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Antimicrobial Resistance Levels of Non-Tuberculous Bacteria Isolates from Sputum of TB Patients in Ghana

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Background: Patients with tuberculosis (TB) often harbor diverse bacteria in their sputum, including both commensal and opportunistic pathogens. This study aimed to characterize the sputum microbiota of TB patients before and after the intensive phase of anti-TB treatment and assess changes in bacterial diversity and antibiotic resistance profiles.

Methods: A total of 162 patients with TB (128 males, 34 females; age range 18–82 years) provided sputum samples at baseline, of which 72 provided follow-up sputum after two months of intensive phase treatment. Sputum samples were cultured on standard agar plates, and distinct colonies were identified by Gram staining and bio-typing using MALDI-TOF mass spectrometry. Antibiotic susceptibility testing of the identified Gram-positive and Gram-negative bacteria was performed using the Kirby–Bauer method according to the CLSI guidelines.

Results: At baseline, 209 bacterial isolates were recovered, dominated by Gram-positive bacteria (GPB), particularly *Streptococcus oralis* (19.6%) and *Staphylococcus aureus* (13.9%). After treatment, the isolation rate significantly decreased (from 129% to 95.8%; p = 0.000002), with a shift towards Gram-negative bacteria (GNB) dominated by *E. coli*. High rates of antibiotic resistance were observed for both the GNB and GPB, notably to ampicillin (86.7%), tetracycline (74%), amoxicillin (70.3%), and sulfamethoxazole (63%) for GNP, and PEN (76.9%) for the GPB. 53% of *S. aureus* isolates were phenotypic Methicillin-resistant *S. aureus* (MRSA) and 57.7% of suspected extended-spectrum *Beta-lactamase* (ESBL) producers were confirmed positive, predominantly carrying the *blaCTX-M-1* gene.

Conclusion: The observed antibiotic resistance among the identified isolates, including MRSA and ESBL, underscores the need for routine antibiotic susceptibility testing and judicious antibiotic use in Ghana. Further research is needed to explore the long-term consequences of these microbiome shifts on TB treatment outcomes and risk of secondary infections.

Plain Language Summary: Tuberculosis (TB) continues to be the leading cause of adult mortality owing to a single infectious disease. The interplay between TB, diabetes, and HIV has become prominent in current TB research. However, the influence of these interactions on lung microflora, other than TB bacilli, has not been extensively explored. Notwithstanding, there is a potential interaction between these non-tuberculous bacteria and TB bacilli, which may impact disease progression and treatment outcomes of TB disease, irrespective of co-infection with HIV or comorbid diabetes. We observed antibiotic resistance among identified non-tuberculous bacteria isolated from the lungs of TB patients in Ghana, irrespective of co-infection with HIV or comorbidity with diabetes, including resistance to the commonly used drugs for treating bacterial diseases. This underscores the need for routine antibiotic susceptibility testing and judicious antibiotic use in Ghana.

Keywords: tuberculosis, sputum microbiota, antibiotic resistance, microbiome, ESBL, MRSA

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Background

Tuberculosis (TB), caused by the *Mycobacterium tuberculosis* complex (MTBC), remains a global health problem with approximately 10 million infections in 2022. [1] In Ghana, TB is the fourth leading cause of death among other communicable and non-communicable diseases [2] Infection usually results in complex immune interactions aimed at clearing the bacteria[3] however, the chemical signals produced can weaken the host humoral and cellular immunity, making the individual prone to other infections and diseases.[4] For example, there is evidence of reduced bactericidal activity of the alveolar surface against other bacteria during active TB.[5] In cases of comorbidities with other immune-disrupting conditions such as diabetes or HIV infection, there is an increased alteration in commensal organisms in the lungs. This allows over-proliferation of bacteria, including pathogenic ones, which can cause complications and/or coinfections in TB patients, especially in regions with a high TB burden.[6–8] Furthermore, some co-infected bacteria may be resistant to antimicrobials, including regular TB drugs, which can lead to unforeseen complications.

