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Concurrent Bacterial and Fungal Co-infections in Smear-Positive Pulmonary Tuberculosis: Multivariate Predictors, Hemoptysis as a Novel Fungal Risk Factor, and Validation of Conventional Sputum Microbiology — A Cross-Sectional Study from North India

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Abstract

Background

Active pulmonary tuberculosis (PTB) predisposes patients to concurrent bacterial and fungal co-infections through immune suppression, structural lung damage, and antibiotic selection pressure. Simultaneous characterisation of both co-infection types in microbiologically confirmed PTB remains rare, with no published data from North India — a region where conventional sputum microbiology is the only routinely available diagnostic modality.

Methods

This cross-sectional study enrolled 100 sputum smear-positive PTB patients at a tertiary care centre in North India. Sputum specimens assessed for adequacy by Bartlett's scoring underwent concurrent Gram staining, pyogenic culture (blood agar and MacConkey agar; pathogenic threshold > 10,000 CFU/mL), 10% KOH wet mount, and Sabouraud dextrose agar fungal culture (> 30 colonies). Binary logistic regression and Firth's penalized likelihood regression identified independent multivariate predictors of bacterial and fungal co-infection respectively.

Results

Bacterial co-infection was identified in 50% and fungal co-infection in 18% of patients; 62% had at least one concurrent co-infection. *Pseudomonas aeruginosa* was the dominant bacterial isolate (40% of positives); *Candida* spp. constituted 88.9% of fungal isolates. Hemoptysis was the strongest independent predictor of fungal co-infection (OR = 20.0, 95% CI 5.28–90.43; adjusted OR = 17.54, 95% CI 5.23–70.33; $p < 0.001$) — a novel, clinically actionable association not previously quantified in this population. Elevated total leukocyte count was the sole independent predictor of bacterial co-infection on multivariate analysis ($p = 0.020$). Type 2 diabetes mellitus (OR = 3.50; $p = 0.021$) and HIV positivity (OR = 7.30; $p = 0.019$) independently predicted fungal co-infection. Gram staining demonstrated 98% sensitivity, 100% specificity, and 99% concordance with culture, validating the diagnostic accuracy of conventional sputum microbiology in this setting.

Conclusions

Concurrent bacterial and fungal co-infections affect the majority of smear-positive PTB patients in this cohort. Hemoptysis is a novel independent predictor of fungal co-infection warranting mandatory fungal workup, while leukocytosis serves as a practical bacterial co-infection signal. Conventional sputum microbiology demonstrates near-perfect diagnostic accuracy and merits routine incorporation into PTB management protocols in resource-limited settings.

INTRODUCTION

Tuberculosis (TB) remains one of humanity's most persistent infectious threats. In 2021, TB ranked as the second leading cause of death from a single infectious agent globally, accounting for an estimated 1.6 million deaths and more than 10 million new cases annually.[1, 2] India bears the single largest national burden, contributing approximately one-quarter of global TB incidence — a challenge further amplified by the country's high prevalence of undernutrition and diabetes mellitus.[1, 3] Pulmonary tuberculosis, particularly sputum smear-positive disease, is the most infectious form of TB; each untreated smear-positive patient may transmit infection to 10–15 contacts annually.[1]

Beyond the direct burden imposed by *Mycobacterium tuberculosis*, active pulmonary TB creates a biological milieu uniquely conducive to secondary microbial co-infections. T-lymphocyte deficiency during active disease impairs cellular immunity, while increased cortisol levels and altered neuroendocrine function further reduce immune competence.[4] Standard anti-tuberculosis therapy (ATT) compounds this vulnerability: Khan et al. demonstrated that ATT-induced gut dysbiosis — particularly with isoniazid and pyrazinamide — impairs alveolar macrophage bactericidal activity through disrupted cellular metabolism, paradoxically increasing pulmonary susceptibility to co-pathogens during treatment.[5] Prolonged exposure to rifampicin and streptomycin exerts selective antibiotic pressure that enriches the respiratory milieu for intrinsically resistant Gram-negative organisms.[6] Structural lung damage in the form of cavitation, bronchiectasis, and parenchymal necrosis further provides anatomical niches for both bacterial colonisation and fungal invasion.[7]

The clinical consequences of these co-infections are substantial. Attia et al. reported that 33% of PTB patients in Cambodia had concurrent bacterial co-infection, with *Klebsiella* and *Pseudomonas* spp. predominating and cavitary radiographic lesions significantly associated.[7] A prospective study from Manila identified bacterial co-infection in 29% of HIV-negative hospitalised TB patients, and co-infection independently increased two-week mortality 1.67-fold.[8] In Nigeria, Iliyasu et al. found Gram-negative bacilli to be as prevalent as *Streptococcus pneumoniae* as secondary pneumonia co-pathogens in PTB patients.[9] Ishikawa et al. demonstrated that co-pathogen isolation at admission in smear-positive PTB was associated with a mortality rate of 39.8% versus 10.2% in those without co-pathogens.[6] Indian data on secondary bacterial co-infection rates of up to 78% have been reported, with *Klebsiella* and *Pseudomonas* spp. most frequently isolated.[10]

Fungal co-infections in PTB have received comparatively less investigative attention despite their clinical significance. Denning et al. highlighted that failure to diagnose chronic pulmonary aspergillosis in PTB patients substantially contributes to diagnostic errors and inappropriate antimicrobial use.[11] Jabbari Amiri et al. in Iran documented invasive fungal co-infection (predominantly *Aspergillus* and *Candida*) in 6.5% of PTB sputum specimens over a decade.[12] From Nigeria, Sani et al. reported a high burden of pulmonary fungal pathogens among presumptive TB patients, with prolonged antibiotic use as a significant risk factor.[13] Mohamed et al. in Kenya demonstrated serological evidence of chronic pulmonary aspergillosis in 19.7% of PTB patients with persistent clinical symptoms.[14] Indian data on

fungal co-infection remains sparse, with a prevalence of 24% reported from Andhra Pradesh.[15] Moreover, the dual burden of type 2 diabetes mellitus and TB — a well-recognised syndemic in India — heightens susceptibility to opportunistic fungal infections by impairing neutrophil function and macrophage-mediated immunity.[16]

Most published studies characterise bacterial or fungal co-infections in PTB in isolation; simultaneous comprehensive characterisation of both types within a single well-defined cohort is uncommon. In resource-limited settings such as India, PCR-based diagnostics remain cost-prohibitive for routine use, making conventional sputum microbiology the primary available modality; however, the diagnostic validity of these tools under rigorous sample adequacy conditions has not been systematically evaluated alongside co-infection prevalence data.[17] No published study has simultaneously characterised the spectrum of bacterial and fungal co-infections in smear-positive PTB patients from North India. This study therefore aimed to determine the prevalence and spectrum of bacterial and fungal co-infections in sputum smear-positive PTB patients at a tertiary care centre in Haryana, identify independent clinical and laboratory predictors of each co-infection type, and evaluate the diagnostic performance of conventional sputum microbiology against culture as the reference standard.

METHODS

Study Design and Setting

This cross-sectional observational study was conducted in the Department of Respiratory Medicine, Pt. B.D. Sharma PGIMS, Rohtak, Haryana, in collaboration with the Department of Microbiology, following Institutional Ethics Committee approval. Written informed consent was obtained from all participants prior to enrolment.

Participants

Patients aged > 18 years with sputum smear-positive PTB attending the outpatient department or admitted to the respiratory medicine ward were eligible. Diagnosis required microbiological confirmation by smear microscopy per NTEP criteria. Patients diagnosed solely on clinicoradiological grounds and those unwilling to consent were excluded. Sample size was calculated using $N = Z^2 \times p \times q / L^2$ (anticipated prevalence p = 50%, Z = 1.96, 20% relative error), yielding a minimum of 96; 100 patients were enrolled.

Data Collection

A structured proforma documented demographics (age, gender, occupation), symptoms (cough, hemoptysis, dyspnoea, weight and appetite loss, fever), personal habits (smoking, alcohol use), comorbidities (type 2 diabetes mellitus [T2DM], hypertension, hypothyroidism), past medical history (prior TB, HIV, HCV, others), family history of TB, vitals, and pulse oximetry. Chest radiography was

reviewed for cavitary lesions, bilateral or unilateral involvement, lobar distribution, consolidation, pneumothorax, and costophrenic angle blunting. AFB smear was graded 1+, 2+, or 3 + per standard criteria.

