Diagnostic Accuracy of Methylene Blue as a Counterstain For LED Fluorescence Microscopy in The Detection of Acid-Fast Bacilli: A Diagnostic Accuracy Study

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ABSTRACT

Objectives

To evaluate the diagnostic accuracy and agreement of methylene blue as a secondary counterstain for auramine O-based light-emitting diode (LED) fluorescence microscopy in the detection and grading of acid-fast bacilli in sputum specimens.

Methods

A prospective diagnostic accuracy study was conducted at the Chest Diseases Laboratory, Lusaka, Zambia, between 6 January and 28 March 2026. The study included 20 direct sputum specimens, 20 concentrated sputum specimens processed using the 4% N-acetyl-L-cysteine-sodium hydroxide method, and 20 proficiency testing slides from the 2025 Round B external quality assessment programme. 2 smears were prepared from each specimen and stained with auramine O followed by either 0.3% methylene blue (index test) or 0.1% potassium permanganate (reference method). The primary outcome measures were sensitivity, specificity, positive predictive value, negative predictive value, and agreement between methylene blue and potassium permanganate for acid-fast bacilli detection and grading.

Results

Compared with potassium permanganate staining, methylene blue demonstrated 100% sensitivity and specificity across all specimen categories. For concentrated sputum specimens, sensitivity was 100% (95% CI 78.2% to 100%) and specificity was 100% (95% CI 47.8% to 100%). For direct sputum specimens, sensitivity was 100% (95% CI 66.4% to 100%) and specificity was 100% (95% CI 71.5% to 100%). Among PT slides, sensitivity and specificity were both 100% (95% CI 69.2% to 100%). Agreement between staining methods was complete across specimen categories (Cohen's κ=1.0). Among positive smears, grading agreement within one grade was observed for all specimen types, with exact grading agreement of 66.7% for concentrated sputum, 88.9% for direct sputum and 100% for PT slides.

Conclusions

Methylene blue demonstrated excellent diagnostic agreement with potassium permanganate when used as a counterstain for auramine O LED fluorescence microscopy.

Keywords: Tuberculosis, acid-fast bacilli, fluorescence microscopy, diagnostic accuracy, methylene blue

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INTRODUCTION

Tuberculosis (TB) remains a major global public health challenge, particularly in low- and middle-income countries where the burden of disease and limitations in diagnostic capacity continue to affect TB control efforts. According to the World Health Organization (WHO) Global Tuberculosis Report 2025, an estimated 10.7 million people developed TB in 2024, resulting in approximately 1.23 million deaths. TB remains one of the leading causes of death from a single infectious disease worldwide (World Health Organization, 2025).

Accurate and timely diagnosis is essential for initiating appropriate treatment, reducing transmission and achieving global TB control targets. Although molecular diagnostic technologies have expanded, sputum smear microscopy remains an important diagnostic method in many resource-limited settings because of its affordability, simplicity and feasibility for decentralised laboratories (Chandra et al., 2015). Conventional Ziehl-Neelsen (ZN) staining using bright-field microscopy has long been the standard method for detecting acid-fast bacilli (AFB). Although it is highly specific in high TB burden settings, its sensitivity is variable, ranging from approximately 20% to 80%, and is further reduced in patients with paucibacillary disease and low bacillary loads. Fluorescence microscopy (FM), on the other hand, offers improved diagnostic performance, with studies showing a 10% increase in sensitivity compared with conventional ZN microscopy while also allowing faster examination of smears due to the use of lower magnification and a wider field of view (World Health Organization, 2011).

The International Union Against Tuberculosis and Lung Disease recommended fluorescence microscopy as a standardised technique for improving smear examination, and in 2011 the WHO endorsed light-emitting diode (LED) fluorescence microscopy as an alternative to conventional light microscopy. LED-FM provides improved sensitivity, faster slide examination, lower energy consumption and greater durability, making it suitable for implementation in peripheral and resource-limited laboratory settings (World Health Organization, 2011).

The diagnostic performance of FM depends not only on the microscope but also on the quality of the staining procedure, including the choice of counterstain used after auramine staining. Potassium permanganate is commonly used as a background counterstain following auramine because it provides a darker background giving it a better contrast and sensitivity as compared to methylene blue (Hooja et al., 2011). However, the availability and supply chain challenges associated with potassium permanganate may affect the sustainability of TB diagnostic services in Zambia.