Non-mycobacterial colonization of the lungs and gut usually includes both Gram-positive (GPB) and Gram-negative (GNB) bacteria.[9,10] Over time, some of these bacteria can become drug-resistant due to untargeted antibiotic exposure and/or misuse.[11] Also, during anti-TB therapy, antibiotics can further destabilize the host microbiome, resulting in the accumulation of resistance,[12] as well as killing susceptible bacteria, including those that are beneficial to gut health.[13]

Among various bacterial families, extended-spectrum *beta-lactamase* (ESBL) genes are acquired either by mutation or horizontal gene transfer of plasmids, leading to resistance to β-lactam antibiotics. bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, and $bla_{\text{CTX-M-9}}$ are common ESBL genes with increased specificity against ceftazidime, cefotaxime, and ceftriaxone substrates.[14,15]

This study aimed to determine the drug resistance pattern of indicator bacteria isolated from the sputum and stool of TB patients[16] as well as identify potential drug resistance-conferring genes. These indicator bacteria included GN *Enterobacteriaceae, Klebsiella sp., E. coli* and *Enterobacter sp*., as well as GP *Staphylococcus* and *Streptococcus species*.

Materials and Methods

Ethical Approval

Ethical approval for the study and its protocols after scientific and technical review was obtained from the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana (Federal wide assurance number: FWA00001824) and the Ethics Review Committee of the Korle-Bu Teaching Hospital. All study procedures were performed according to approved protocols in compliance with the declaration of Helsinki.

Study Area and Recruitment of Study Participants

The targets for this cross-sectional study were microbiologically confirmed TB cases who were 18 years old and above who were not on any immunosuppressive treatment. The purpose of the study was explained to the target population in a language understood by them after which consent was sought before recruitment. We excluded patients younger than 18 years of age and pregnant women.

The diagnosis of pulmonary TB was based on the positive Gene Xpert MTB-RIF (Cepheid, USA) and chest radiography. A structured questionnaire was used to record demographic and clinical data including age, sex, history of TB, TB bacterial load, alcohol and cigarette use, previous medications, and other underlying conditions confirmed TB participants were screened at baseline for diabetes (fasting blood sugar above 7 mmol/L and HbA1c confirmatory test above 6.4%), HIV (positive rapid First Response HIV1-2.0 card test, Premier Medical Corporation Limited, India), and positive confirmatory OraQuick rapid test (OraSure Technologies, USA)). Sputum samples were collected following standard procedures at baseline (before starting anti-TB therapy) and at 2 months follow-up (after the intensive phase of anti-TB therapy) using clean sterile plastic containers.

Isolation and Identification of Non-Mycobacteria from Sputum Samples

Sputum samples from recruited individuals were aseptically inoculated immediately in the field within 5–10 mins after sample collection on chocolate agar (CA), mannitol salt agar (MSA), gentamicin blood agar (GBA), and Potato Dextrose agar (PDA) to isolate fastidious organisms. Colonies distinct from all primary plates were purified, characterized, and identified by Gram staining and MALDI-TOF mass spectrometry (Bruker Daltonics, Germany).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method[17] (Figure S1) following the CLSI guidelines.[18] Quality control for antibiotics and other reagents was performed using the reference standard strains of *Staphylococcus aureus* ATCC 3556 and *Escherichia coli* ATCC 25922. For GNB, 30 µg of Meropenem (MEM), 30 µg of cefotaxime (CRX), 30 µg of cefuroxime, 30 µg of amikacin (AMK), 10 µg of gentamicin (GEN), 1.25/23.75 µg of trimethoprim/sulfamethoxazole (SXT), 10 µg of ampicillin (AMP), 30 µg of tetracycline (TET), 30 µg of chloramphenicol (CHL), 5 µg of ciprofloxacin (CIP), 30 µg of ceftazidime (CAZ), and 30 µg pf amoxicillin/clavulanic acid (AMC) all from Oxoid (Oxoid, USA) were used. Similarly, GPB were tested against 10 µg of GEN, 10 µg of TET, 30 µg of CHL, 1.25/23.75 µg of SXT, 5 µg of CIP, 30 µg of cefoxitin (FOX), 10 units of penicillin (PEN), 1 µg of oxacillin (OX), 15 µg of erythromycin (ERY), 30 µg of vancomycin (VA), 30 µg of azithromycin (AZM), 30 µg of chloramphenicol (CHL), 30 µg of linezolid (LZD), and 2 µg of clindamycin (DA) from Oxoid (Oxoid, USA). *Staphylococcus aureus* isolates with FOX zone sizes ≤21 mm (at 33–35 ◦C ambient air incubation for 16–18 h) were considered presumptive methicillin-resistant *Staphylococcus aureus* (MRSA)[19] and were phenotypically confirmed by Microscan MIC detection (Pos Breakpoint Combo Panel 28) (Beckman Coulter, USA). Multidrug resistance (MDR) was defined as the resistance to at least three groups of antibiotics: ESBL and MRSA.[20]

