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Research Article

# QUANTITATIVE ASSESSMENT OF MYCOBACTERIAL LOAD THROUGH SPUTUM SMEAR GRADING AND TRUENAT CFU/mL MEASUREMENT

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## ABSTRACT

This study assessed mycobacterial load in sputum samples using smear microscopy grading and Truenat CFU/mL quantification, and evaluated the correlation between these diagnostic methods. Sputum specimens were collected from suspected pulmonary tuberculosis patients. Prepared sputum smears were stained using the Ziehl-Neelsen method, and the same sputum samples were tested using the Truenat platform. 85 (14.2%) tested positive for mycobacterium tuberculosis using Truenat. The infection rate was higher in males (61.2%), with the highest prevalence observed in the 21–30 age group. Of the 85 Truenat-positive cases, 66 (77.6%) were also positive on smear microscopy. Notably, adult males were more likely to present with high-grade smears (3+), indicating greater infectivity. The correlation between smear grade and Truenat CFU/mL was statistically significant (Chi-square = 4.2736, p = 0.0387), meeting the threshold for significance at $p < 0.05$. Bacterial loads ranged from $2.7 \times 10^1$ to $5.6 \times 10^7$ CFU/mL. A high bacterial load ($\geq 10^6$ CFU/mL) was observed in over 21% of patients, reflecting advanced disease stages. However, Truenat demonstrated superior diagnostic performance, achieving 100% sensitivity and specificity, compared to smear microscopy, which showed 61.67% sensitivity and 76% specificity.

**Keywords:** Microscopy, Mycobacterial load, Truenat, Ziehl-neelsen staining, Diagnostic sensitivity, Specificity.

## INTRODUCTION

The "World Health Organization (WHO)" reported 10.6 million TB cases in 2021, leading to 1.6 million deaths (Akhtar *et al*., 2022; Ali *et al*., 2012;). India launched the "National Strategic Plan 2017-2025" to combat TB, focusing on Test, Treat, Prevent, and Build pillars. The goal is to make India TB-free by 2025 (Brahmapurkar *et al*., 2017; Khutade *et al*., 2023; WHO, 2022). For rapid diagnosis, automated, cartridge-based nucleic acid amplification tests (NAATs) are now widely used (Hai *et al*., 2021). However, conventional sputum smear microscopy continues to play a crucial role, especially in high-burden settings. It remains a cost-effective and accessible diagnostic tool that categorizes bacterial load into semi-quantitative grades (e.g., 1+, 2+, 3+), offering a quick, though somewhat limited, estimate of bacillary burden (Hazra *et al*., 2019).

Timely evaluation of treatment response through the detection of viable bacteria is critical for assessing therapeutic efficacy and predicting clinical outcomes (Imam & Oyeyi, 2010). Such evaluations improve the accuracy of patient management. However, reliable follow-up in patients with TB remains challenging ( Kassa *et al*., 2021). Bacillary load in TB patients is commonly estimated using automated culture systems like the Mycobacterial Growth Indicator Tube (MGIT) or molecular methods such as Truenat. While culture-based techniques are considered the gold standard, they are inherently slow and susceptible to contamination. Moreover, the presence of non-culturable Mtb populations limits the reliability of culture-based

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assays for evaluating treatment response (Khan *et al*., 2006). Although DNA-based molecular diagnostics offer faster results, they cannot distinguish between live and dead bacteria, as bacterial DNA may persist even after cell death. In contrast, the Molecular Bacterial Load Assay (MBLA)—which targets 16S rRNA—provides a rapid and accurate quantification of viable Mtb. Because rRNA degrades quickly after cell death, MBLA offers a more precise reflection of bacterial viability and holds promise as a valuable tool for early prediction of treatment failure or disease progression (Khutade *et al*., 2024).