Laboratory Investigations

Haematological investigations included haemoglobin (Hb; anaemia classified per WHO severity grades), total leukocyte count (TLC; categorised as leukopenia < 4,000/cumm, normal, or leukocytosis > 11,000/cumm), and absolute platelet count (APC). Liver function tests (SGOT, SGPT, ALP, serum protein) and kidney function tests (blood urea, serum creatinine) were performed using standard automated methods.

Sputum Processing

Early-morning sputum (3–5 mL) was collected in sterile, leak-proof containers following oral gargling with plain water. Specimen adequacy was assessed by Bartlett's scoring and microscopic criteria (> 25 pus cells and < 10 squamous epithelial cells per low-power field); inadequate specimens were discarded and repeat samples collected. Isolates identified as oropharyngeal commensals or contaminants were excluded from analysis.

Gram staining was performed using the standard four-step technique. Adequate specimens were inoculated onto blood agar and MacConkey agar plates, incubated at 37°C, and examined at 48-hour intervals. Bacterial growth exceeding 10,000 CFU/mL by semi-quantitative method was considered pathogenic. Organisms were identified using standard biochemical methods.

A 10% KOH wet mount was prepared and examined for budding yeast cells, pseudohyphae, and septate hyphae. Specimens were inoculated onto Sabouraud dextrose agar and incubated at 28°C for seven days; growth exceeding 30 colonies was considered significant. Identification was based on macroscopic colony morphology and microscopic characteristics using standard mycological methods.

Statistical Analysis

All analyses were performed using R version 4.5.2. Continuous variables are expressed as mean ± SD and median (IQR); categorical variables as frequency and percentage. Group comparisons used the independent samples t-test or Mann-Whitney U test, and Chi-square or Fisher's exact test, as appropriate following Shapiro-Wilk normality testing. Spearman's rank correlation assessed trend analyses. Predictors of bacterial co-infection were assessed by binary logistic regression (covariates: age, gender, smoking, alcohol, T2DM, old PTB, HIV, AFB grade, haemoglobin, TLC). Firth's penalized likelihood logistic regression was used for fungal co-infection predictors given low events-per-variable (EPV = 4.5, events = 18) to prevent separation bias. Gram stain diagnostic performance was calculated against culture as the reference standard. Statistical significance was set at $p < 0.05$ (two-tailed).

RESULTS

Baseline Demographic and Clinical Profile

One hundred patients were enrolled; their characteristics are presented in Table 1. The cohort was predominantly male (67%), with a mean age of 45.4 ± 16.4 years. Housewives (30%) and farmers (24%) comprised the majority occupational groups. Smoking and alcoholism were prevalent in 67% and 51% respectively. T2DM was the most common comorbidity (27%); HIV was present in 7%. Prior history of pulmonary or extrapulmonary TB was recorded in 27%; family history of TB was rare (3%). All patients had cough with sputum and fever; weight and appetite loss were present in 99%; dyspnoea in 98%. Hemoptysis was present in 22% of patients.

Laboratory Parameters and Radiological Profile

Detailed laboratory and radiological data are presented in Table 1. Anaemia was present in 87% of patients, with moderate severity predominating (47%). Leukocytosis was documented in 64% of patients. SGOT was elevated in 69% and SGPT in 55%; serum protein was low in 38%, reflecting widespread hypoalbuminaemia. AFB 3 + grade was present in 46% of patients. Chest radiography demonstrated cavitary lesions in 67%, infiltrates/opacities in 68%, and bilateral lung involvement in 64%, with upper zone predominance — consistent with a severely diseased, far-advanced cohort (Fig. 1).

Prevalence and Spectrum of Bacterial Co-infections

Microbiological findings are detailed in Table 2. Bacterial co-infection was identified in 50% of patients on pyogenic culture. Gram staining identified Gram-negative organisms in 34% and Gram-positive in 14% of specimens. Among 50 culture-positive cases, Pseudomonas aeruginosa was the dominant isolate (40%), followed by Klebsiella spp. and S. aureus (18% each) and Acinetobacter spp. (12%). Gram-negative organisms collectively accounted for 68% of all positive cultures. The diagnostic performance of Gram staining against culture is presented in Table 2; a concordance rate of 99% with sensitivity of 98% and specificity of 100% confirms its outstanding reliability in this setting (Fig. 2A-C).

Prevalence and Spectrum of Fungal Co-infections

Fungal co-infection was identified in 18% of patients (Table 2). Candida spp. constituted 88.9% of fungal isolates, with Aspergillus fumigatus accounting for the remainder. KOH mount morphology was consistent with these identifications — branching pseudohyphae and budding yeast in Candida cases, and hyaline septate hyphae in both Aspergillus cases. Considering both infection types together, 62% of the cohort had at least one concurrent microbial co-infection (bacterial only 44%, fungal only 12%, both 6%). Bacterial and fungal co-infections did not co-occur more frequently than expected by chance (Fisher's exact $p = 0.192$) (Fig. 2D).

Predictors of Bacterial Co-infection

All bivariate and multivariate data are consolidated in Table 3. Female patients demonstrated a significantly higher rate of bacterial co-infection compared to males (66.7% vs 41.8%; p = 0.019). Non-smokers and non-alcoholics paradoxically showed higher bacterial co-infection rates (p = 0.006 and p = 0.009 respectively); these associations were not sustained on multivariate analysis, indicating confounding. A significant positive trend was observed between AFB grade and bacterial co-infection prevalence (Spearman rho = 0.206, p = 0.040), with rates rising progressively from AFB 1 + through 3+. TLC was significantly higher in bacterially co-infected patients (p = 0.007), as was serum urea (p = 0.042). Age, occupation, T2DM, hypertension, old PTB, HIV, hemoptysis, cavitary lesions, bilateral involvement, anaemia severity, deranged liver function tests, and deranged kidney function tests were not significantly associated with bacterial co-infection (Table 3). On binary logistic regression adjusting for all clinical and demographic covariates, TLC was the sole independent predictor of bacterial co-infection (p = 0.020; Table 3, Panel F; Fig. 3).

Predictors of Fungal Co-infection

Hemoptysis emerged as the dominant bivariate predictor of fungal co-infection, with 59.1% of patients with hemoptysis testing positive versus 6.4% without (OR = 20.0, 95% CI 5.28–90.43; p < 0.001) (Table 3). HIV positivity (OR = 7.30, 95% CI 1.11–55.34; p = 0.019) and T2DM (OR = 3.50, 95% CI 1.06–11.70; p = 0.021) were independently associated. A significant positive trend was found between AFB grade and fungal co-infection prevalence (Spearman rho = 0.222, p = 0.027). Old PTB history demonstrated a near-significant trend (OR = 2.62; p = 0.082). Age, gender, smoking, alcoholism, cavitary lesions, and bilateral involvement were not significantly associated with fungal co-infection. None of the laboratory parameters — including haemoglobin, TLC, liver or kidney function tests — differed significantly between fungal co-infected and non-infected patients; anaemia severity was similarly non-predictive (Table 3). On Firth's penalized logistic regression, hemoptysis retained overwhelming independent significance (adjusted OR = 17.54, 95% CI 5.23–70.33; p < 0.001). T2DM and smoking showed non-significant trends (Table 3, Panel F; Fig. 4).

Subgroup Analysis: Bacterial Pathogen Distribution

Organism-level subgroup data are presented in Table 3 (Panel E). *Pseudomonas aeruginosa* was proportionally more common among T2DM patients (29.6%) than non-diabetics (16.4%). *Pseudomonas* prevalence was substantially lower in old PTB patients (7.4%) compared to new PTB (24.7%), while *S. aureus* and *Acinetobacter* were distributed comparably. HIV-positive patients had disproportionately elevated rates of both bacterial (57.1%) and fungal (57.1%) co-infection compared to HIV-negative patients (49.5% and 15.1% respectively). Old PTB patients showed a non-significant trend toward higher fungal co-infection (29.6% vs 13.7%; p = 0.082).