Phenotypic ESBL Detection and Molecular Confirmation

The simultaneous phenotypic resistance of GNB *Enterobacteriaceae* to *β*-lactam antibiotics, cefotaxime, and ceftazidime (Figure S1) was confirmed as ESBLs using PCR to detect the presence of six different ESBL-conferring genes in *Enterobacterales:* bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, and $bla_{\text{CTX-M-9}}$. A simple DNA extraction protocol as previously described[21] was used for crude DNA extraction from the presumptive isolates, followed by multiplex PCR for the six different ESBL genes.[22] Amplicons were visualized after resolution on 2% agarose gel. The expected band sizes for the various genes were bla_{TEM} (800 bp), bla_{SHV} (718 bp), $bla_{\text{OXA-48}}$ (564 bp), $bla_{\text{CTX-M-1}}$ (688 bp), $bla_{\text{CTX-M-2}}$ (404 bp), $bla_{\text{CTX-M-9}}$ (561 bp).

For quality control, each batch of the medium and reagent was subjected to sterility and performance testing. During the antibiotic susceptibility test, quality control was done using the control strains of *E. coli* ATCC 25922 and *S. aureus* 3556.

Data Analysis

Categorical data are presented as numbers and proportions and were compared using the chi-square (χ^2) test, Fisher's exact test, and two-sample proportion tests when applicable. The antibiogram of each isolate was interpreted according to CLSI guidelines and specifications as resistant, intermediate, or susceptible. The percentages of cases in each category were then computed and compared using graph pad Prism and R. Descriptive statistical analysis was conducted, and associations were determined at 95% confidence level.

Results

The study involved a total of 162 TB cases, comprising 128 (79.0%) males and 34 (21%) females. Sputum samples were obtained from all participants whose ages ranged–18–82 years (mean, 42 years; median, 44 years) (Table 1). Results from Gene Xpert-MTB showed 85 (52.1%) of the participants had high TB bacterial loads, whereas 30 (18.4%) and 38 (23.3%) had medium and low bacterial loads, respectively. Resistance to rifampicin was observed in two patients with low MTB and one with high MTB load.

Table 1 Biographical and Clinical Data of Participants

Parameter	Number of Patients	Male	Female
Mean Age		42.4	38.8
Diabetics	23	17 (74%)	6 (26%)
HIV positive	16	11 (68.7)	5 (31.3%)
Total Primary cases	162	128 (79%)	34 (21%)
TB-Only	123	101 (82%)	22 (18%)
TB-Diabetes	23	16 (70%)	7 (30%)
TB-HIV	16	11 (69%)	5 (31%)
Follow-up	72	56 (77.8%)	17 (22.2%)
TB-Only	57	45 (79%)	12 (21%)
TB-Diabetes	10	7 (70%)	3 (30%)
TB-HIV	5	3 (60%)	2 (40%)
Deceased	*7*	*4 (57%)*	*3 (43%)*
Bacterial load (GeneXpert)			
High	85	66 (77.6%)	19 (22.4%)
Medium	30	24 (80%)	6 (20%)
Low	38	31 (82%)	7 (18%)
Healthy controls	15	5 (33.3%)	10 (66.7%)

The study participants comprised 123 (75.4%) TB-only, 23 (14.1%) TB–diabetes, and 16 (9.8%) TB–HIV cohorts. At follow-up, 73 (44.8%) of the primary cases were re-recruited: 52 (80%) TB-Only, 8 (12%) TB-DM, and 5 (8%) TB-HIV. Eighty-two of the primary cases were lost to follow-up, whereas the deceased patients included 4 males and 3 females (Table 1).

We found 81% of participants had low BMI, 66.7% had chest pain, 65.4% had extreme cough, and 64.2% had night sweats during recruitment. Symptoms at follow-up were, however, reduced such as chest pain (25%), persistent extreme cough (6.9%) and night sweats (16.7%) (Table S1).

Identified Bacterial Species from Sputum Samples

At baseline, a total of 162 sputum samples were cultured for isolation of bacteria, with an average isolation frequency of 1.3 organisms per sample resulting in 209 organisms from sputum samples. The isolated bacteria were mostly GPB (137; 65.6%), with relatively high abundance of *Streptococcus oralis* (19.6%), *Staphylococcus aureus* (13.9%), *Staphylococcus epidermidis* (12%), and *Streptococcus mitis* (6.2%). After the 2 follow-up, 69 isolates were recovered from the 72 sputum samples collected from the study participants, dominated by 71.1% GNB (49/69), which were mostly *E. coli* (36; 53%). We observed from the pairwise comparison of sputum from the three TB cohorts before and after intensive phase anti-TB therapy that most of the organisms isolated from sputum samples 55.7% (54/97), irrespective of the TB-cohort, were not isolated after the intensive phase of treatment (Figure 1), and that there was a significant difference in isolation rate ($p = 0.000002$) between baseline (209/162) and follow-up samples (69/72) using two-sample proportion test as described.[23,24]

Antibiotic Susceptibility Profile of Indicator Organisms

A total of 279 bacterial isolates from both baseline and follow-up samples, comprising 157 GPB (137 baseline and 20 follow-up isolates) and 122 GNP (72 baseline and 49 follow-up isolates), were screened for antimicrobial susceptibility based on the Gram staining results (Figure 2).

