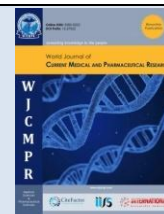




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EVALUATION OF MOLECULAR DIAGNOSTIC ASSAYS IN COMPARISON WITH ACID FAST STAINING FOR DETECTION OF TUBERCULOSIS IN PATIENTS ATTENDING A TERTIARY CARE HOSPITAL, UTTARAKHAND.

ISHAN PANDITA^{1*} , SHASHIKANT N. TAYADE¹ , TARUNA G SHIRODKER¹ , ANKIT KHANDURI¹

¹Department of Microbiology, Graphic Era Institute of Medical Sciences, Dehradun, Uttarakhand, India.

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ARTICLE HISTORY	ABSTRACT
Received on: 14-07-2026 Revised on: 21-08-2026 Accepted on: 30-09-2026	Background: Acid-fast bacilli (AFB) microscopy has limited sensitivity at low bacillary burden. Cartridge-based nucleic acid amplification testing (CBNAAT) detects <i>Mycobacterium tuberculosis</i> (MTB) directly and provides rifampicin-resistance information. Aim: To compare AFB microscopy with CBNAAT for TB detection in clinical specimens at a tertiary care hospital in Uttarakhand. Materials and methods: This specimen-based cross-sectional study included 304 specimens from 3,160 specimens received for presumptive TB evaluation during December 2025 to August 2026. AFB microscopy and CBNAAT (GeneXpert MTB/RIF Ultra) were compared in specimens, where both results were available. Molecular-load and rifampicin-resistance results were analyzed among CBNAAT-detected specimens. Results: Of 304 specimens, 217 (71.4%) were respiratory and rest 87 (28.6%) were non-respiratory. AFB stain was positive for 92 and negative for 163 specimens. CBNAAT detected MTB in 301 specimens; 3 were not detected and were reported as NTM. Among 255 specimens with both results, 163 (63.9%) were AFB-negative/CBNAAT-detected. AFB positivity increased from 0/14 (Trace) to 37/39 (High) across molecular-load categories ($P < 0.001$). Rifampicin resistance was detected in 13/274 determinate results (4.7%). Conclusion: CBNAAT detected MTB in many AFB-negative specimens, while AFB positivity increased with molecular load. These findings describe concordance and discordance in the studied specimen set rather than diagnostic accuracy.
Keywords: Tuberculosis; AFB microscopy; CBNAAT; GeneXpert MTB/RIF Ultra; molecular load; rifampicin resistance.	
Corresponding Author Dr. Ishan Pandita Department of Microbiology, Graphic Era Institute of Medical Sciences, Dehradun, Uttarakhand, India.	

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1. INTRODUCTION

Bacteriological confirmation is central to tuberculosis (TB) diagnosis and helps guide appropriate treatment and early assessment of drug resistance. India accounts for a substantial proportion of the global TB burden [1]. Rapid and reliable laboratory methods are therefore important for timely diagnosis, particularly in patients with paucibacillary disease. Ziehl–Neelsen (ZN) smear microscopy remains widely used because it is inexpensive, rapid and requires relatively limited infrastructure. However, its sensitivity is limited when the bacillary burden is low, and detection can be particularly difficult in paucibacillary disease and extrapulmonary specimens [2]. In addition, microscopy cannot distinguish *Mycobacterium tuberculosis* complex from other acid-fast organisms or provide information on drug resistance. Cartridge-based nucleic acid amplification testing (CBNAAT), including the GeneXpert platform, uses automated real-time PCR to detect *M. tuberculosis* complex DNA and mutations

associated with rifampicin resistance directly from clinical specimens. WHO recommends rapid molecular tests as initial diagnostic tests in appropriate TB diagnostic pathways [3]. In a multicentre study, a single Xpert MTB/RIF test detected 72.5% of smear-negative, culture-positive TB cases [4]. Systematic reviews have also reported high diagnostic performance in respiratory specimens, although sensitivity varies according to specimen type, particularly in extrapulmonary disease [5,6]. Xpert MTB/RIF Ultra, a later version of the assay, provides semi-quantitative molecular-load categories and includes a "Trace" category for specimens with very low levels of MTB DNA [8]. Discordance between smear microscopy and molecular testing is common, particularly when the bacillary burden is low. A specimen may be AFB-negative while MTB is detected by CBNAAT, whereas AFB-positive specimens may occasionally be negative for MTB by molecular testing because acid-fast organisms other than *M. tuberculosis* can be present. Characterising these patterns in routine clinical