Laboratory Parameters by AFB Grade and TLC Category

Table 3 (Panels C and D) presents stratified analyses. TLC increased significantly across AFB grades (AFB 1+: 11,166 → 3+: 17,974/cumm; p = 0.004), demonstrating a dose-response relationship between

bacillary burden and systemic leukocytosis. Serum urea rose similarly across grades (p = 0.011). Analysis by TLC category revealed the highest bacterial co-infection rate in leukocytosis (57.8%) compared to normal TLC (34.4%), approaching but not reaching significance (p = 0.091) (Fig. 5A-C).

Laboratory Correlations

Detailed Spearman correlations are presented in the Supplementary Table. Key significant correlations included a strong positive relationship between SGOT and SGPT (rho = 0.659; p < 0.001), an inverse correlation between ALP and haemoglobin (rho = − 0.327; p = 0.001), and a strong positive correlation between serum creatinine and serum urea (rho = 0.557; p < 0.001). Age showed significant positive correlations with serum urea (rho = 0.551; p < 0.001) and serum creatinine (rho = 0.590; p < 0.001), and an inverse correlation with APC (rho = − 0.214; p = 0.033).

DISCUSSION

This study reports bacterial co-infection in 50%, fungal co-infection in 18%, and at least one concurrent co-infection in 62% of smear-positive PTB patients at a North Indian tertiary care centre. These findings emerged from a profoundly disease-burdened cohort characterised by AFB 3 + in 46%, cavitary lesions in 67%, anemia in 87%, leukocytosis in 64%, and SGOT elevation in 69%, emphasising that co-infections represent a near-universal clinical reality in far-advanced PTB rather than incidental microbiological events.

Anemia of chronic disease predominated, present in 87% of patients with moderate severity in 47%. These figures exceed the pooled global estimate from a meta-analysis by Barzegari et al. reporting anemia in 61.53% of TB patients with moderate-to-severe anemia in 43%,[18] yet are consistent with an Indian tertiary care study from Ludhiana reporting anemia in 88.5% of PTB patients, with anemia of chronic disease accounting for 97.17% of cases.[19] Hepatic involvement was extensive, with SGOT and SGPT elevated in 69% and 55% respectively. This likely reflects a dual mechanism: granulomatous hepatic infiltration from systemic MTB dissemination, and early ATT-induced hepatocellular injury. Thomas et al. demonstrated that ATT-induced LFT elevation begins within the first month of therapy — particularly in slow NAT2 acetyltransferase phenotypes — and is clinically significant in a substantial proportion of Indian patients.[20] Hypoalbuminaemia (serum protein < 6 g/dL) in 38% further corroborates the severe nutritional depletion characteristic of far-advanced PTB.[19]

The 50% bacterial co-infection prevalence falls within the wide global range of 10.9–78%, reflecting methodological heterogeneity across studies in culture thresholds, sample adequacy criteria, and referral complexity.[7, 8, 10, 21] Bir et al. from Faridabad, India, using a comparable culture-based protocol in confirmed PTB patients, reported a prevalence of 10.91% — substantially lower than ours, possibly because their cohort was assembled over a shorter observation window with more outpatient presentations.[21] Shashi Bhushan et al. from Bangalore reported 78%, likely reflecting a lower culture threshold and different patient selection.[10] *Pseudomonas aeruginosa* dominated the bacterial isolate

spectrum (40%), consistent with Bir et al. who similarly identified *Pseudomonas* as the leading co-pathogen (36.84%) at an Indian tertiary centre, both series confirming its predilection for structurally damaged, cavitary lungs in hospital-based PTB cohorts.[21] *Klebsiella* spp. and *S. aureus* were equally prevalent (18% each). A broad respiratory coinfection study at a national tertiary care centre in India identified *S. aureus* as the most common bacterial co-pathogen in immunocompromised respiratory patients, followed by *Klebsiella pneumoniae*, confirming the clinical relevance of these organisms at Indian tertiary care settings.[22] *Acinetobacter* spp. (12%) — synonymous with hospital-acquired multidrug resistance — was also prominent in the Faridabad series (26.31%), together suggesting that bacterial co-infection in hospitalised PTB increasingly mirrors hospital-acquired infection ecology rather than community-acquired pneumonia.[21] The consistent Gram-negative dominance (68%) across our data and Iliyasu et al.'s Nigerian cohort reinforces that intrinsically drug-resistant Gram-negative organisms constitute the primary bacterial co-infection threat in hospitalised PTB.[9] Strikingly, *Pseudomonas* was substantially less prevalent in old PTB patients (7.4%) than in new cases (24.7%) — possibly reflecting prior antibiotic or ATT exposure altering the lung microbial ecology, a hypothesis warranting prospective investigation.

Fungal co-infection (18%), dominated by *Candida* spp. (88.9%), is consistent with Gandi et al. (24%, India) and Mohamed et al. (19.7%, Kenya), while exceeding Jabbari Amiri et al. (6.51%, Iran), where *Aspergillus* rather than *Candida* predominated.[12, 14, 15] The *Candida* predominance in culture-based studies is expected in malnourished, antibiotic-exposed, and hyperglycaemic hosts. Critically, our KOH-based *Aspergillus* detection likely underestimates the true *Aspergillus* burden: Page et al. demonstrated that chronic pulmonary aspergillosis — identified only by serology and CT — occurs at an annual incidence of 6.5% in PTB survivors with residual cavitation, far exceeding culture-based estimates.[23] Volpe-Chaves et al. found serological evidence in 10.9% of active and previous PTB patients, with prevalence increasing progressively with time from TB diagnosis.[24] Old PTB patients in our cohort showed a clinically meaningful, though non-significant, trend towards higher fungal co-infection (29.6% vs 13.7%; OR = 2.62; p = 0.082), corroborating the longitudinal fungal burden accumulation pattern documented by Volpe-Chaves et al.[24]

The association between hemoptysis and fungal co-infection (OR = 20.0, 95% CI 5.28–90.43; adjusted OR = 17.54 on Firth's regression) is the most clinically actionable finding of this study, and it is mechanistically well-grounded. Page et al. demonstrated that the combination of raised *Aspergillus*-specific IgG, hemoptysis, and cavitary chest radiography achieved 85.7% sensitivity and 99.6% specificity for chronic pulmonary aspergillosis in treated PTB patients.[23] Volpe-Chaves et al. independently identified hemoptysis as an independent predictor of this condition ($p < 0.001$), consistent with our adjusted OR.[24] Alastruey-Izquierdo et al. emphasise that hemoptysis complicating pulmonary aspergillosis can be fatal, arising from *Aspergillus* invasion of pulmonary blood vessels within cavities; prompt antifungal intervention is critical.[25] Korukanti et al. demonstrated that early-stage aspergilloma formation in post-TB cavities produces the greatest parenchymal inflammation and abnormal vascularity, directly accounting for hemoptysis.[26] Mei et al. confirmed hemoptysis as a hallmark clinical feature distinguishing pulmonary aspergillosis superimposed on TB.[27] Even *Candida*

pseudohyphae may invade bronchial mucosa, contributing to mucosal bleeding. No prior study from a North Indian smear-positive PTB cohort has quantified this hemoptysis–fungal association — this study provides robust evidence that hemoptysis should be a mandatory trigger for sputum fungal KOH mount and culture, given the 59.1% fungal co-infection rate in our hemoptysis subgroup.