Among the GNB, the species with the highest resistance was *E. coli* irrespective of whether they were isolated from baseline or follow-up samples, with TET, AMP, SXT, and AMC being the most affected drugs. *E. coli, Enterobacter* and *Citrobacter species* were highly resistant to all four drugs. The *K. pneumoniae* isolates were highly resistant to AMP,

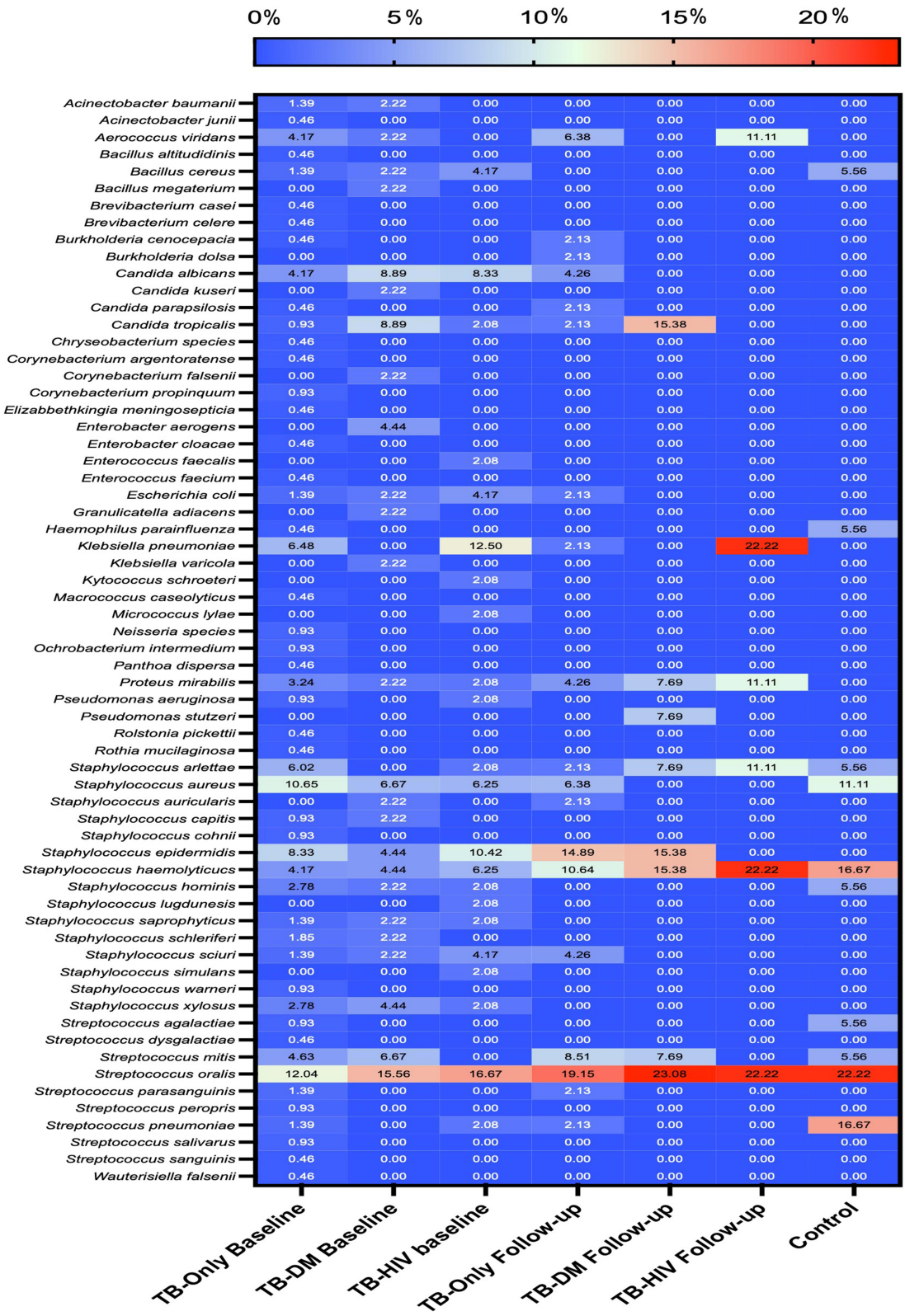

Figure 1 Abundance of microorganisms isolated from sputum samples at baseline and 2- months follow-up among the three TB cohorts (TB-only, TB-DM, TB-HIV) and healthy controls.