specimens may help clarify the complementary roles of microscopy and molecular testing. However, data describing these findings from Uttarakhand remain limited. The primary objective of this study was to compare AFB microscopy with CBNAAT for the detection of MTB in clinical specimens evaluated for TB. Secondary objectives were to describe MTB detection among AFB-negative specimens, assess the relationship between AFB microscopy results and CBNAAT molecular-load categories, examine findings according to specimen type, and describe rifampicin-resistance results.

2. MATERIALS AND METHODS

2.1 Study design and setting

This was a specimen-based cross-sectional comparison of AFB microscopy and CBNAAT conducted in the Department of Microbiology, Graphic Era Institute of Medical Sciences, Dehradun, Uttarakhand. The study was conducted over nine months, from December 2025 to August 2026. Laboratory results recorded during this period were analyzed.

2.2 Study population and specimens

The study included specimens received from patients of any age or sex with presumptive pulmonary or extrapulmonary TB. During the study period, 3,160 specimens were received for evaluation of presumptive TB, of which 304 constituted the final study dataset, which included specimens with either CBNAAT and/or AFB microscopy positive.

AFB microscopy and CBNAAT were performed on the specimen collected. Specimen types were recorded and grouped as respiratory or non-respiratory. Respiratory specimens included sputum, bronchoalveolar lavage, endotracheal or tracheal secretions, RT/gastric aspirate and bronchoscopic biopsy. Non-respiratory specimens included specimen types, such as pus, pleural fluid, FNAC aspirate and tissue.

2.3 Inclusion and exclusion criteria

As specified in the protocol, specimens from patients with presumptive TB were eligible when suitable for AFB staining and molecular testing. Inadequate or contaminated specimens were excluded.

2.4 AFB microscopy

AFB microscopy was performed using Ziehl–Neelsen staining according to the study protocol. Positive smears were graded as scanty, 1+, 2+ or 3+ for sputum and tracheal/endotracheal samples. AFB microscopy was not ordered by the treating clinician for 49 specimens, but CBNAAT result came as detected in these specimens.

2.5 CBNAAT

Molecular testing was performed using the Xpert MTB/RIF Ultra assay (Cepheid) on the GeneXpert platform. The assay detects MTB-complex DNA and mutations associated with rifampicin resistance [7,8]. Results were recorded as MTB detected or not detected, with detected specimens assigned a semi-quantitative molecular-load category (Trace, Very low, Low, Medium or High). Rifampicin results were recorded as resistance detected, not detected or indeterminate. The assay is designed to detect MTB complex and does not identify non-tuberculous mycobacteria.

2.6 Data collection

The analysed variables included age, sex, specimen type, AFB result and grade, CBNAAT result, molecular-load category and rifampicin result.

2.7 Statistical analysis

Categorical variables were expressed as n (%), with the relevant denominator stated. Age was summarized as mean $\pm$ standard deviation and median (interquartile range). The AFB–CBNAAT comparison included specimens for which both results were available. Wilson 95% confidence intervals were calculated for selected proportions. The association between AFB positivity and ordered CBNAAT molecular-load categories was assessed using the Cochran–Armitage trend test. Respiratory and Non-respiratory groups were compared using chi-square or Fisher's exact test, as appropriate. Rifampicin-resistance findings were summarized descriptively, with indeterminate results excluded from the denominator.

Because some patients contributed more than one specimen, a sensitivity analysis using one record per UHID was performed for key comparisons. A P value <0.05 was considered statistically significant. Given the potential non-independence of repeated specimens, inferential results were interpreted cautiously.

2.8 Ethical considerations

Ethical approval was obtained from the Institutional Ethics Committee, Graphic Era Institute of Medical Sciences, Dehradun (approval no. GEU-GEIMS/IEC/2025/3875, dated 02/12/2025) before initiation of the study.