Female patients demonstrated higher bacterial co-infection (66.7% vs 41.8%; p = 0.019), which is counterintuitive given that males are broadly more susceptible to active PTB through both hormonal and behavioural pathways.[29] Females who nonetheless develop severe PTB in this high-burden context may represent a particularly immunologically vulnerable subset, a phenomenon consistent with the predominantly housewife occupational profile (30%) and the known differential in healthcare-seeking behaviour. The association was lost on multivariate adjustment, confirming confounding. Similarly, the paradoxical inverse associations between smoking/alcoholism and bacterial co-infection (both lost on multivariate) are attributable to confounding: in this cohort, smokers and alcoholics are predominantly male farmers and labourers whose co-infection risk profile differs substantially from non-smoking females and housewives. T2DM independently predicted fungal co-infection (OR = 3.50; p = 0.021) through hyperglycemia-mediated neutrophil dysfunction, reduced oxidative killing, and RAGE receptor-mediated suppression of inflammatory signalling.[28] Notably, T2DM was not significantly associated with bacterial co-infection (p = 0.822), consistent with prior observations that co-pathogen isolation in PTB correlates more strongly with nutritional markers and performance status than T2DM per se.[6] The numerically higher Pseudomonas prevalence in diabetic patients (29.6% vs 16.4%) merits further investigation in larger cohorts. HIV positivity (OR = 7.30; p = 0.019) led to simultaneous bacterial and fungal co-infection in 57.1% of HIV-positive patients — consistent with the established elimination of cell-mediated antifungal immunity by progressive T-cell depletion in HIV-TB co-infection.[11]

Cavitary lesions were not significantly associated with bacterial (p = 0.524) or fungal (p = 0.104) co-infection. Attia et al. in Cambodia found bacterial co-infection in 43% of cavitary patients, reaching statistical significance.[7] Our non-significant results most plausibly reflect a ceiling effect — with 67% of the entire cohort having cavitary disease, the non-cavitary group (n = 33) is too small for adequate discriminatory power. All cavity-related odds ratios were directionally consistent with prior literature (OR = 1.31 for bacterial; OR = 2.88 for fungal), suggesting larger cohorts would likely achieve significance. Bilateral involvement showed a near-significant trend for bacterial co-infection (p = 0.096, OR = 2.02) that similarly did not reach significance, again consistent with this ceiling effect interpretation.

TLC was the sole independent predictor of bacterial co-infection on multivariate analysis (p = 0.020). A dose-response relationship between AFB grade and TLC (AFB 1+: 11,166 → 3+: 17,974/cumm; p = 0.004) demonstrates that higher bacillary burden drives escalating systemic leukocyte activation. Serum urea rose similarly across AFB grades (p = 0.011), indicating progressive systemic catabolic and inflammatory stress. The significant positive AFB grade trend for both bacterial (rho = 0.206, p = 0.040) and fungal (rho = 0.222, p = 0.027) co-infection corroborates that disease severity is the primary driver of co-infection vulnerability. The strong SGOT–SGPT correlation (rho = 0.659; p < 0.001) reflects parallel hepatocellular enzyme release during granulomatous or ATT-induced liver injury, a relationship consistent with the

pharmacogenomic hepatotoxicity data of Thomas et al.[20] The inverse ALP–haemoglobin correlation (rho = − 0.327; p = 0.001) reflects the co-occurrence of cholestatic or granulomatous hepatic disease (elevating ALP) and anaemia of chronic disease (suppressing haemoglobin) driven by the same systemic inflammatory burden.[19] The strong serum creatinine–urea correlation (rho = 0.557; p < 0.001) reflects consistent co-tracking of renal filtration function, while the strong age correlations with serum urea (rho = 0.551) and serum creatinine (rho = 0.590; both p < 0.001) reflect the expected age-related progressive decline in glomerular filtration rate.

The near-perfect Gram stain performance (sensitivity 98%, specificity 100%, concordance 99%) far exceeds the 54% sensitivity reported under non-standardised conditions, where inconsistent sample quality limited accuracy.[17] The decisive factor in our results was rigorous Bartlett's-based sample adequacy assessment — the single false negative (Gram stain−, culture+) was the only exception across 100 specimens. Yoshimura et al., in a multicentre randomised trial, demonstrated that Gram stain-guided antibiotic therapy was non-inferior to guideline-based broad-spectrum treatment in ventilator-associated pneumonia, significantly reducing antipseudomonal and anti-MRSA agent use.[30] Together, these data provide strong policy-level evidence that Gram staining on adequately processed specimens is both diagnostically accurate and capable of guiding rational, narrower antibiotic therapy in resource-limited settings where molecular diagnostics are inaccessible.

Strengths of this study include simultaneous characterisation of both co-infection types, rigorous Bartlett's-based sample adequacy, appropriate Firth's penalized regression for rare outcomes (EPV = 4.5), and comprehensive multivariable adjustment for a single cohort. Key limitations are the single-centre cross-sectional design (precluding causal inference), small HIV subgroup (n = 7) limiting stratified analyses, absence of species-level *Candida* identification and antimicrobial susceptibility data, no mortality or treatment outcome follow-up, and the high baseline cavitary prevalence that created a ceiling effect limiting detection of cavitary-co-infection associations.

CONCLUSIONS

This study demonstrates a substantial burden of concurrent microbial co-infections in sputum smear-positive PTB patients at a North Indian tertiary care centre, with bacterial co-infection in 50%, fungal co-infection in 18%, and at least one concurrent co-infection in 62% of patients. *Pseudomonas aeruginosa* dominated the bacterial spectrum, reflecting the tertiary referral and nosocomial context, while *Candida* spp. accounted for the majority of fungal isolates.

Three clinically actionable conclusions emerge. First, hemoptysis in a PTB patient is a novel, powerful independent predictor of fungal co-infection (adjusted OR = 17.54) and should routinely prompt mandatory sputum fungal KOH mount and culture. Second, leukocytosis is the only independent predictor of bacterial co-infection, making a routine total leukocyte count a practical and universally accessible screening signal. Third, patients with T2DM or HIV represent a high-risk group warranting early proactive microbiological evaluation for both co-infection types.

Conventional sputum microbiology — Gram staining, pyogenic culture, and KOH mount — demonstrated near-perfect diagnostic accuracy when applied with rigorous sample adequacy standards, and is recommended for routine incorporation into PTB management protocols in resource-limited settings. Future multi-centre studies with species-level fungal identification and antimicrobial susceptibility profiling are warranted.

Table 1
Baseline Clinical, Demographic and Laboratory Profile (n = 100)

Variable	n (%) or Mean ± SD
DEMOGRAPHIC CHARACTERISTICS	
Age (years): Mean ± SD	45.4 ± 16.4
Median (IQR)	45 (31–57); Range 18–82
18–30 / 31–45 / 46–60 / >60 years	25 / 29 / 25 / 21
Male / Female	67 (67.0) / 33 (33.0)
Housewife / Farmer / Labourer / Others / Student / Shopkeeper	30 / 24 / 16 / 12 / 11 / 7
PERSONAL HISTORY	
Smoking / Alcoholism	67 (67.0) / 51 (51.0)
COMORBIDITIES	
Type 2 Diabetes Mellitus / Hypertension / Hypothyroidism	27 (27.0) / 8 (8.0) / 1 (1.0)
PAST HISTORY	
Nil	60 (60.0)
Old PTB/EPTB	27 (27.0)
HIV positive	7 (7.0)
HCV positive	3 (3.0)
Pneumothorax/Hydropneumothorax	3 (3.0)
Others (COVID/HBsAg/Abscess)	3 (3.0)
Family history of PTB	3 (3.0)
CLINICAL SYMPTOMS	
Cough with sputum / Fever	100 (100.0) / 100 (100.0)
Loss of weight / Loss of appetite	99 (99.0) / 99 (99.0)
Shortness of breath / Hemoptysis	98 (98.0) / 22 (22.0)
HAEMATOLOGICAL PARAMETERS	
Haemoglobin (g/dL): Mean ± SD; Median (IQR)	9.7 ± 2.2; 9.9 (8.0–11.0)
Anaemia severity: None / Mild / Moderate / Severe	13 / 17 / 47 / 23

PTB: Pulmonary tuberculosis; EPTB: Extrapulmonary tuberculosis; TLC: Total leukocyte count; APC: Absolute platelet count; IQR: Interquartile range

Variable	n (%) or Mean ± SD
TLC (/cumm): Mean ± SD	15216 ± 8157
Leukopenia (< 4,000) / Normal / Leukocytosis (> 11,000)	4 (4.0) / 32 (32.0) / 64 (64.0)
APC (Lac/cumm): Mean ± SD	2.3 ± 1.1
BIOCHEMICAL PARAMETERS	**Mean ± SD**
SGOT (U/L)	62.9 ± 58.9
SGPT (U/L)	53.9 ± 61.1
ALP (U/L)	144.8 ± 72.0
Serum Protein (g/dL)	6.2 ± 0.9
Serum Urea (mg/dL)	36.9 ± 19.0
Serum Creatinine (mg/dL)	0.9 ± 0.5
AFB SMEAR GRADE	
1+ / 2+ / 3+	23 (23.0) / 31 (31.0) / 46 (46.0)
CHEST X-RAY FINDINGS	
Infiltrates/Opacities	68 (68.0)
Cavitary lesions	67 (67.0)
Bilateral involvement	64 (64.0)
Right-sided / Left-sided predominance	46 (46.0) / 25 (25.0)
Upper / Middle / Lower zone	47 (47.0) / 20 (20.0) / 12 (12.0)
Pneumothorax / CP angle blunting	4 (4.0) / 4 (4.0)
Consolidation / Fibrocavitary / White-out lung	3 (3.0) / 3 (3.0) / 2 (2.0)