Recently introduced molecular diagnostics, such as the Truenat MTB/MTB-RIF test, utilize chip-based real-time PCR technology and have been endorsed by the WHO as reliable tools for TB diagnosis (MacLean *et al*., 2020). One of the key advantages of Truenat is its ability to quantify bacillary load in colony-forming units per milliliter (CFU/mL), offering a more sensitive and objective assessment compared to conventional methods (Magar *et al*., 2020). Although both smear microscopy and molecular diagnostics are widely used in clinical practice, there is a lack of systematic evaluation correlating smear grading with quantitative CFU/mL results obtained from Truenat. Exploring this relationship can help identify potential synergies and complementarities between the two methods. Establishing this correlation could enhance diagnostic accuracy, particularly in resource-limited settings where rapid, reliable tools are essential for effective TB control (Mistry *et al*., 2016). The aim of this study was to objectively evaluate the mycobacterial load in sputum samples by quantifying bacillary burden through both smear microscopy grading and Truenat CFU/mL measurements, and to determine the correlation between these two diagnostic methods.

## MATERIALS AND METHOD

### Ethics statement

A prospective study was conducted at the "Vedantaa Institute of Medical Sciences and Research Centre, Palghar, Maharashtra, India", over a one-year period from January to December 2024. Sputum samples were collected and processed in accordance with institutional protocols, ensuring adherence to relevant biosafety and ethical guidelines (Approval number: EC/VIMS/12/2023).

### Study design and setting

The study involved the analysis of 599 sputum samples. Inclusion criteria: Samples were collected from patients suspected of Mycobacterium tuberculosis, meeting WHO criteria for TB suspicion, including cough, chronic fever, night sweats, or unintentional weight loss. Exclusion criteria: Participants with extra-pulmonary tuberculosis were not included in the research study (WHO, 2024).

### Sample Collection

Sputum specimens were collected from patients who met the WHO inclusion criteria. Clear instructions on proper sputum collection techniques were provided to each patient to ensure the quality of the sample. Patients were instructed to take a deep breath, hold it for a few seconds, and then forcefully cough up sputum (not saliva) from deep in the lungs. The sputum was to be spat into a clean, sterile container with a tightly fitting screw cap. Patients were also advised to avoid any contact between the mouthpiece and saliva, food remnants, or any other potential contaminants. Both microscopy and Truenat analysis were performed on the morning sputum samples, as bacillary concentration tends to be higher in specimens collected in the morning.

### Z-N Staining

Our research center is accredited as a Designated Microscopy Centre (DMC) and is equipped for Truenat testing under the RNTCP in Palghar, Maharashtra. In accordance with RNTCP guidelines, Z-N staining was utilized to detect acid-fast bacilli (AFB). Under the microscope, acid-fast bacilli appear bright red against a light blue background, enabling easy identification.

### Truenat MTB Assay

Sample handling was performed using the Trueprep AUTO MTB Sample Pre-treatment Pack, which facilitates the homogenization and concentration of sputum samples. This step enhances the efficient lysis of *Mycobacterium tuberculosis* cells while also eliminating potential PCR inhibitors. Following this, DNA was extracted and purified using the "Trueprep AUTO Universal Cartridge-based Sample Preparation Kit," a miniaturized, handheld system that operates effectively at room temperature. Molecular testing was then carried out using the "Truenat MTB micro-PCR chip". This highly sensitive test detects bacterial DNA by measuring the cycle threshold (Ct) value, which represents the cycle number at which fluorescence crosses the detection threshold. Once the test is completed, the system provides a qualitative result of "Detected" or "Not Detected," along with an Internal Positive Control (IPC) to validate the testing process. For samples positive for *M. tuberculosis*, further testing was conducted to assess rifampicin resistance using the "Truenat MTB-RIF micro-PCR chip," performed on the same analytical platform (Wagh *et al*., 2024; Molbio, 2025).

### Statistically analysis

The relationship between gender (male/female) and AFB result (positive/negative) was evaluated using a Chi-Square Test of Independence. The test was conducted online tools "https://www.socscistatistics.com/tests/chisquare2/default2.aspx". Additionally, data analysis for diagnostic performance was determinate using "MedCalc Statistical Software https://www.medcalc.org/calc/diagnostic_test.php."