Methylene blue is widely used as a biological counterstain in microbiological staining procedures and provides adequate background contrast for the detection of AFB during LED-FM. However, evidence regarding its performance as an alternative counterstain for auramine O-based LED-FM remains limited in Zambia. Evaluation of alternative staining reagents is therefore important to ensure reliable diagnostic performance, maintain laboratory quality standards and support sustainable TB diagnostic services.

This study aimed to evaluate the diagnostic accuracy of methylene blue as a secondary counterstain for auramine O LED-FM in the detection and grading of AFB in sputum specimens. The performance of methylene blue was compared with potassium permanganate as the reference counterstain by assessing sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and agreement between staining methods.

Study objective

To evaluate the diagnostic accuracy and agreement of methylene blue as a secondary counterstain for auramine O-based LED-FM in the detection and grading of AFB in sputum specimens.

Research question

Does methylene blue provide comparable diagnostic performance to potassium permanganate when used as a secondary counterstain for auramine O-based LED-FM for the detection and grading of AFB?

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METHODS

Study design

This was a laboratory-based prospective diagnostic accuracy study evaluating the performance of methylene blue as a secondary counterstain for auramine O-stained smears examined using LED-FM for the detection of AFB. The study compared methylene blue with potassium permanganate, the routine counterstain used at Chest Diseases Laboratory (CDL) as the reference method. The study was conducted and reported in accordance with the Standards for Reporting Diagnostic Accuracy Studies (STARD) 2015 guidelines.

Study setting

The study was conducted at CDL, a tuberculosis reference laboratory in Zambia responsible for TB microscopy services, quality assurance activities and diagnostic testing support. The laboratory performs routine TB smear microscopy using auramine O staining followed by LED-FM according to standardized laboratory procedures. Specimens and PT slides included in the study were evaluated between 6 January 2026 and 28 March 2026. All laboratory procedures were performed according to the CDL procedure ID: CDL-TB-TECH-004 (Preparation, Staining and Examination of Smears Using Fluorescence Microscopy).

Study specimens

Purposive sampling was used to select sputum specimens and proficiency testing (PT) slides for evaluation. A minimum of 20 specimens per specimen category was selected based on recommendations from the Clinical and Laboratory Standards Institute (CLSI) for method verification and validation studies, which recommend the use of an appropriate number of representative specimens to assess method performance characteristics (Clinical and Laboratory Standards Institute, 2014). The study included three specimen categories:

Direct sputum specimens

20 direct sputum specimens collected from clinically suspected pulmonary TB patients were included. 2 smears were prepared from each specimen resulting in 40 slides for comparative staining. The specimens were submitted from various health facilities across the country for routine TB diagnosis as part of the CDL national mandate.

Concentrated sputum specimens

20 concentrated sputum specimens processed using the 4% N-acetyl-L-cysteine-sodium hydroxide (4% NALC-NaOH) digestion and decontamination method were included. 2 smears were prepared from each processed specimen resulting in 40 slides for comparative evaluation. The specimens were processed according to the CDL procedure ID: CDL-TB-TECH-003 (Decontamination and Re-decontamination of Sputum and Non-sputum Specimens).

PT slides

20 slides from the Round B 2025 external quality assessment programme were included. These consisted of 10 AFB-positive slides with different bacillary grades and 10 AFB-negative slides.

The final analysis included 60 specimen-slide sets comprising direct sputum specimens, concentrated sputum specimens, and PT slides.