PTB: Pulmonary tuberculosis; EPTB: Extrapulmonary tuberculosis; TLC: Total leukocyte count; APC: Absolute platelet count; IQR: Interquartile range

Table 2
Microbiological Findings and Diagnostic Performance

Variable	**n (%)**
BACTERIAL CO-INFECTION	
Overall culture positivity	50 (50.0)
Gram stain: Gram-negative / Gram-positive / Mixed / No pathogen	34 (34.0) / 14 (14.0) / 1 (1.0) / 51 (51.0)
Bacterial Isolate Spectrum (n = 50 culture positives)	**n**
Pseudomonas aeruginosa	20
Klebsiella spp.	9
Staphylococcus aureus	9
Acinetobacter spp.	6
Other/Polymicrobial	3
Haemophilus influenzae	1
S. epidermidis	1
S. pneumoniae	1
Gram-negative total / Gram-positive total	34 (68.0%) / 14 (28.0%)
FUNGAL CO-INFECTION	
KOH/culture positivity	18 (18.0)
Candida spp. (of 18 positives) / *Aspergillus fumigatus*	16 (88.9%) / 2 (11.1%)
KOH morphology: Budding yeast / Pseudohyphae / Septate hyphae / Unspecified	3 / 5 / 2 / 13
OVERALL INFECTION PATTERN	
Neither / Bacterial only / Fungal only / Both	38 (38.0) / 44 (44.0) / 12 (12.0) / 6 (6.0)
At least one concurrent co-infection	**62 (62.0)**
GRAM STAIN DIAGNOSTIC PERFORMANCE	
Gram stain positive	
Gram stain negative	
Sensitivity / Specificity / PPV / NPV / Concordance	98.0% / 100.0% / 100.0% / 98.0% / 99.0%

Table 3
Predictors of Bacterial and Fungal Co-infection — Bivariate, Subgroup and Multivariate Analyses Panel A: Demographic and Risk Factor Associations

Variable	Bacterial + n (%)	OR (95% CI)	p	Fungal + n (%)	OR (95% CI)	p
Age group (overall)	—	—	0.692	—	—	0.899
Male (n = 67) / Female (n = 33)	28 (41.8) / 22 (66.7)	0.36 (0.15–0.88) / Ref	0.019*	15 (22.4) / 3 (9.1)	2.86 / Ref	0.165
Smoking Yes (n = 67) / No (n = 33)	27 (40.3) / 23 (69.7)	0.29 (0.12–0.71) / Ref	0.006**	15 (22.4) / 3 (9.1)	2.88 / Ref	0.104
Alcohol Yes (n = 51) / No (n = 49)	19 (37.3) / 31 (63.3)	0.34 (0.15–0.78) / Ref	0.009**	11 (21.6) / 7 (14.3)	1.65 / Ref	0.343
T2DM Yes (n = 27) / No (n = 73)	13 (48.1) / 37 (50.7)	0.90 (0.37–2.19) / Ref	0.822	9 (33.3) / 9 (12.3)	3.50 (1.06–11.70) / Ref	0.021*
Hypertension Yes (n = 8) / No (n = 92)	5 (62.5) / 45 (48.9)	1.73 (0.32–11.80) / Ref	0.715	—	—	—
Old PTB Yes (n = 27) / No (n = 73)	10 (37.0) / 40 (54.8)	0.49 (0.20–1.20) / Ref	0.115	8 (29.6) / 10 (13.7)	2.62 (0.78–8.64) / Ref	0.082
HIV Yes (n = 7) / No (n = 93)	4 (57.1) / 46 (49.5)	1.36 (0.22–9.78) / Ref	1.000	4 (57.1) / 14 (15.1)	7.30 (1.11–55.34) / Ref	0.019*

Panel B: Clinical and Radiological Associations

Variable	Bacterial + n (%)	OR (95% CI)	p	Fungal + n (%)	OR (95% CI)	p
Hemoptysis Yes (n = 22) / No (n = 78)	12 (54.5) / 38 (48.7)	1.26 (0.49–3.26) / Ref	0.629	13 (59.1) / 5 (6.4)	20.00 (5.28–90.43) / Ref	< 0.001***
Cavitary Yes (n = 67) / No (n = 33)	35 (52.2) / 15 (45.5)	1.31 (0.57–3.03) / Ref	0.524	15 (22.4) / 3 (9.1)	2.88 (0.77–10.78) / Ref	0.104
Bilateral Yes (n = 64) / No (n = 36)	36 (56.2) / 14 (38.9)	2.02 (0.88–4.64) / Ref	0.096	14 (21.9) / 4 (11.1)	2.24 (0.68–7.41) / Ref	0.179
AFB Grade (trend)	—	Spearman rho = 0.206	0.040*	—	Spearman rho = 0.222	0.027*
AFB 1+ (n = 23) / 2+ (n = 31) / 3+ (n = 46)	7 (30.4%) / 16 (51.6%) / 27 (58.7%)	—		1 (4.3%) / 5 (16.1%) / 12 (26.1%)	—	

Panel C: Laboratory Parameter Comparison

Parameter	Bact+ Mean ± SD	Bact− Mean ± SD	p	Fung+ Mean ± SD	Fung− Mean ± SD	p
Haemoglobin (g/dL)	9.9 ± 2.2	9.5 ± 2.2	0.420	10.0 ± 2.4	9.6 ± 2.1	0.585
TLC (/cumm)	17572 ± 9430	12860 ± 5846	0.007**	13950 ± 7912	15494 ± 8231	0.606
APC (Lac/cumm)	2.4 ± 1.2	2.2 ± 1.1	0.460	2.4 ± 1.3	2.3 ± 1.1	0.918
SGOT (U/L)	71.0 ± 79.6	54.8 ± 23.4	0.951	55.0 ± 25.1	64.6 ± 64.0	0.975
SGPT (U/L)	62.9 ± 82.3	44.9 ± 24.7	0.565	43.0 ± 22.6	56.3 ± 66.5	0.597
ALP (U/L)	161.0 ± 87.7	128.6 ± 47.4	0.071	133.5 ± 54.6	147.3 ± 75.3	0.560
Serum Protein (g/dL)	6.3 ± 1.0	6.1 ± 0.7	0.215	6.4 ± 0.8	6.1 ± 0.9	0.301
Serum Urea (mg/dL)	40.6 ± 19.6	33.3 ± 17.7	0.042*	41.9 ± 21.1	35.9 ± 18.4	0.270
Serum Creatinine (mg/dL)	0.9 ± 0.5	0.9 ± 0.5	0.309	1.0 ± 0.6	0.9 ± 0.5	0.857
Deranged LFT	39/82 (47.6%)	11/18 (61.1%)	0.436	17/82 (20.7%)	1/18 (5.6%)	0.183
Deranged KFT	25/41 (61.0%)	25/59 (42.4%)	0.103	9/41 (22.0%)	9/59 (15.3%)	0.435
Anaemia severity (overall p)			0.241			0.629
TLC category (overall p)			0.091			0.126

Panel D: Laboratory Parameters by AFB Grade (Kruskal-Wallis)

Parameter	AFB 1+ (n = 23)	AFB 2+ (n = 31)	AFB 3+ (n = 46)	p
TLC (/cumm)	11166 ± 4103	14129 ± 7223	17974 ± 9295	0.004**
Serum Urea (mg/dL)	28.0 ± 12.4	33.6 ± 12.1	43.6 ± 22.9	0.011*
ALP (U/L)	124.7 ± 31.5	125.6 ± 50.0	167.8 ± 90.8	0.055
Haemoglobin (g/dL)	10.3 ± 1.9	9.4 ± 2.3	9.6 ± 2.2	0.277
Serum Creatinine (mg/dL)	0.8 ± 0.4	0.8 ± 0.3	1.0 ± 0.6	0.113
Bacterial co-infection	7 (30.4%)	16 (51.6%)	27 (58.7%)	0.084
Fungal co-infection	1 (4.3%)	5 (16.1%)	12 (26.1%)	0.086