irrespective of the cohort. MEM followed by AMK, GEN and cefepime (bacterial). On the other hand, we observed on average the highest resistance against by GNB to AMP (86.7%), followed by TET (74%), AMC (70.3%), and SXT (63%) cohorts (Figure 2).

In the case of the two GPB species, *Staphylococcus sp* and *Streptococcus sp* (Figure 3), penicillin was the drug with least potency irrespective of the species and/or cohort, with at least 63% up to 89% (76.9% on average) resistance. Although all *Streptococcus sp* isolates were sensitive to CIP, AZM, GEN, OX, and FOX, irrespective of the sampling period, Staphylococcus isolates were mostly resistant to these drugs, with every follow-up isolate being resistant to CIP, AZM, GEN, and OX, and only FOX showed little activity against them (Figure 3). The most potent drug against GPB was chloramphenicol, followed by ceftriaxone (Figure 3).

Out of the total *S. aureus* isolates at both baseline and follow-up, 53% were phenotypic MRSA, using cefoxitin screening as a substitute marker for detection of MRSA when genotypic detection of *mecA* gene was not possible.[25] A total of 104 suspected ESBLs among the isolates (from cefoxitin screening) were tested for PCR detection of the common ESBL genes: bla_{TEM}, bla_{SHV}, bla_{OXA-48}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, and $bla_{CTX-M-9}$. Of these, 60 (57.7%) were confirmed as ESBL-positive, including 45 baseline isolates and 15 follow-up isolates. The confirmed ESBLs included *E. coli, K. pneumoniae, E. cloacae*, and *C. freundii*. As shown below (Figure 4), the 45 confirmed ESBLs at baseline