Specimen flow

Specimens were assessed for eligibility before inclusion in the study. Eligible specimens included direct sputum specimens submitted for routine TB smear microscopy, concentrated sputum specimens processed using the 4% NALC-NaOH method and Round B 2025 PT slides with established reference results. Specimens were required to have sufficient material to prepare paired smears and meet routine laboratory quality standards for FM. Specimens with insufficient volume, damaged or poorly prepared smears, incomplete identification or technical

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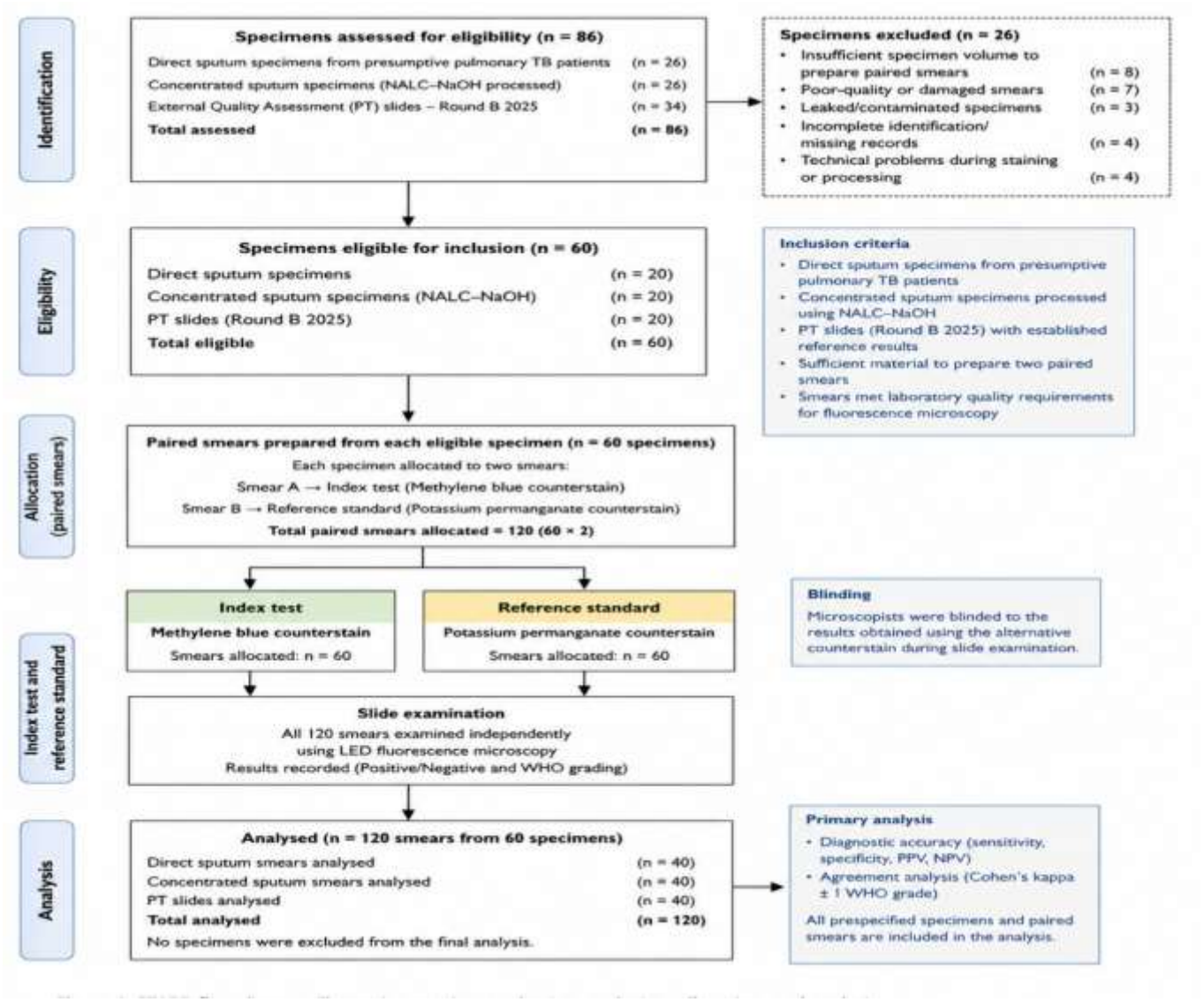
issues affecting microscopic interpretation were excluded. Each eligible specimen was used to prepare paired smears. 1 smear was counterstained with methylene blue and the other with potassium permanganate. All stained slides were examined independently by LED-FM and included in the final analysis (figure 1). A detailed specimen selection and eligibility flow diagram is provided in Supplementary Figure 1.