Panel E: Bacterial Isolate Distribution and Co-infection by Clinical Subgroup

Organism / Outcome	T2DM+ (n = 27)	T2DM− (n = 73)	Old PTB (n = 27)	New PTB (n = 73)	HIV+ (n = 7)	HIV− (n = 93)
Pseudomonas aeruginosa	8 (29.6%)	12 (16.4%)	2 (7.4%)	18 (24.7%)	—	—
Klebsiella spp.	2 (7.4%)	7 (9.6%)	2 (7.4%)	7 (9.6%)	—	—
S. aureus	1 (3.7%)	8 (11.0%)	3 (11.1%)	6 (8.2%)	—	—
Acinetobacter spp.	2 (7.4%)	4 (5.5%)	2 (7.4%)	4 (5.5%)	—	—
Fisher's p (organism distribution)	0.699		—		—	
Bacterial co-infection	13 (48.1%)	37 (50.7%)	10 (37.0%)	40 (54.8%)	4 (57.1%)	46 (49.5%)
Fungal co-infection	9 (33.3%)	9 (12.3%)	8 (29.6%)	10 (13.7%)	4 (57.1%)	14 (15.1%)
p (fungal)	0.021*		0.082		0.019*	

Panel F: Multivariate Analysis

Binary Logistic Regression — Bacterial Co-infection (AIC = 138.5)

Variable	Adjusted OR	95% CI	p
TLC (per unit)	1.00	1.00–1.00	0.020*
AFB grade 2 + vs 1+	2.81	0.78–10.08	0.113
AFB grade 3 + vs 1+	2.70	0.76–9.53	0.123
Haemoglobin	1.18	0.94–1.48	0.146
Smoking	0.26	0.04–1.91	0.186
Old PTB	0.53	0.18–1.55	0.248
HIV	2.52	0.39–16.17	0.331
Alcoholism	0.61	0.16–2.34	0.466
Gender (Male)	1.69	0.23–12.47	0.605
T2DM	1.06	0.35–3.16	0.918
Age	1.00	0.96–1.03	0.769

Firth's Penalized Regression — Fungal Co-infection (events = 18; EPV = 4.5)

Variable	Adjusted OR	95% CI	p
Hemoptysis	17.54	5.23–70.33	< 0.001***
T2DM	2.72	0.78–9.92	0.116
Smoking	3.29	0.76–18.26	0.114
Old PTB	1.75	0.47–6.33	0.396

*OR: Odds ratio; CI: Confidence interval; T2DM: Type 2 diabetes mellitus; TLC: Total leukocyte count; EPV: Events per variable; * p < 0.05; ** p < 0.01; *** p < 0.001*

Declarations

Ethics Approval and Consent to Participate

This study was approved by the Biomedical Research Ethics Committee (BREC) of Pt. B.D. Sharma Post Graduate Institute of Medical Sciences (PGIMS)/University of Health Sciences (UHS), Rohtak, Haryana, India (Approval Letter No. BREC/24/596), dated 13 May 2024. Written informed consent was obtained from all participants prior to enrolment. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent for Publication: Not applicable.

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

SB conceptualized and designed the study, performed data collection and formal analysis, and wrote the original draft. RG contributed to conceptualization, supervised the study, and critically reviewed the manuscript. PP performed microbiological analysis, contributed to methodology, and reviewed the manuscript. AG contributed to methodology and data interpretation, and reviewed the manuscript. NS assisted with data collection and reviewed the manuscript. RaG performed data analysis and reviewed the manuscript. All authors read and approved the final manuscript.

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Figures

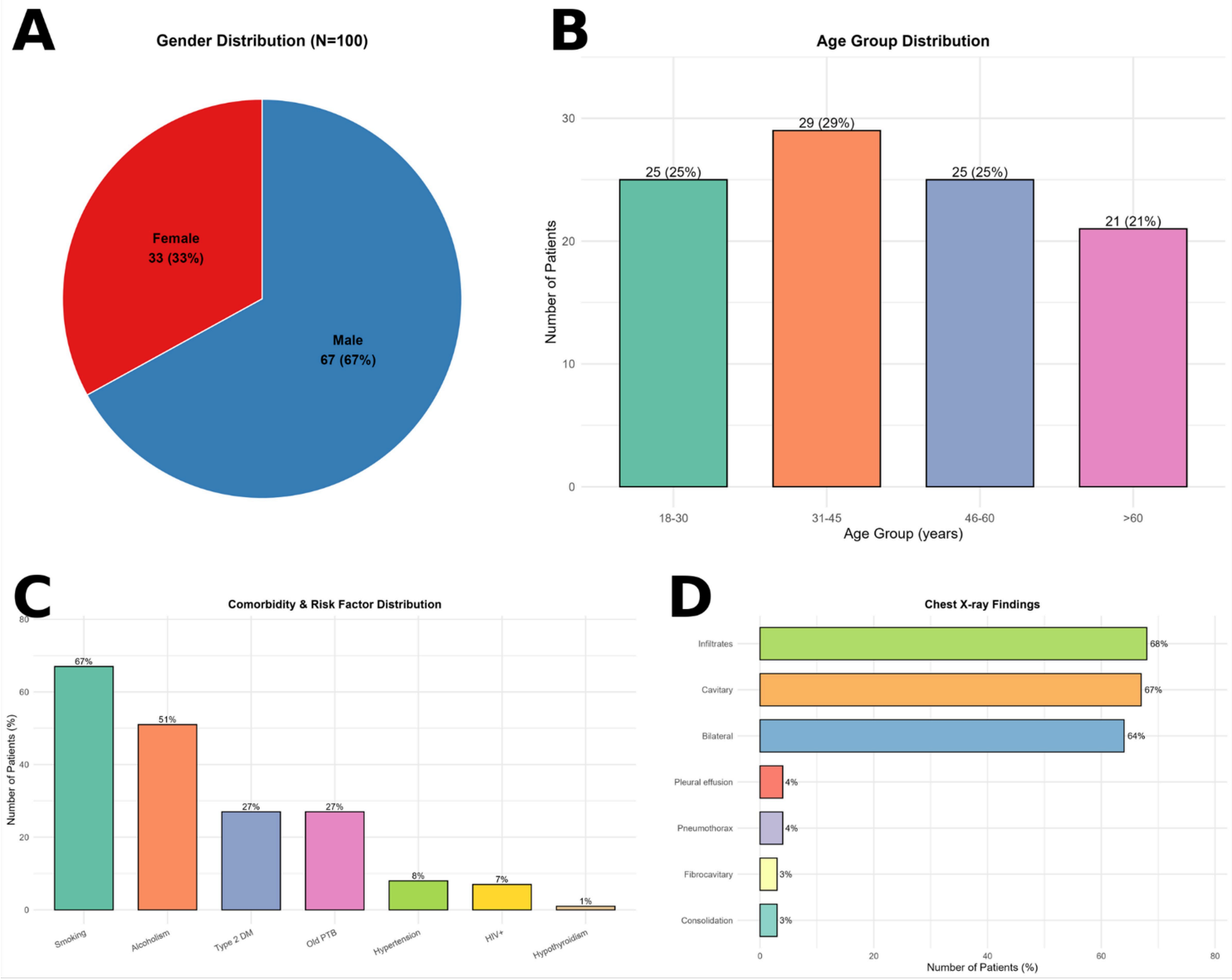


Figure 1

Baseline demographic, clinical and radiological profile of the study cohort (N=100). (A) Gender distribution; (B) Age group distribution; (C) Comorbidity and risk factor burden, demonstrating that smoking, alcoholism, type 2 diabetes mellitus and prior tuberculosis predominate in this cohort; (D) Chest X-ray findings showing high prevalence of infiltrates, cavitary lesions and bilateral involvement consistent with far-advanced disease.