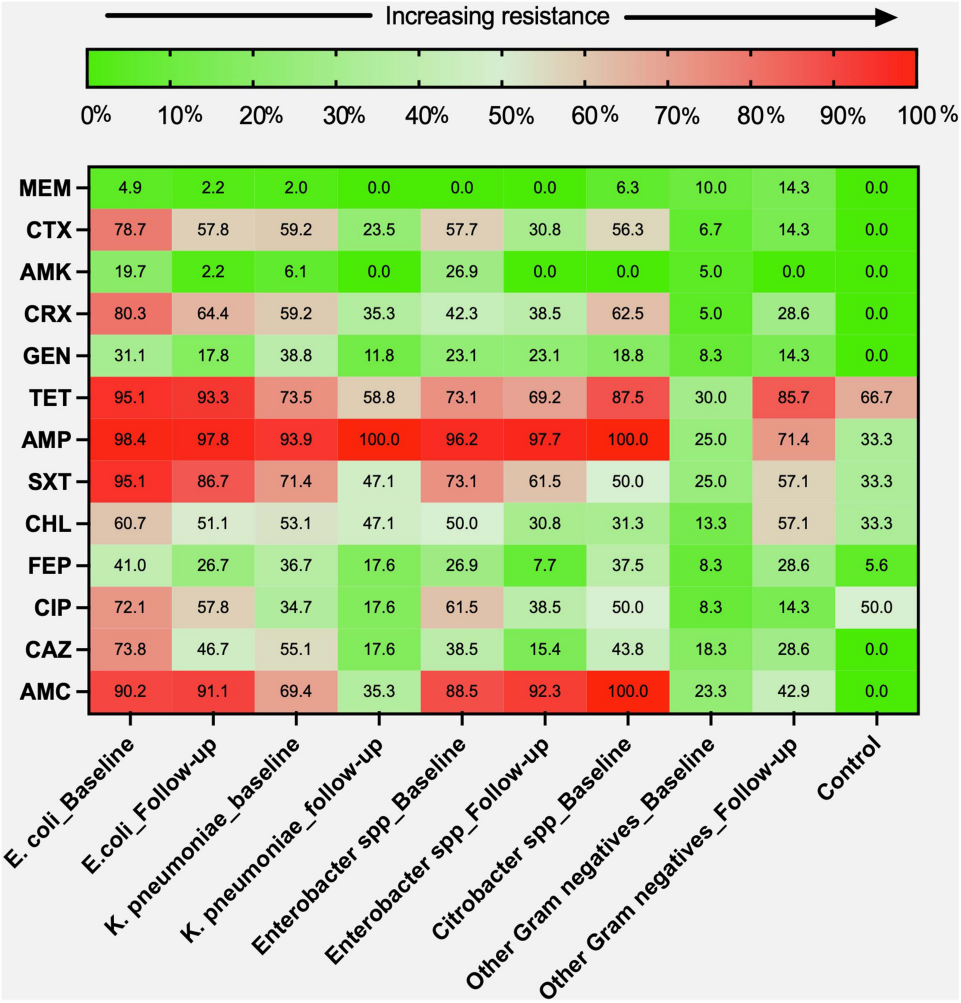

Figure 2 Antibiotic sensitivity of different gram-negative organisms at baseline and follow-up to selected antibiotics.
Abbreviations: MEM, Meropenem; CTX, Cefotaxime; AMK, Amikacin; CRX, Cefuroxime; GEN, Gentamicin; TET, Tetracycline; AMP, Ampicillin; SXT, Sulfamethoxazole-trimethoprim; CHL, Chloramphenicol; FEP, Cefepime; CIP, Ciprofloxacin; CAZ, Ceftazidime; AMC, Amoxicillin/clavulanic acid.

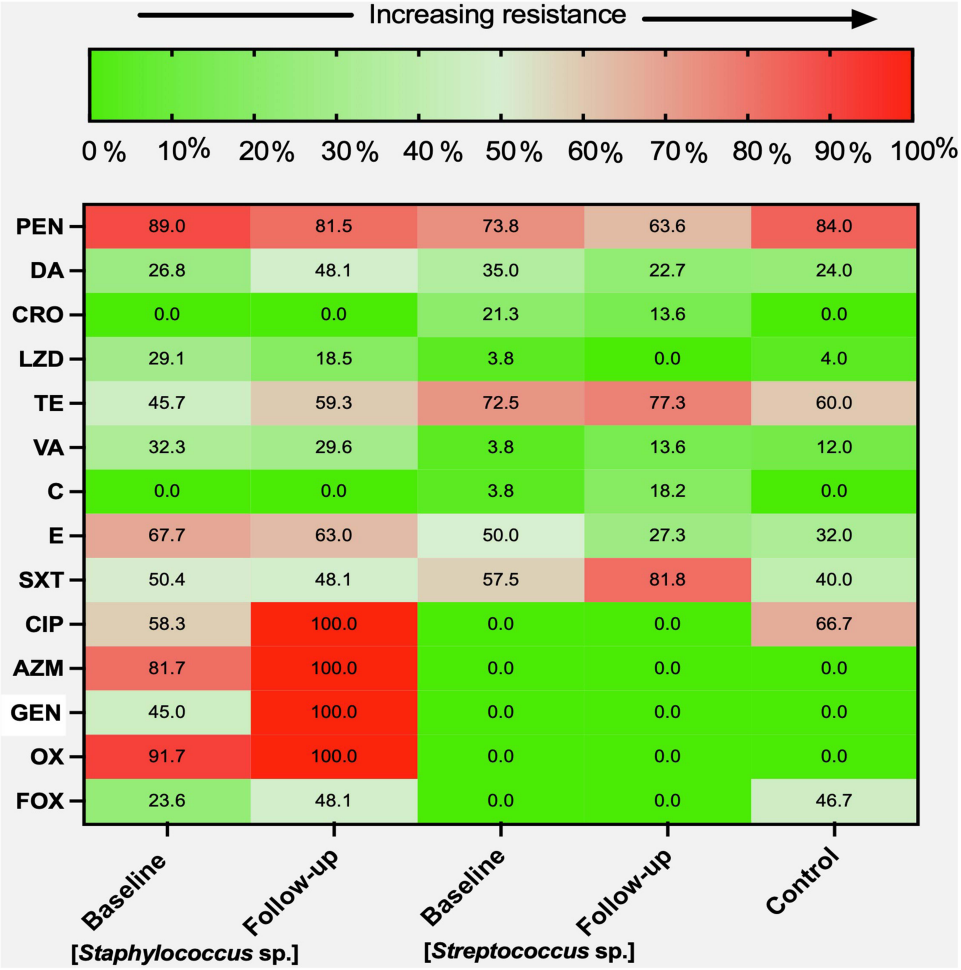

Figure 3 Antibiotic sensitivity of different gram-positive organisms at baseline and follow-up to selected antibiotics.
Abbreviations: PEN, Penicillin; DA, Clindamycin; CRO, Ceftriaxone; LZD, Linezolid; TE, Tetracycline; VA, Vancomycin; C, Chloramphenicol; ERY, Erythromycin; SXT, Sulfamethoxazole-trimethoprim; CIP, Ciprofloxacin; AZM, Azithromycin; GEN, Gentamicin; OX, Oxacillin; FOX, Cefoxitin.

were mostly *E. coli* (28; 62.2%), *K. pneumoniae* were (8; 17.8%), *E. cloacae* (8; 17.8%), and *C. freundii* (1; 2.2%). However, the follow-up isolates were mainly *E. coli* ESBLs making up 93.3% of the 15 ESBLs isolated.