Figure 1. **Diagram illustrating specimen selection, exclusion, allocation and analysis**



Index test

The index test was auramine O fluorescence microscopy using 0.3% methylene blue as the secondary counterstain. For each specimen, smears were prepared on grease-free frosted glass slides, air-dried and heat-fixed. Slides were stained with freshly prepared 0.1% auramine O for 15 minutes, followed by gentle rinsing with distilled water. Smears were decolorized using 0.5% acid alcohol for not more than two minutes and rinsed with distilled water. Counterstaining was performed using 0.3% methylene blue for two minutes. Slides were immediately rinsed, air-dried and examined using a Zeiss Primo Star LED fluorescence microscope (GRZ/MOH/CDL-260). Smears were examined for the presence of AFB and graded according to the WHO FM grading guidelines (CDL-TB-JA-003). Microscopists examining slides stained with methylene blue were blinded to the results obtained from the corresponding potassium permanganate-stained slides to minimize interpretation bias. A positive index test result was defined as the presence of at least one acid-fast bacillus detected by LED-FM after methylene blue counterstaining. A negative result was defined as the absence of AFB in the examined smear. The criteria were pre-specified before data analysis based on standard microscopy interpretation guidelines (CDL-TB-JA-003).

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Reference standard

The reference standard was auramine O fluorescence microscopy using 0.1% potassium permanganate as the secondary counterstain, which is the routinely used counterstaining method at CDL. The reference slides underwent the same staining procedure as the index test except that potassium permanganate was used instead of methylene blue. Reference standard slides were examined independently from the index test slides. Microscopists were blinded to the methylene blue results during interpretation. Results from both methods were compared based on; detection of AFB (positive or negative result) and AFB grading category among positive slides. A positive reference result was defined as the observation of at least one acid-fast bacillus using LED-FM, while a negative result was defined as no AFB observed after examination of the smear. The criteria were pre-specified before data analysis based on standard microscopy interpretation guidelines (CDL-TB-JA-003).

Outcome measures

The primary outcome was the diagnostic performance of methylene blue as a counterstain for LED fluorescence microscopy. The following measures were calculated using potassium permanganate as the reference standard:

- Sensitivity: proportion of reference-positive slides correctly identified by methylene blue.
- Specificity: proportion of reference-negative slides correctly identified by methylene blue.
- PPV: probability that a methylene blue positive result represented a true positive.
- NPV: probability that a methylene blue negative result represented a true negative.

Agreement between methylene blue and potassium permanganate methods was assessed based on:

- Agreement in AFB detection (positive/negative classification)
- Agreement in grading of positive smears

Agreement within ±1 grading category was considered acceptable according to laboratory interpretation criteria.

Statistical analysis

Data were entered into Microsoft Excel and analysed using Stata version 17. Descriptive statistics were used to summarize specimen characteristics and microscopy findings. Categorical variables were presented as frequencies and percentages. Diagnostic accuracy measures, including sensitivity, specificity, PPV and NPV were calculated using two-by-two contingency tables, with potassium permanganate fluorescence microscopy used as the reference standard. Corresponding 95% confidence intervals (CIs) for all diagnostic accuracy estimates were computed to assess the precision of the measures. Agreement between methylene blue and potassium permanganate methods was assessed using Cohen's kappa statistic (κ). Kappa values were interpreted according to the following classification:

- <0.20: Poor agreement
- 0.21-0.40: Fair agreement
- 0.41-0.60: Moderate agreement
- 0.61-0.80: Substantial agreement
- 0.81-1.00: Almost perfect agreement

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RESULTS

Specimen selection and study population

A total of 86 specimens were initially assessed for eligibility. These comprised direct sputum specimens from presumptive pulmonary TB patients (n = 26), concentrated sputum specimens processed using NALC-NaOH (n = 26) and PT slides from Round B 2025 (n = 34). Of these, 26 specimens were excluded prior to analysis due to insufficient specimen volume for preparation of paired smears (n = 8), poor-quality smears (n = 7), leaked specimens (n = 3), incomplete identification (n = 4) and technical issues during staining or processing (n = 4).