## RESULTS AND DISCUSSION

Of the 599 registered cases, 85 (14.2%) tested positive and 514 (85.8%) tested negative, using Truenat as the reference method. Among the confirmed cases, male - 52 (61.2%) and female- 33 (38.8%). Across all age groups, males were consistently more affected, especially in the 41–60 years range. This suggests that the infection is more prevalent in males, particularly in the working-age population (Figure 1).

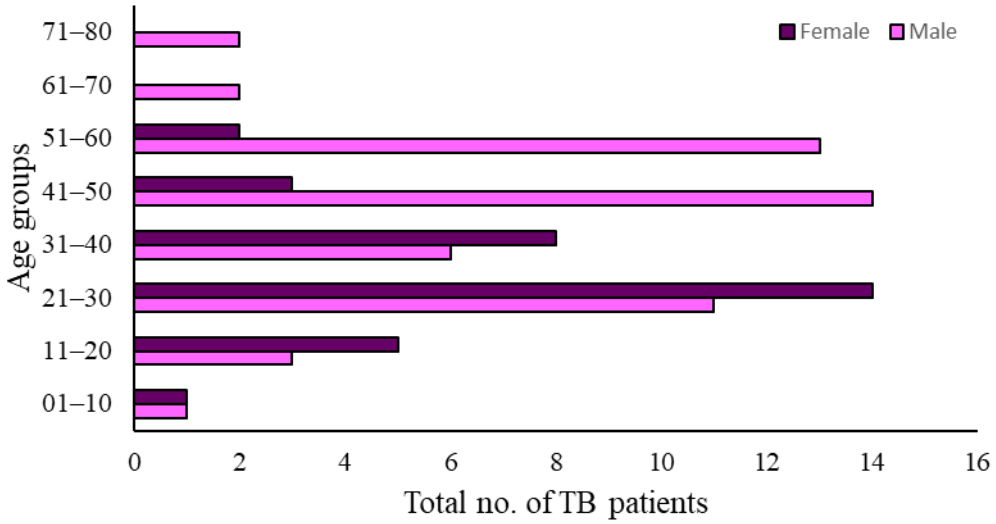


**Figure 1.** Age and gender wise distribution of TB patients (n=85). The highest proportion of confirmed cases were in the 21–30 years age group, accounting for 29.4% of the cases, with 11 males and 14 females. The 41–50 years age group was the second most affected, contributing 17 cases (20.0%), predominantly male (14 cases). The 51–60 years age group accounted for 15 cases (17.6%), again with a male predominance. There were 14 cases (16.5%) in the 31–40 years age range. Fewer cases were observed in both the ≤20 years and ≥60 years age groups.

A total of 85 samples were tested for AFB, with results analyzed by age and sex distribution. Among these, 66 (77.6%) tested positive for AFB, while 19 (22.4%) were negative. The majority of AFB-positive cases (29, 43.9%) had a bacillary load of 3+, indicating high infectivity. The 45–54 years male age group had the highest number of positives (15, 22.7%), with 9 of them showing a 3+ grade. Across all age groups, the positive rate and bacillary load were consistently higher in males.

Notably, there were no positive AFB results in the younger age group (0–14 years). Males dominated the significant positive cases in the 35–44 and 25–34 years age groups. In contrast, 15–24 and 55–64 years age groups showed scantier and 1+ bacillary grades. Females had fewer high-grade positive results (2+ or 3+) compared to males, which suggests a greater exposure and intensity of infection among adult males, particularly those of working age (Table 1). The chi-square value, p-value and determination of significance is displayed under the table. To measure the relationship between gender and swimming, a chi square for independence was used. A significant relationship was observed between these factors. The chi-square value was 4.2736. The p-value was. 038709. The result was statistically significance at $p < 0.05$.