The $bla_{\text{CTX-M-1}}$ was the most detected *β-lactamase* gene among 20 (28.75%) isolates followed by $bla_{\text{OXA-48}}$ (17.5%), $bla_{\text{CTX-M-9}}$ (16.25%), bla_{TEM} (15%) $bla_{\text{CTX-M-2}}$ (13.75%), and bla_{SHV} (8.75) (Figure 5).

Discussion

The presence of other bacterial infections in the lungs of pulmonary tuberculosis patients (PTB) is one of the most common complications of PTB.[26] This study examined the bacteriological aspects of sputum samples from TB patients with or without comorbidities of either diabetes or HIV before TB treatment (baseline) and after the intensive phase of TB treatment (follow-up). This included the isolation rates of bacteria other than members of the *Mycobacterium tuberculosis* complex, the composition and abundance of the isolated bacteria, and the antibiotic susceptibility profiles of the isolated bacteria.

Bacterial Identification and Isolation Rates

The predominance of *Streptococcus* and *Staphylococcus species* in the lungs of TB patients aligns with the common microbiota of the upper respiratory tract.[27] However, the significant reduction in isolation rates after the intensive phase of TB therapy could be due to the effect of the administered TB drugs (isoniazid, rifampicin, ethambutol, and

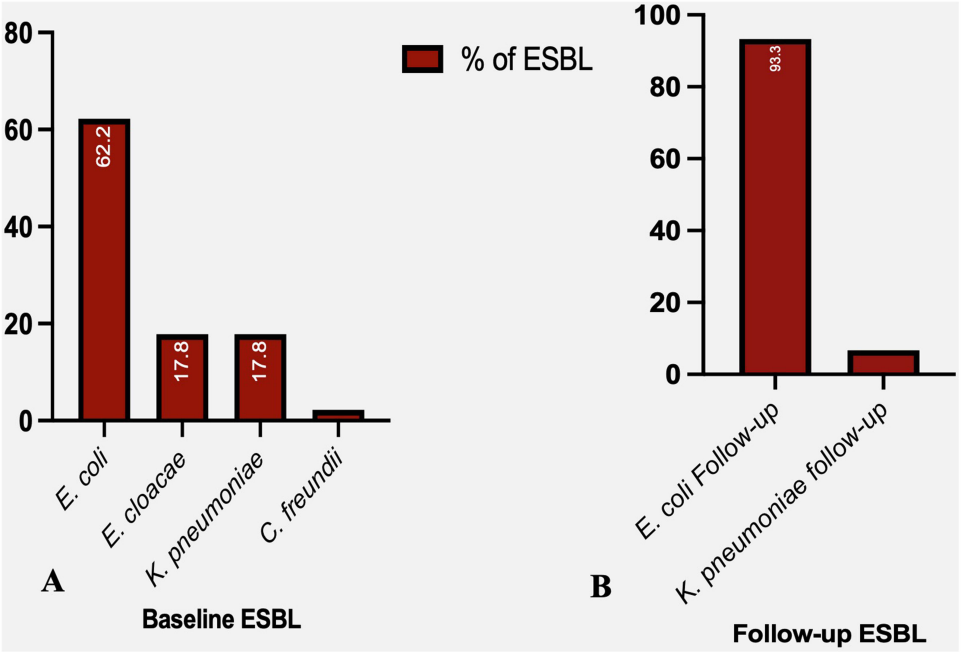

Figure 4 Percentage of ESBLs distribution amongst the different organisms. (**A**) Baseline; (**B**) Follow-up).