3. RESULTS

3.1 Demographic characteristics

The final dataset comprised 304 specimens, including 184 (60.5%) from males and 120 (39.5%) from females. The mean age was 51.2 ± 21.0 years, with a median of 52.5 years (IQR, 32–70; range, 8–94 years). Twelve specimens (3.9%) were from individuals aged <18 years, 164 (53.9%) from those aged 18–59 years, and 128 (42.1%) from those aged ≥ 60 years. Overall demographic and specimen characteristics are presented in Table 01.

Table 01: Demographic and specimen characteristics (N = 304 specimens)

Characteristic	n (%)
Sex	
Male	184 (60.5)
Female	120 (39.5)
Age, years	
Mean $\pm$ SD	51.2 ± 21.0
Median (IQR); range	52.5 (32–70); 8–94
<18 years	12 (3.9)
18–59 years	164 (53.9)
≥ 60 years	128 (42.1)
Specimen type	
Respiratory specimens	217 (71.4)
— Sputum	113 (37.2)
— Bronchoalveolar lavage	79 (26.0)
— Endotracheal/tracheal secretions	18 (5.9)
— Bronchoscopic biopsy	4 (1.3)
— Gastric/RT Aspirate	3 (1)
Non-respiratory specimens	87 (28.6)
— Pus	28 (9.2)

— Pleural fluid	22 (7.2)
— FNAC aspirate	10 (3.3)
— Tissue	8 (2.6)
— Others	19 (6.3)

3.2 Specimen characteristics

Respiratory specimens accounted for 217 (71.4%) of the 304 specimens, with sputum (113, 37.2%) and bronchoalveolar lavage (79, 26.0%) being the most frequent. Non-respiratory specimens accounted for 87 (28.6%), most commonly pus (28, 9.2%) and pleural fluid (22, 7.2%). (Table 01).

3.3 AFB microscopy findings

AFB microscopy was positive in 92 specimens (30.3%), negative in 163 (53.6%), and was not ordered in 49 (16.1%). Among the 92 AFB-positive specimens, 18 were graded 3+, 10 were 2+, 14 were 1+, and 13 were scanty; the grade was not recorded in 37 specimens. AFB was not ordered in 31 of 217 respiratory specimens (14.3%) and 18 of 87 non-respiratory specimens (20.7%). Among specimens for which an AFB result was available, AFB positivity was higher in respiratory specimens than in non-respiratory specimens [81/186 [43.5%] vs. 11/69 [15.9%].

3.4 CBNAAT findings

CBNAAT using Xpert MTB/RIF Ultra detected MTB in 301 of 304 specimens (99.0%) and did not detect MTB in three (1.0%). All three CBNAAT-not-detected specimens were AFB-positive, with smear grades of 2+, 1+ and 1+, respectively, and were reported by the laboratory as non-tuberculous mycobacterial (NTM) specimens. The Xpert MTB/RIF Ultra assay specifically targets the MTB complex and does not identify NTM species. The overall flow of specimens through the study is shown in **Figure 01**.

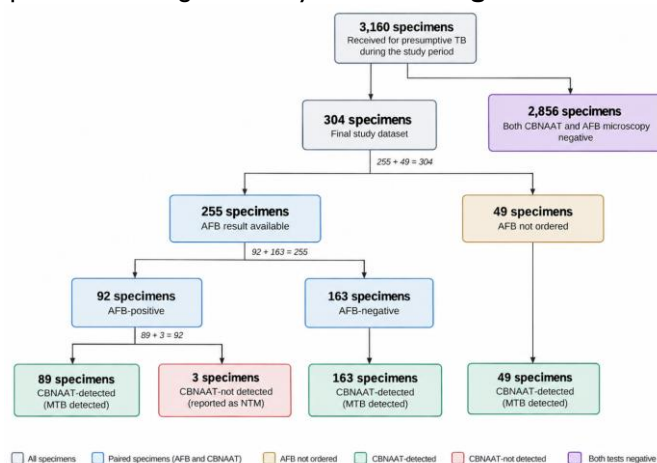


Figure 01: Flow of specimens through the study. Of 3,160 specimens received, 304 constituted the final study dataset; 255 had AFB results and 49 had AFB not ordered. Among the 255 specimens, 92 were AFB-positive and 163 AFB-negative. Of the AFB-positive specimens, 89 were CBNAAT-detected and 3 were CBNAAT-not detected and reported as NTM. All AFB-negative and AFB-not-ordered specimens were CBNAAT-detected.