**Table 1.** Stratification of AFB positivity by demographics in sputum samples (n=85).

| Age Group | Gender | Total Samples Examined | AFB Negative | Scanty (1–9/100 fields) | 1+ (10–99/100 fields) | 2+ (1–10/field) | 3+ (>10/field) | Total AFB Positive |
|---|---|---|---|---|---|---|---|---|
| 0–14 yrs | Male | 2 (2.4%) | 2 (2.4%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| | Female | 1 (1.2%) | 1 (1.2%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| 15–24 yrs | Male | 5 (5.9%) | 0 (0.0%) | 3 (4.5%) | 1 (1.5%) | 0 (0.0%) | 1 (1.5%) | 5 (7.6%) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Female | 13 (15.3%) | 5 (5.9%) | 1 (1.5%) | 1 (1.5%) | 3 (4.5%) | 3 (4.5%) | 8 (12.1%) |
| 25–34 yrs | Male | 10 (11.8%) | 2 (2.4%) | 1 (1.5%) | 1 (1.5%) | 1 (1.5%) | 5 (7.6%) | 8 (12.1%) |
| | Female | 8 (9.4%) | 4 (4.7%) | 0 (0.0%) | 0 (0.0%) | 2 (3.0%) | 2 (3.0%) | 4 (6.1%) |
| 35–44 yrs | Male | 9 (10.6%) | 0 (0.0%) | 1 (1.5%) | 0 (0.0%) | 4 (6.1%) | 4 (6.1%) | 9 (13.6%) |
| | Female | 5 (5.9%) | 0 (0.0%) | 0 (0.0%) | 1 (1.5%) | 2 (3.0%) | 2 (3.0%) | 5 (7.6%) |
| 45–54 yrs | Male | 15 (17.6%) | 0 (0.0%) | 0 (0.0%) | 2 (3.0%) | 4 (6.1%) | 9 (13.6%) | 15 (22.7%) |
| | Female | 2 (2.4%) | 1 (1.2%) | 0 (0.0%) | 0 (0.0%) | 1 (1.5%) | 0 (0.0%) | 1 (1.5%) |
| 55–64 yrs | Male | 9 (10.6%) | 4 (4.7%) | 2 (3.0%) | 1 (1.5%) | 1 (1.5%) | 1 (1.5%) | 5 (7.6%) |
| | Female | 2 (2.4%) | 0 (0.0%) | 0 (0.0%) | 1 (1.5%) | 0 (0.0%) | 1 (1.5%) | 2 (3.0%) |
| 65+ yrs | Male | 3 (3.5%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 2 (3.0%) | 1 (1.5%) | 3 (4.5%) |
| | Female | 1 (1.2%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 1 (1.5%) | 0 (0.0%) | 1 (1.5%) |
| Total (Statistical significant) | Male | | 8 (11.85) [1.25] | | | | | 45 (41.14) [0.36] |
| | Female | | 11 (7.15) [2.07] | | | | | 21 (24.85) [0.60] |
| Total | | 85 (100%) | 19 (22.4%) | 8 (12.1%) | 8 (12.1%) | 21 (31.8%) | 29 (43.9%) | 66 (100%) |

Bacterial loads among the 85 patients varied significantly, ranging from $2.7 \times 10^1$ to $5.6 \times 10^7$ CFU/mL, reflecting the diverse severity of the disease. Moderate bacterial loads, ranging from $10^4$ to $10^5$ CFU/mL, were seen in 30 patients (35.3%), with common values being $4.2 \times 10^4$ CFU/mL and $3.9 \times 10^4$ CFU/mL. Low bacterial counts ($\leq 10^3$ CFU/mL) were found in 15 cases It is noteworthy that some patients exhibited very low bacterial loads (e.g., $2.7 \times 10^1$ CFU/mL), which may suggest latent infection. The overall distribution of bacterial loads was skewed toward higher values, consistent with active disease. These findings suggest that quantifying bacterial load could serve as a reliable surrogate marker for both infectious burden and clinical status (Figure 2).