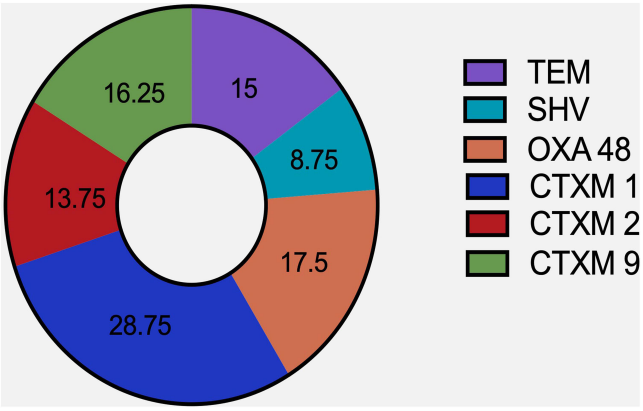

Figure 5 Distribution of ESBL genes detected from the total confirmed positive ESBLs.

pyrazinamide), which may have broad-spectrum collateral activity against non-tuberculous bacteria in the respiratory tract.[28] It also suggests that these bacteria are potential colonizers rather than playing a direct role in TB pathogenesis. Additionally, the improvement in immunity accompanying the clearance of TB bacteria after the intensive phase of treatment can also contribute to a reduction in the overall bacterial load and diversity of the non-tuberculous bacterial population.[29] Nevertheless, the increase in the proportion of GNB, predominantly *E. coli*, post-treatment is alarming.[30] This could be due to antibiotic-induced dysbiosis of the respiratory microbiome or development of secondary bacterial infections. However, further investigation is required to determine the clinical significance of this potential dysbiosis.

Antibiotic Susceptibility Trends

The widespread resistance of GNB and GPB to ampicillin, tetracycline, and sulfamethoxazole is alarming, highlighting a crucial public health concern.[31,32] This emphasizes the need for routine antibiotic susceptibility testing (AST) for bacteria-associated infections to guide treatment choices for better outcomes. Meropenem, amikacin, gentamicin, and cefepime were the most effective, especially against the isolated GNB. These might be considered as alternatives for

empirical therapy in settings with high resistance rates while awaiting AST results that may take between a couple of days to weeks, depending on the settings.[33,34] The observed resistance patterns for commonly used antibiotics in this study generally mirror the trends observed globally, especially in areas with high antibiotic use and potentially less regulated prescribing practices.[32,35] The research sheds valuable light on changes in the sputum microbiome after anti-TB treatment. While GNB dominance after tuberculosis treatment has been mentioned in some studies,[30] a more in-depth analysis of these shifts is needed, especially considering their potential impact on treatment response, TB recurrence, or development of non-tuberculous lung pathologies. The observed ESBL rate (57.7%) is comparable to the rates (41.5% to 57.8%) reported by earlier studies from different parts of Ghana[36–38] and remains higher than 36.1% in South Africa[39] and 38.5% Tunisia[40] while being lower than the 89% reported in Uganda.[41] The dominance of the $bla_{\text{CTX-M-1}}$ gene (28.78%), followed by $bla_{\text{OXA-48}}$ (17.5%), and $bla_{\text{CTX-M-9}}$ (16.25%) among the identified ESBLs agrees with earlier reports from other parts of Africa and Asia.[42,43] This finding is alarming because the $bla_{CTX\text{-}M}$ product can hydrolyze both third- and fourth-generation cephalosporins and is implicated in cefotaxime resistance.[44] The notable prevalence of MRSA and ESBL producers highlights the additional challenge of multidrug-resistant (MDR) organisms complicating TB management[45] which may require alternative antibiotics, such as vancomycin.[46] Aggressive infection control measures and tailored antibiotic regimens based on AST, including DNA-based diagnostics targeting specific AMR genes and novel rapid phenotypic assays, such as bioluminescence-based AST that can be performed in a matter of hours,[47,48] are crucial.

Limitations

Our findings may be limited by the sample size, which may limit their generalizability. Additionally, the absence of a healthy control group makes it difficult to definitively distinguish between normal flora and potential secondary pathogens in post-treatment stages.

Conclusion

This study underscores the value of antibiotic susceptibility testing throughout TB treatment, for both initial antibiotic selection and ongoing therapeutic decision-making. We tested a substantial panel of antibiotics to provide a broad overview of bacterial resistance patterns, which is valuable in determining treatment options. We highlight drugs with the highest and lowest potential for the treatment of upper respiratory bacterial colonization and/or infections in Ghana. The observed ESBL rate (57.7%) is alarming, as ESBL bacteria can be resistant to multiple classes of antibiotics. Further studies are warranted to elucidate the long-term consequences of anti-TB treatment on the respiratory microbiome and its association with clinical outcomes. This could potentially inform strategies to mitigate dysbiosis-related complications. The high proportion of MDR non-tuberculous bacteria underscores the need for improved detection and surveillance as well as antibiotic stewardship in Ghana.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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