After exclusions, 60 specimens met the inclusion criteria and were included in the final analysis, comprising 20 direct sputum specimens, 20 concentrated sputum specimens, and 20 PT slides. These formed 120 paired smears for comparative analysis. The distribution of positive and negative specimens according to specimen type is presented in table 1**.**

Table 1. Specimen characteristics

Specimen type	Positive	Negative	Total
Direct sputum	9	11	20
Concentrated sputum	15	5	20
PT slides	10	10	20

concentrated sputum specimens

Among the 20 concentrated sputum specimens included in the analysis, methylene blue staining was compared with potassium permanganate staining for the detection of AFB. The diagnostic accuracy measures, including sensitivity, specificity, predictive values and agreement statistics, are presented in table 2.

Table 2. Diagnostic accuracy of methylene blue compared with potassium permanganate (concentrated sputum, n = 20)

Measure	Estimate (%)	95% CI
Sensitivity	100	78.2 – 100
Specificity	100	47.8 – 100
PPV	100	78.2 – 100
NPV	100	47.8 – 100
Cohen's kappa (κ)	1.0	

Methylene blue staining correctly identified all 15 potassium permanganate-positive specimens and all 5-potassium permanganate-negative specimens. The sensitivity was 100% (95% CI 78.2% to 100%), specificity was 100% (95% CI 47.8% to 100%), PPV was 100% (95% CI 78.2% to 100%), and NPV was 100% (95% CI 47.8% to 100%). The agreement between methylene blue and potassium permanganate staining was κ=1.0 (table 2).

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Direct sputum specimens

Among the 20 direct sputum specimens, methylene blue staining was compared with potassium permanganate staining for the detection of AFB. The diagnostic accuracy measures for direct sputum specimens are presented in table 3.

Table 3. **Diagnostic accuracy of methylene blue compared with potassium permanganate (direct sputum, n = 20)**

Measure	**Estimate (%)**	**95% CI**
Sensitivity	100	66.4 – 100
Specificity	100	71.5 – 100
PPV	100	66.4 – 100
NPV	100	71.5 – 100
Cohen's kappa (κ)	1.0	

Methylene blue staining identified all 9 potassium permanganate-positive specimens and all 11 potassium permanganate-negative specimens. The sensitivity was 100% (95% CI 66.4% to 100%), specificity was 100% (95% CI 71.5% to 100%), PPV was 100% (95% CI 66.4% to 100%), and NPV was 100% (95% CI 71.5% to 100%). The agreement between methylene blue and potassium permanganate staining was κ=1.0 (table 3).

proficiency testing slide specimens

Among the 20 PT slide specimens, methylene blue staining was compared with potassium permanganate staining for the detection of acid-fast bacilli. The diagnostic accuracy measures for PT slide specimens are presented in table 4.

Table 4. **Diagnostic accuracy of methylene blue compared with potassium permanganate (PT slides, n = 20)**

Measure	**Estimate (%)**	**95% CI**
Sensitivity	100	69.2 – 100
Specificity	100	69.2 – 100
PPV	100	69.2 – 100
NPV	100	69.2 – 100
Cohen's kappa (κ)	1.0	

Methylene blue staining identified all 10 potassium permanganate-positive PT slide specimens and all 10 potassium permanganate-negative PT slide specimens. The sensitivity was 100% (95% CI 69.2% to 100%), specificity was 100% (95% CI 69.2% to 100%), PPV was 100% (95% CI 69.2% to 100%), and NPV was 100% (95% CI 69.2% to 100%). The agreement between methylene blue and potassium permanganate staining was κ=1.0 (table 4).

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Grading Agreement

The agreement between methylene blue and potassium permanganate staining based on AFB grading categories was assessed across the three specimen types. The distribution of exact agreement, agreement within one grading category (±1 grade), and disagreements greater than one grade is presented in table 5.

