00	90.07

TP, true positive; FP, false positive; FN, false negative; TN, true negative; PPV, positive predictive value; NPV, negative predictive value.

Definitions:

- **Sensitivity = TP / (TP + FN) × 100**
- **Specificity = TN / (TN + FP) × 100**
- **PPV = TP / (TP + FP) × 100**
- **NPV = TN / (TN + FN) × 100**

Table 4. Statistical comparison of Ziehl–Neelsen microscopy and LED fluorescence microscopy across direct and pretreated sputum smear methods

Sputum smear method	Chi-square	df	p-value	Statistical significance
Direct smear	2.35	1	0.124	Not significant
Modified Petroff's method	0.517	1	0.472	Not significant
5% bleach sedimentation	0.484	1	0.486	Not significant
5% bleach centrifugation	2.45	1	0.117	Not significant
PhAS sedimentation	0.078	1	0.779	Not significant
Chitin sedimentation	0.020	1	0.887	Not significant

Data were compared between Ziehl–Neelsen microscopy and LED fluorescence microscopy for each processing method using the chi-square test. A p-value of <0.05 was considered statistically significant.

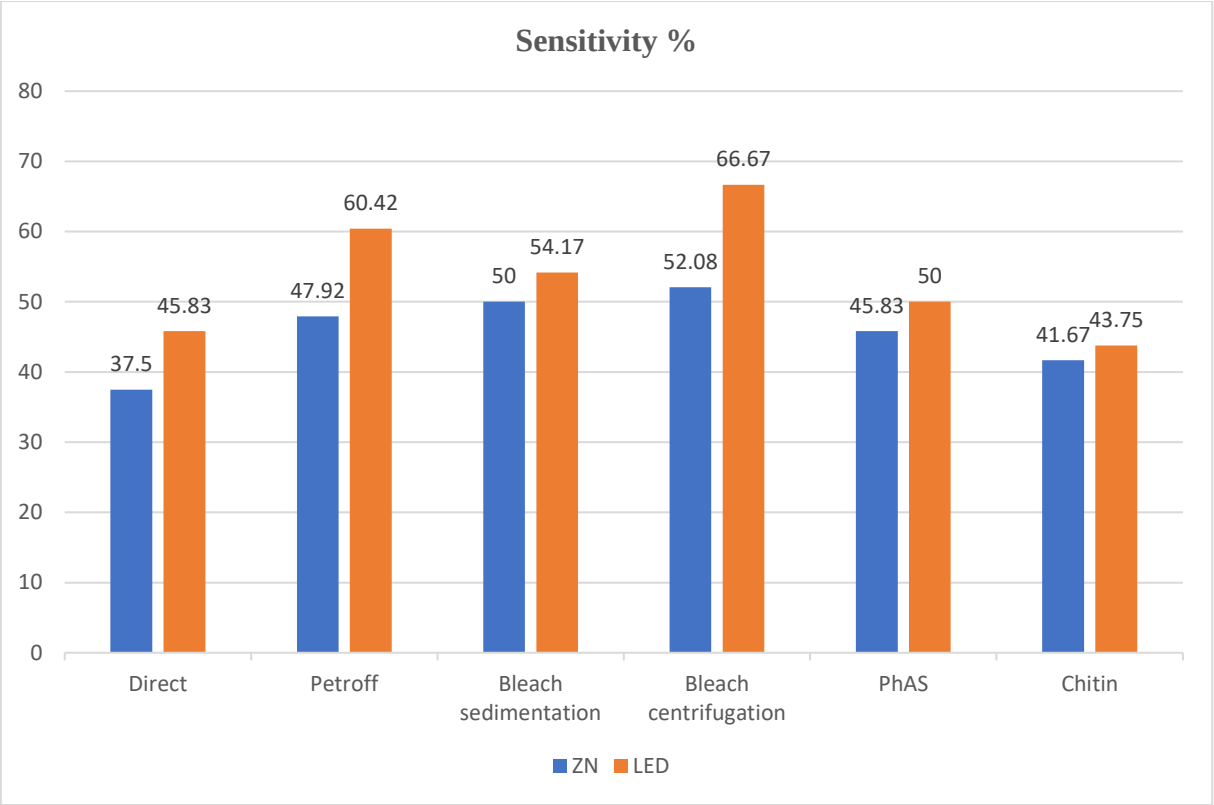

Figure 1. Comparison of sensitivity of direct and pretreated sputum smear microscopy methods using Ziehl–Neelsen and LED fluorescence microscop

DISCUSSION

The present study evaluated the diagnostic performance of direct and pretreated sputum smear microscopy using both Ziehl–Neelsen (ZN) staining and LED fluorescence microscopy (LED-FM). Overall, LED-FM showed higher sensitivity than conventional ZN microscopy across most processing methods, while specificity remained consistently high. The highest sensitivity was observed with 5% bleach centrifugation using LED-FM (66.67%). However, despite this improvement, the difference between ZN and LED-FM was not statistically significant. Similarly, Chang et al. (2016) reported in their meta-analysis that LED-FM improved sensitivity while maintaining high specificity, and Coulibaly et al. (2023) found LED-FM to be a useful alternative to conventional microscopy for sputum examination in resource-limited settings [6,10]. Tapia-Sequeiros et al. (2025) also reported better diagnostic performance with fluorescence-based staining than with conventional ZN microscopy [11].