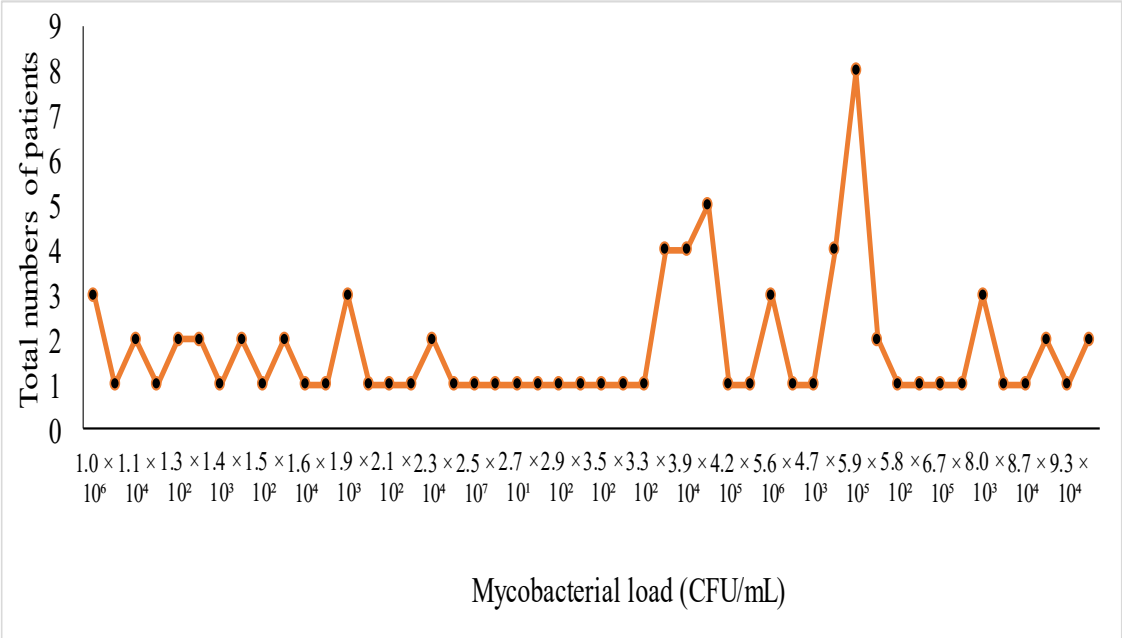


**Figure 2.** Frequency of bacterial loads detected in sputum samples correlation between smear grading and truenat CFU/mL**.** The most common bacterial load observed was $5.9 \times 10^5$ CFU/mL, found in 8 patients (9.4%). High bacterial loads ($\geq 10^6$ CFU/mL) were detected in 18 patients (21.2%), indicating advanced stages of infection in this subset. The highest reported bacterial load was $5.6 \times 10^7$ CFU/mL, observed in 4 patients.

Bacteriological load (CFU/mL) was compared with microscopic smear grading to assess bacillary load. In the cohort of 85 patients, a significant proportion exhibited high-grade smears (3+), which also correlated with peak bacterial loads ($5.9 \times 10^5$ and $5.6 \times 10^7$ CFU/mL). This smear grading system provides a reliable means of quantifying infectious burden, even in resource-limited settings. The quantitative measurement of CFU/mL enhances the interpretive accuracy of conventional smear microscopy (Figure 3).