Table 5. Grading agreement between methylene blue and potassium permanganate staining across specimen types

Specimen type	n (positive)	Exact agreement n (%)	±1 grade agreement n (%)	>1 grade disagreement n (%)
Concentrated sputum	15	10 (66.7%)	5 (33.3%)	0 (0%)
Direct sputum	9	8 (88.9%)	1 (11.1%)	0 (0%)
PT slides	10	10 (100%)	0 (0%)	0 (0%)

Across all specimen types, methylene blue and potassium permanganate demonstrated high concordance in smear grading intensity among positive smears. For concentrated sputum, 10 of the 15 positive smears (66.7%) showed exact agreement in grading between the two staining methods, while the remaining 5 (33.3%) showed a one-grade difference. Of these 5 discordant smears, 3 were graded as 2+ with methylene blue; 1 of these was graded as 1+ and 2 were graded as 3+ with potassium permanganate. The remaining 2 discordant smears were graded as 1+ with methylene blue and as 2+ with potassium permanganate. No grading differences exceeded one grade. For direct sputum, 8 of the 9 positive smears (88.9%) showed exact agreement in grading, while one smear (11.1%) showed a one-grade difference. This smear was graded as 1+ with methylene blue and 2+ with potassium permanganate. No grading differences greater than one grade were observed. For PT slides, all 10 positive slides (100%) showed exact agreement in grading between methylene blue and potassium permanganate, with no grading discrepancies observed. Consequently, agreement under the predefined criterion of ±1 grade was also 100%, and no disagreements exceeding one grade were identified (table 5).

DISCUSSION

This study demonstrated that methylene blue is diagnostically equivalent to potassium permanganate as a counterstain for LED fluorescence microscopy in the detection of AFB. Across direct sputum, concentrated sputum and PT slides, methylene blue correctly classified all positive and negative specimens, yielding sensitivity, specificity, PPV and NPV of 100%, with perfect agreement ($\kappa = 1.0$). These findings show that substituting potassium permanganate with methylene blue did not compromise the diagnostic performance of LED fluorescence microscopy under the conditions of this study. These findings are consistent with the principle underlying auramine O - based fluorescence microscopy, in which AFB retain the fluorochrome and fluoresce brightly against a dark background. The primary role of the counterstain is to reduce background fluorescence and improve contrast, thereby facilitating the visualisation of fluorescent bacilli rather than influencing fluorochrome uptake by the organisms themselves. Consequently, provided that background quenching is adequate, the choice of counterstain is unlikely to substantially affect the qualitative detection of AFB (Forbes et al., 2018; World Health Organization, 2021).

Although diagnostic agreement was perfect across all specimen types, variation was observed in the semi-quantitative grading of positive smears. Exact grading agreement was highest for PT slides (100%), followed by direct sputum (88.9%) and concentrated sputum (66.7%). However, all grading discrepancies were confined to a single grading category, and no specimen showed a difference greater than one grade. According to the predefined agreement criterion used in this study, all positive smears therefore demonstrated acceptable grading agreement. The greater grading variability observed among concentrated sputum specimens is likely attributable to characteristics of specimen processing rather than the counterstain itself. Digestion and concentration using

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the NALC-NaOH method increase bacillary recovery but may also produce uneven clustering of bacilli on the smear. Because FM grading is based on counting fluorescent bacilli within selected microscopic fields, slight differences in bacillary distribution or the fields examined may result in counts crossing the threshold between adjacent grading categories. In this study, 5 concentrated sputum smears differed by one grading category. 3 smears graded as 2+ with methylene blue were graded as either 1+ (1 smear) or 3+ (2 smears) with potassium permanganate, while 2 smears graded as 1+ with methylene blue were graded as 2+ with potassium permanganate. The bidirectional nature of these differences suggests random variation in semi-quantitative grading rather than systematic bias favouring either counterstain.

Similarly, only 1 direct sputum smear differed in grading, being reported as 1+ with methylene blue and 2+ with potassium permanganate. Minor variation of this nature is expected in FM because bacillary counts close to grading thresholds may be influenced by differences in bacillary distribution, smear thickness, microscopic field selection and normal observer variability. Previous studies have demonstrated that semi-quantitative AFB grading is inherently subject to small inter-reader and intra-reader variations, particularly for smears with low to moderate bacillary loads (Steingart et al., 2006; World Health Organization, 2021). Importantly, none of the observed grading differences resulted in a change in qualitative diagnosis.