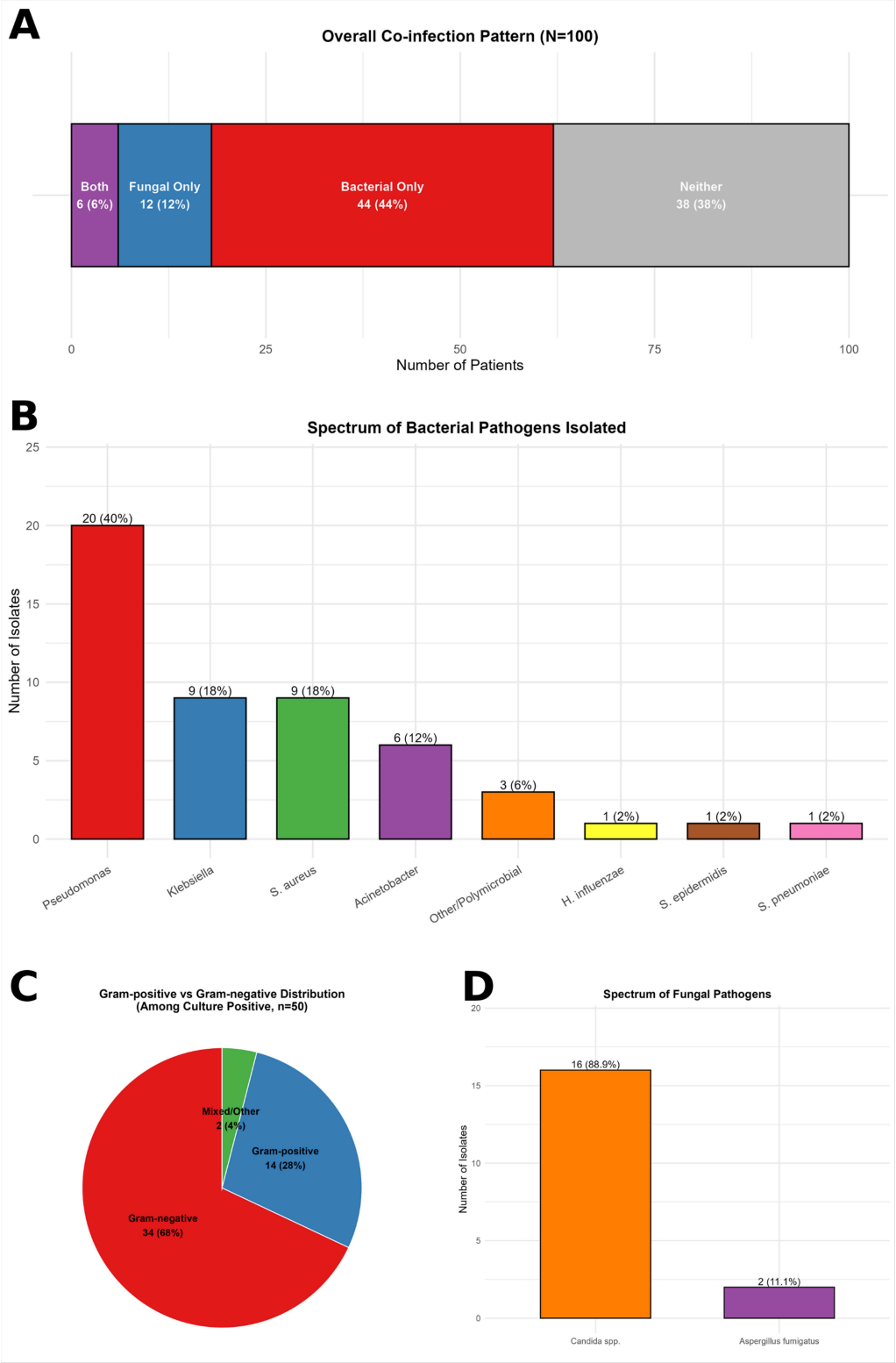


Figure 2

Prevalence and microbial spectrum of concurrent co-infections. (A) Overall co-infection pattern: 62 of 100 patients had at least one co-infection (bacterial only 44%, fungal only 12%, both 6%, neither 38%); (B) Bacterial isolate spectrum showing dominance of Pseudomonas aeruginosa (40%) followed by Klebsiella spp. and Staphylococcus aureus (18% each); (C) Gram stain distribution among culture-positive specimens (Gram-negative 68%, Gram-positive 28%, mixed 4%); (D) Fungal isolate spectrum:

Candida spp. accounted for 88.9% of fungal isolates with Aspergillus fumigatus comprising the remainder.

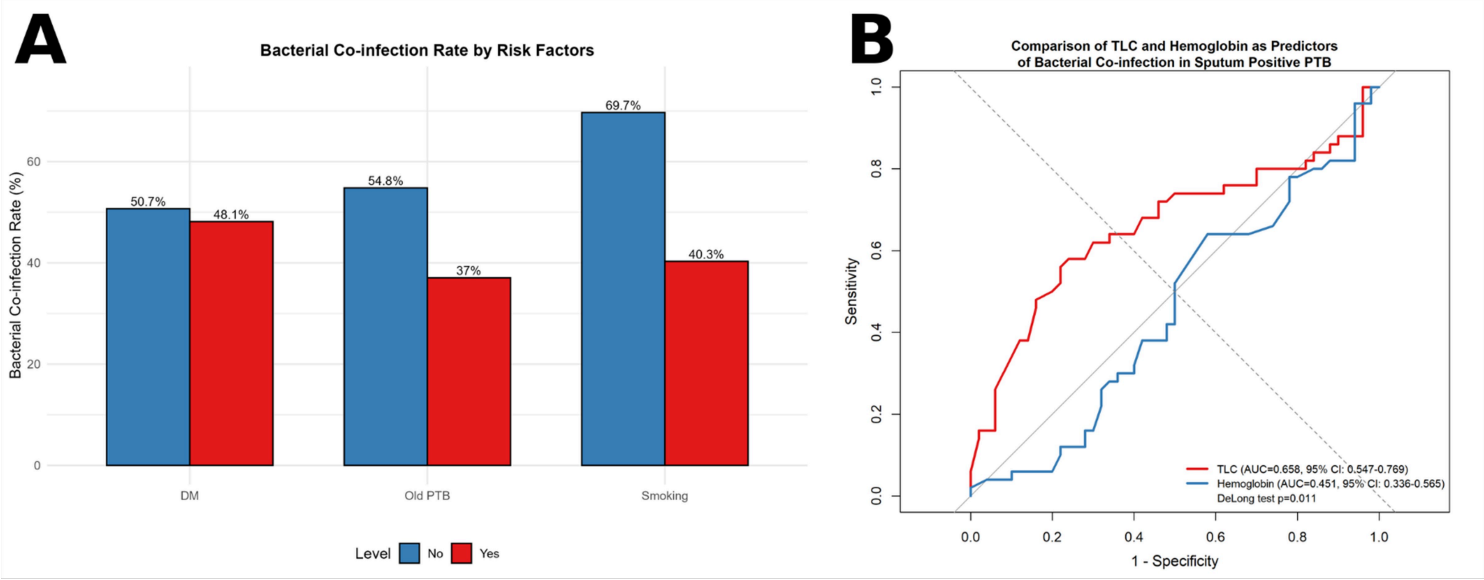


Figure 3

Predictors of bacterial co-infection. (A) Bivariate bacterial co-infection rates stratified by major risk factors (diabetes mellitus, prior pulmonary tuberculosis, smoking); (B) Receiver operating characteristic (ROC) curves comparing total leukocyte count (TLC) and haemoglobin as predictors of bacterial co-infection. TLC achieved a significantly higher discriminative performance (AUC=0.658, 95% CI 0.547-0.769) than haemoglobin (AUC=0.451, 95% CI 0.336-0.565); DeLong test p=0.011. TLC was the sole independent predictor on multivariate logistic regression (p=0.020).