3.5 AFB-CBNAAT concordance and discordance

Among the 255 specimens for which both AFB microscopy and CBNAAT results were available, 89 (34.9%) were AFB-positive/CBNAAT-detected, three (1.2%) were AFB-positive/CBNAAT-not detected, and 163 (63.9%; 95% CI, 57.9–69.6) were AFB-negative/CBNAAT-detected. No specimen was negative by both methods (**Table 2**). All 49

specimens for which AFB microscopy was not ordered were CBNAAT-detected and were therefore excluded from the paired concordance table. Among the 252 CBNAAT-detected specimens with an available AFB result, 89 (35.3%; 95% CI, 29.7–41.4) were AFB-positive. The proportion of AFB-negative/CBNAAT-detected specimens were 105/186 (56.5%) among respiratory specimens and 58/69 (84.1%) among non-respiratory specimens. The overall pattern remained unchanged in the sensitivity analysis restricted to one record per UHID ($P < 0.001$).

Table 02: AFB microscopy versus CBNAAT results among specimens with both results available ($n = 255$)

AFB result	CBNAAT: MTB detected	CBNAAT: MTB not detected	Total
Positive	89 (34.9%)	3 (1.2%)	92
Negative	163 (63.9%)	0 (0.0%)	163
Total	252	3	255

The 49 specimens for which AFB microscopy was not ordered were not included in this paired analysis. All 49 were CBNAAT MTB-detected. The three CBNAAT-not-detected specimens were AFB-positive and reported by the laboratory as NTM specimens.

3.6 Relationship between AFB microscopy and molecular load

Among the 301 CBNAAT-detected specimens, the most frequent molecular-load category was Low (100, 33.2%), followed by Very low (83, 27.6%), Medium (53, 17.6%), High (45, 15.0%) and Trace (20, 6.6%) (**Table 03**). Among the 252 specimens with both CBNAAT detection and an available AFB result, AFB positivity increased progressively across the molecular-load categories, from 0/14 (0%) in the Trace category to 37/39 (94.9%) in the High category (Cochran–Armitage trend $z = 10.51$, $P < 0.001$).

When molecular-load categories were grouped, AFB was positive in 23/165 (13.9%) specimens with Trace, Very low or Low load compared with 66/87 (75.9%) with Medium or High load. Among the 163 AFB-negative/CBNAAT-detected specimens, 142 (87.1%) had Trace, Very low or Low molecular load. Among AFB-positive specimens with a recorded smear grade ($n = 52$), a High molecular load was observed in 15/18 (83.3%) specimens with 3+ smears and none of the 13 specimens with scanty smears. The trend remained significant in the one-record-per-UHID sensitivity analysis ($P < 0.001$).

Table 03: CBNAAT molecular-load category according to AFB microscopy result among CBNAAT MTB-detected specimens ($n = 301$)

Molecular-load category	AFB positive ($n = 89$)	AFB negative ($n = 163$)	AFB not ordered ($n = 49$)	Total ($n = 301$)
Trace	0 (0.0%)	14 (8.6%)	6 (12.2%)	20 (6.6%)
Very low	1 (1.1%)	62 (38.0%)	20 (40.8%)	83 (27.6%)
Low	22 (24.7%)	66 (40.5%)	12 (24.5%)	100 (33.2%)
Medium	29 (32.6%)	19 (11.7%)	5 (10.2%)	53 (17.6%)
High	37 (41.6%)	2 (1.2%)	6 (12.2%)	45 (15.0%)

Percentages are column percentages.

3.7 Rifampicin-resistance findings

Among the 301 CBNAAT-detected specimens, rifampicin resistance was not detected in 261 (86.7%), detected in 13 (4.3%), and indeterminate in 27 (9.0%) (Table 04). After excluding the 27 indeterminate results, 13 of 274 determinate results (4.7%; 95% CI, 2.8–7.9) showed rifampicin resistance. All 20 Trace-category specimens and seven of 83 Very low-category specimens had indeterminate rifampicin results, whereas no indeterminate result occurred among specimens in the Low, Medium or High categories.