The complete grading agreement observed for PT slides was expected because PT slides are prepared under standardized conditions with controlled bacillary concentrations and uniform smear quality. Such slides minimise variability arising from specimen heterogeneity and therefore provide highly reproducible results irrespective of the counterstain used. The findings indicate that methylene blue performs comparably to potassium permanganate under optimal laboratory conditions. The perfect diagnostic accuracy observed in this study should nevertheless be interpreted alongside the confidence intervals. Although all point estimates were 100%, the CIs were relatively wide, reflecting the modest sample size and absence of discordant observations.

Strengths of the study

This study compared methylene blue and potassium permanganate counterstains using three different specimen types: direct sputum, concentrated sputum and PT slides. Evaluating the staining methods across multiple specimen types provided a comprehensive assessment of their performance under different laboratory conditions.

A further strength of the study was the use of potassium permanganate as the reference staining method, allowing direct comparison of diagnostic performance and smear grading between the two counterstains. In addition to qualitative smear results (positive or negative), the study also assessed semi-quantitative smear grading, providing a more detailed evaluation of agreement between the methods.

The use of standardized laboratory procedures for specimen preparation, staining, and slide reading minimized procedural variation and enhanced the reliability of the findings. Furthermore, diagnostic performance was evaluated using established measures, including sensitivity, specificity, PPV, NPV and Cohen's kappa, together with 95% CIs, providing a comprehensive assessment of agreement between the two staining methods.

Limitations of the study

This study had several limitations. First, the sample size was relatively small, with only 20 specimens evaluated for each specimen type. Although perfect agreement was observed between the two staining methods, the small sample size resulted in relatively wide confidence intervals and may limit the precision of the diagnostic accuracy estimates.

Second, the study was conducted at a single laboratory, which may limit the generalizability of the findings to other settings with different laboratory conditions, equipment, or levels of staff experience.

Third, the study evaluated only smear microscopy specimens and did not compare the staining methods using mycobacterial culture or molecular diagnostic methods as an independent reference standard. Consequently, the study assessed agreement between the two staining methods rather than the absolute diagnostic accuracy of methylene blue against a definitive reference standard.

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Finally, the study focused on laboratory performance under controlled conditions. Operational factors such as staining time, long-term reagent stability, cost-effectiveness, user acceptability and performance under routine high-workload conditions were not evaluated and warrant further investigation.

CONCLUSION

Methylene blue demonstrated comparable performance to potassium permanganate as a counterstain for LED fluorescence microscopy in direct sputum, concentrated sputum, and PT slide specimens. It achieved high sensitivity, specificity, PPV, and NPV across all specimen types, with perfect agreement between the two staining methods. Smear grading also showed high concordance, with all observed differences limited to within one grading level.

Recommendations and future perspectives

Based on the findings of this study, methylene blue demonstrated diagnostic performance comparable to potassium permanganate as a counterstain for LED fluorescence microscopy, with perfect agreement in qualitative AFB detection and only minor variations in smear grading. However, before widespread implementation, larger multicentre studies involving diverse laboratory settings should be conducted to confirm the reproducibility and generalisability of these findings. Future research should also evaluate the long-term stability of methylene blue-stained smears, the effect of methylene blue on background fluorescence and ease of slide interpretation, inter- and intra-observer agreement among microscopists with varying levels of experience, and the cost-effectiveness and operational feasibility of adopting methylene blue within national tuberculosis laboratory programmes.

Declarations

Ethics considerations

This study was conducted as part of routine laboratory method verification activities at the CDL, Zambia. The study used residual sputum specimens and PT slides generated through routine laboratory services. No additional specimens were collected specifically for the study, no direct patient contact occurred and no patient identifiers were accessed during data collection or analysis. Permission to access and analyse routinely collected laboratory data and anonymised specimens was obtained from the CDL. The study was conducted in accordance with the laboratory quality management system and the ethical principles of the Declaration of Helsinki.

Consent to participate

Not applicable.

Clinical trial number

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare no competing interests.

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Author contributions

PF, WN, JMu, DKM and MJ conceived and designed the study, developed the study protocol, coordinated data collection, analyzed and interpreted the data and drafted the manuscript. WM, MJ and AM contributed to specimen processing, staining procedures, and slide examination. EKN, BMM, JS, JMz, KN, PM and WN participated in data collection, laboratory quality checks, and review of results. All authors reviewed the manuscript critically for important intellectual content, contributed to revisions, and approved the final version for submission.

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