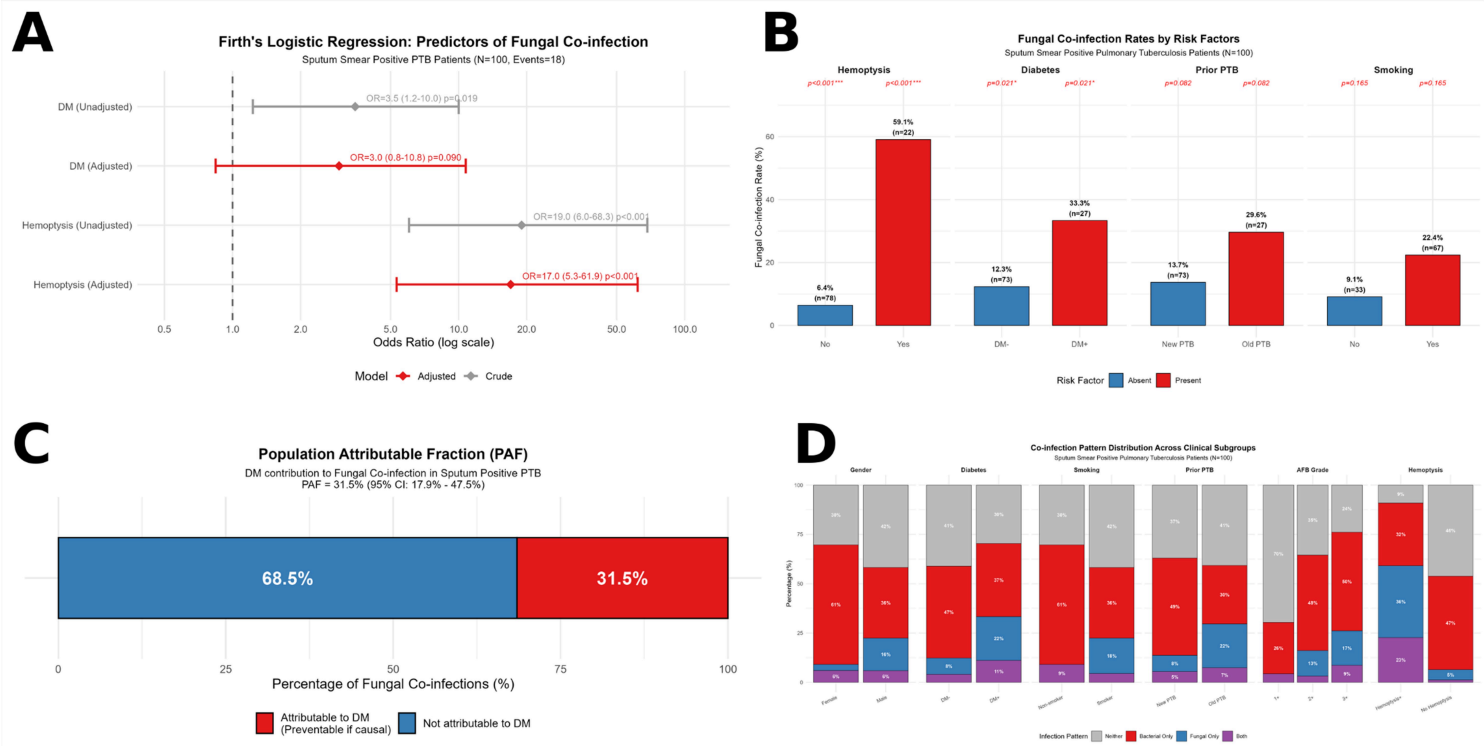


Figure 4

Predictors of fungal co-infection - hemoptysis as a novel risk factor. (A) Firth's penalized logistic regression forest plot: hemoptysis retained an overwhelming independent association (adjusted OR=17.0, 95% CI 5.3-61.9; p<0.001) while T2DM showed a non-significant trend (adjusted OR=3.0, 95% CI 0.8-10.8; p=0.090); (B) Fungal co-infection rates stratified by hemoptysis, T2DM, prior PTB and smoking; patients with hemoptysis had a fungal co-infection rate of 59.1% versus 6.4% in those without (p<0.001); (C) Population attributable fraction (PAF) for type 2 diabetes contribution to fungal co-infection (31.5%, 95% CI 17.9-47.5%); (D) Co-infection pattern across all six clinical subgroups (gender, diabetes, smoking, prior PTB, AFB grade and hemoptysis), illustrating disproportionately elevated fungal and combined burden in the hemoptysis subgroup.

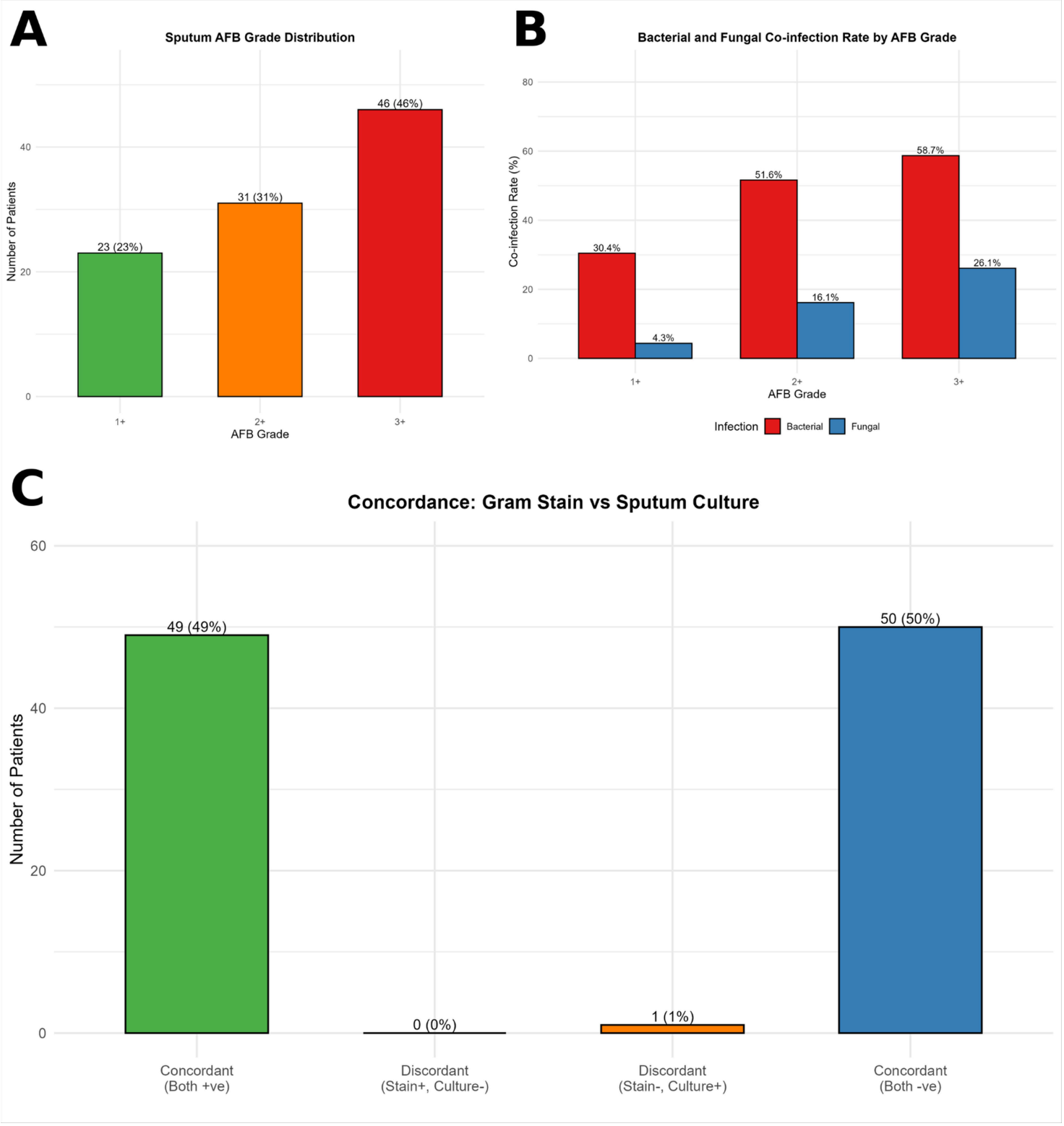


Figure 5

Bacillary burden dose-response relationships and Gram stain diagnostic validation. (A) Distribution of AFB smear grades (1+: 23%, 2+: 31%, 3+: 46%); (B) Bacterial and fungal co-infection rates rising progressively across AFB grades (bacterial 30.4%-->51.6%-->58.7%; fungal 4.3%-->16.1%-->26.1%; Spearman trends p=0.040 and p=0.027 respectively); (C) Concordance between Gram stain and sputum

culture: 49 (49%) Both positive, 50 (50%) Both negative, 1 (1%) discordant - corresponding to 98% sensitivity, 100% specificity and 99% overall concordance.

Supplementary Files

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- SUPPLEMENTARYTABLE.docx