The low sensitivity of direct smear microscopy observed in the present study is consistent with the known limitations of conventional sputum microscopy. In contrast, LED-FM showed improved detection, which may be attributed to better contrast, faster slide screening, and easier recognition of bacilli. Similar observations have been reported by Ojha et al. (2020), who highlighted the practical advantages of LED-based fluorescence microscopy, by Bhadade et al. (2015), who demonstrated its utility in pulmonary tuberculosis diagnosis, and more recently by Tshokey et al. (2026), who also reported useful diagnostic performance of auramine O LED microscopy in routine hospital settings [12, 13, 14].

Pretreatment of sputum samples improves the diagnostic yield of smear microscopy by reducing background debris and enhancing bacillary concentration. In the present study, all pretreatment methods showed higher sensitivity than direct smear microscopy, with 5% bleach centrifugation demonstrating the best performance. Similar improvements in smear positivity following chemical pretreatment have been reported by Githui et al. (2007), who showed enhanced detection in smear-negative tuberculosis cases using sodium hypochlorite sedimentation [15]. Hepple et al. (2010) also demonstrated that bleach sedimentation improves diagnostic yield compared to direct microscopy [16]. Furthermore, newer approaches focusing on sample processing and concentration techniques, such as those described by Liu et al. (2025) and Wang et al. (2022), emphasize the importance of efficient bacillary recovery for improving diagnostic accuracy. These findings collectively support the role of sputum pretreatment in enhancing smear microscopy performance [17, 18].

Among the evaluated pretreatment methods, Modified Petroff's method and PhAS sedimentation showed moderate improvement in sensitivity, whereas chitin-based pretreatment did not outperform bleach-based methods in the present study. This observation highlights the importance of effective sputum processing for optimizing smear microscopy, as emphasized by Steingart et al. (2007) [19]. Recent reviews by Zaporojan et al. (2024) and Kontsevaya et al. (2024) have also underscored the continuing need to refine conventional diagnostic approaches in resource-limited settings [20, 21]. In

addition, Liu et al. (2025) demonstrated that improved purification and concentration strategies can enhance mycobacterial recovery, supporting the rationale for continued exploration of novel processing methods such as chitin [17].

Improving the sensitivity of smear microscopy has important implications for tuberculosis control, particularly in resource-limited settings where it remains the primary diagnostic tool. Enhancing smear-based detection through simple modifications such as sputum pretreatment and the use of LED fluorescence microscopy can contribute to earlier diagnosis and reduced transmission. Studies by Davis et al. (2013) have highlighted that optimizing smear microscopy strategies can improve case detection without increasing patient visits, while Ramsay et al. (2009) and Cambanis et al. (2007) emphasized the importance of operational improvements in microscopy services to maximize diagnostic yield [22, 23, 24]. In this context, the present findings support the integration of effective pretreatment methods with LED-FM to strengthen existing diagnostic frameworks, in line with global tuberculosis control priorities outlined by the World Health Organization (2024) [25].

The present study has several strengths, including the comparative evaluation of multiple sputum processing methods within the same study population, the use of both Ziehl–Neelsen and LED fluorescence microscopy, and the use of culture as the reference standard. However, certain limitations should be acknowledged. The study was conducted at a single center, which may limit generalizability, and the sample size of specific subgroups was limited. Additionally, although improvements in sensitivity were observed with pretreatment methods, the differences were not statistically significant, and cost-effectiveness analysis was not performed.

CONCLUSION

In conclusion, sputum pretreatment enhances the diagnostic performance of smear microscopy, with LED fluorescence microscopy consistently showing higher sensitivity than Ziehl–Neelsen staining. Among the evaluated methods, 5% bleach centrifugation combined with LED-FM demonstrated the best overall performance. These findings support the use of simple, cost-effective modifications to improve tuberculosis detection in resource-limited settings, although further multicentric studies are required to validate these results.

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Conflict of Interest Disclosure

The authors declare no conflict of interest.

Funding Source Statement

This study received no external funding.

Authors' Contribution

All authors contributed to the conception, study design, data collection, analysis, interpretation, manuscript drafting, and final approval of the manuscript.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval Statement

The study was approved by the Institutional Ethics Committee prior to commencement.

Informed Consent Statement

Written informed consent was obtained from all participants before sample collection.

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