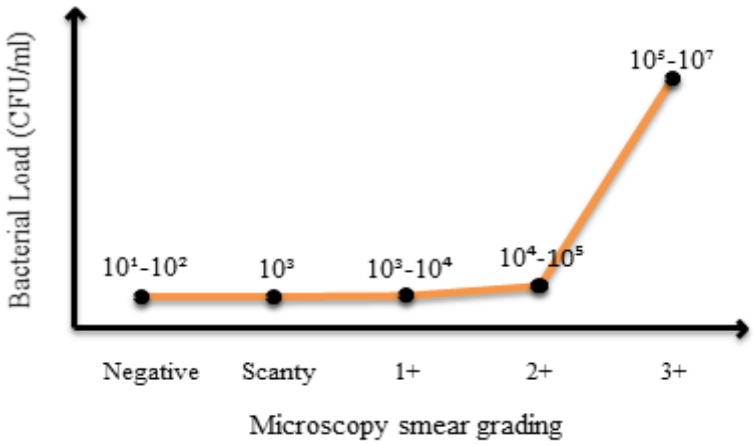


**Figure 3.** Smear grading based on bacterial concentration. Loads in the $10^1$–$10^2$ CFU/mL range were typically associated with negative smears, found in individuals with low bacteria. Sparse smears were linked to bacterial loads around $10^3$ CFU/mL, suggesting early or low infection. 1+ smears were generally observed in patients with bacterial loads between $10^3$–$10^4$ CFU/ml, while 2+ smears were seen in those with loads ranging from $10^4$–$10^5$ CFU/mL. The majority of cases with high bacterial burdens ($10^5$–$10^7$ CFU/mL) corresponded to 3+ smear grades, indicative of a high bacilli load.

The performance of microscopy was assessed against the reference standard method, Truenat. Among the 85 confirmed MTB cases, 66 individuals were True Positives (TP). Truenat identified all 66 TP cases without any False Negatives (FN), yielding a clinical sensitivity rate of 100% (95% CI: 94.56%–100%). Truenat also demonstrated 100% specificity (95% CI: 82.35%–100%), correctly identifying 19 true negatives (TNs) with 0 false positives (FPs). In contrast, microscopy detected only 37 true positives (TPs), missing 23 cases (false negatives, FN), resulting in a sensitivity of just 61.67% (95% CI: 48.21%–73.93%). The specificity for microscopy was 76% (95% CI: 54.87%–90.64%), based on 19 TNs and 6 FPs. These findings highlight the superior accuracy of Truenat over routine microscopy (Table 2).

**Table 2.** Sensitivity and specificity of truenat for *mycobacterium tuberculosis* diagnosis in 85 sputum samples.

| Method | TP | FN | TN | FP | Clinical Sensitivity (95% CI) | Clinical Specificity (95% CI) |
|---|---|---|---|---|---|---|
| Microscopy | 37 | 23 | 19 | 6 | 61.67% (48.21% to 73.93%) | 76% (54.87% to 90.64%) |
| Truenat | 66 | 0 | 19 | 0 | 100% (94.56% to 100%) | 100% (82.35% to 100.00%) |

The diagnostic accuracy of micro real-time PCR and microscopy for pulmonary tuberculosis diagnosis. The most significant prevalence of tuberculosis was studied in the 21–30-year age group, while the lowest was seen in the 0–20 and 60+ years categories. Similarly, the "Xpert MTB/RIF method" identified the highest rate of infection among individuals aged 21–30 in Jumlah[14]. At Patan Hospital, the oldest age group had a TB rate of 32.54%, while the youngest group contributed only 5.79% (Sah *et al*., 2020). In Ethiopia, the highest TB prevalence was found in the 15–24 and 25–34 age groups (Banti *et al*., 2023), while in Nigeria, the 30–43 age group showed the highest prevalence at 17% (Ukoaka *et al*., 2024). In Malaysia, the 21–40 age group had the highest TB prevalence at 37% (Ahmad *et al*., 2021), whereas in Pakistan, the <20 years group had the highest prevalence at 48.08% (Jawad *et al*., 2023). A steady increase in TB prevalence was also observed by age in Satara district, where men were found to be twice as affected as women ((Mohite *et al*., 2014). Our study yielded similar findings, with a comparable pattern reported at Lumbini Provincial Hospital (Paudel & Maharjan, 2018). Across much of Nepal, men had a higher prevalence of tuberculosis than women (Smith, 2024).