The 13 rifampicin-resistant specimens represented 12 UHIDs and included five sputum, four bronchoalveolar lavage, one endotracheal secretion, one RT aspiration and two cerebrospinal fluid specimens. AFB was positive in four, negative in seven and not ordered in two.

Table 04: Rifampicin-resistance results among CBNAAT MTB-detected specimens (n = 301)

Rifampicin result	n (%) of 301	n (%) of 274 determinate results
Resistance not detected	261 (86.7%)	261 (95.3%)
Resistance detected	13 (4.3%)	13 (4.7%)
Indeterminate	27 (9.0%)	—
Total	301 (100%)	274 (100%)

95% CI for resistance detected among determinate results: 2.8–7.9% (Wilson method).

4. DISCUSSION

The main finding was that 163 of 255 specimens with both results (63.9%) were AFB-negative but had MTB detected by CBNAAT, while only three were AFB-positive and CBNAAT-not detected; these three were reported by the laboratory as non-tuberculous mycobacteria (NTM). Without an independent reference standard, the AFB-negative/CBNAAT-detected specimens cannot be classified as false-negative smears, and MTB detection cannot by itself be equated with active disease.

The discordance was largely associated with molecular load. Xpert MTB/RIF Ultra improves detection at low bacillary loads and includes a Trace category [8,9], while microscopy generally requires a higher bacillary burden [2]. In this study, 87.1% of AFB-negative/CBNAAT-detected specimens had Trace, Very low or Low molecular load. However, sampling variation, smear preparation and detection of DNA from non-viable bacilli may also contribute [10].

AFB positivity increased from 0% in Trace specimens to 94.9% in High-load specimens, consistent with previous reports linking higher molecular burden with greater smear positivity [11]. However, 21 AFB-negative specimens had Medium or High loads, indicating that factors other than molecular load may also influence microscopy results.

AFB positivity was lower in non-respiratory than respiratory specimens (15.9% vs. 43.5%), while AFB-negative/CBNAAT-detected results were more frequent among non-respiratory specimens (84.1% vs. 56.5%). This may reflect the paucibacillary nature of extrapulmonary disease [6,12].

Rifampicin resistance was detected in 13 of 274 determinate results (4.7%), but this should not be interpreted as a population-level estimate. Twenty-seven

results (9.0%) were indeterminate, occurring in the Trace and Very low categories.

Previous Indian studies have also reported MTB detection among smear-negative specimens [15,16]. Direct comparison is limited because those studies evaluated broader presumptive-TB populations. For laboratory practice, the findings support the complementary use of microscopy and molecular testing. CBNAAT detected MTB in many AFB-negative specimens, while AFB positivity increased with molecular load. The three AFB-positive/CBNAAT-not-detected specimens reported as NTM also show that AFB positivity alone does not establish MTB infection.

The study had several limitations. There was no independent reference standard, so diagnostic accuracy could not be assessed. Clinical history and follow-up were unavailable and smear-processing details were limited. Finally, this was a single-centre study.

5. CONCLUSION

AFB microscopy and CBNAAT (Xpert MTB/RIF Ultra) showed substantial discordance in this specimen set. CBNAAT detected MTB in 163 AFB-negative and 89 AFB-positive specimens out of 255 specimens with both results, while three AFB-positive/CBNAAT-not-detected specimens were reported as NTM. AFB positivity increased with molecular-load category, and rifampicin resistance was detected in 4.7% of determinate results. These findings support the complementary role of molecular testing alongside microscopy, while interpretation remains limited by the selected specimen set and absence of an independent reference standard.

6. FUNDING

Nil

7. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest

8. AUTHOR CONTRIBUTIONS

I.P.: Conceptualization, methodology, data collection, data analysis, and manuscript drafting.
S.N.T.: Data collection, laboratory investigation, and critical review of the manuscript.
T.G.S.: Data analysis, interpretation of findings, and critical review of the manuscript.
A.K.: Literature review, interpretation of findings, and manuscript editing.

All authors: Reviewed and approved the final version of the manuscript.

9. ETHICAL STATEMENT

Ethical approval for the study was obtained from the Institutional Ethics Committee of Graphic Era Institute of Medical Sciences, Dehradun, Uttarakhand, India (Approval No. GEU-GEIMS/IEC/2025/3875, dated: 2nd December 2025). The study was conducted in accordance with the approved protocol and applicable ethical standards.

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