Among 520 pulmonary TB cases, "16.35% (95% CI: 13.40%, 19.79%) tested sputum smear-negative. Of the remaining patients, 15.19% (95% CI: 13.40%, 19.79%) had scanty (1 to 9 bacilli)" results, 18.27% (95% CI: 12.35%, 18.55%) had 1+, 15.77% (95% CI: 12.87%, 19.17%) had 2+, and 34.42% (95% CI: 30.45%, 38.65%) had 3+ smear grades, respectively. In total, the sputum smear-positive rate was 83.65% (95% CI: 80.21%, 86.60%) ( Kassa *et al*., 2021). Among the positive cases, 40.1% had a 3+ grade, while 6.8% had a scanty grade. The treatment success rate was lowest in the scanty group (71.4%), followed by 1+ (80.2%), with the highest success rate observed in sputum smear-negative cases (84.1%). The overall failure rate was 16.9%. Increased risks of poor outcomes were observed in patients over 40 years old, males, and those undergoing retreatment. The TB-MBLA assay quantified a "bacterial load of 4.8 log10 eCFU per mL (SD 1.5)". HIV-positive participants had a lower mean bacterial load than HIV-negative participants "(3.8 log10 eCFU per mL [SD 1.6] vs 5.2 log10 eCFU per mL [SD 1.3]; $p = 0.0002$). The median MGIT time to positivity was 7 days (IQR 5–10), which was associated with both Xpert-Ultra and TB-MBLA ($r = 0.5$, $p = 0.021$)" (Sabiiti *et al*., 2020). Notably, the time to obtain TB bacillary load results was significantly reduced from days to hours, with early treatment responses detected by the TB-MBLA assay. By week 12, 58% of patients showed a reduction in bacillary load. This reduction was linked to culture time-to-positivity ($r = -0.51$, $p < 0.0001$). Patients with a higher bacillary burden before therapy were less likely to convert to negative by week 8 ($p = 0.0005$) ( Neumann *et al*., 2025; Musisi *et al*., 2024). For smear-negative TB, the sensitivities of both tests were similar (Truenat MTB Plus: 55% vs Xpert: 53%). Regarding specificity, Truenat MTB Plus showed 96% (95% CI: 94-97%), while Xpert had a slightly higher specificity of 99% (95% CI: 97-99%) (Ngangue *et al*., 2022).

In a cohort of 175 patients, 92.6% tested positive by Mini PCR, while 84.6% were positive by smear microscopy. The performance metrics of Z-N staining were as follows: "sensitivity" 86.31%, "specificity" 57.14%, "positive predictive value" (PPV) 97.97%, "negative predictive value" (NPV) 14.81%, and accuracy 85.14%. In comparison, the performance of TrueNat was as follows: "sensitivity" 94.05%, "specificity" 42.86%, PPV 97.53%, NPV 23.08%, and accuracy 92.00% (Akhtar *et al*., 2022). This study was performed at a single tertiary care centre, so the outcome result may not be directly applicable to regions with different TB burdens or healthcare infrastructures. Additionally, Truenat was only compared to microscopy, not to MGIT culture systems or other advanced molecular diagnostic methods, which could provide a more comprehensive evaluation of its performance. The study also did not account for potential co-infections or comorbidities, such as HIV, which can influence both TB diagnosis and treatment outcomes.

## CONCLUSION

This study highlights the higher prevalence of pulmonary TB among adult males, particularly those in the working-age group, with a significant association between gender and bacillary load. A strong correlation was observed between smear grading and Truenat CFU/mL values, validating smear microscopy as a practical indicator of infectivity while confirming Truenat's superior sensitivity and specificity in TB diagnosis. The findings emphasize the value of bacterial load quantification in assessing disease severity and underscore Truenat's potential as a more accurate diagnostic tool, especially in settings where early and reliable detection is critical for TB control.

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## CONFLICT OF INTERESTS

The authors declare no conflict of interest

## ETHICS APPROVAL

Sputum samples were collected and processed in accordance with institutional protocols, ensuring adherence to relevant biosafety and ethical guidelines (Approval number: EC/VIMS/12/2023).

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## AI TOOL DECLARATION

The authors declares that no AI and related tools are used to write the scientific content of this manuscript.

## DATA AVAILABILITY

Data will be available on request

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