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Molecular Detection and Antibiotic Susceptibility of Respiratory Pathogens in Patients at Abubakar Tafawa Balewa University Teaching Hospital Bauchi, Bauchi State, Nigeria

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Abstract

Microbial coinfections are a major health concern around the world. With the increasing incidence of tuberculosis, diagnosing and treating the disease has become more difficult and time-consuming. This study was conducted in Bauchi to test individuals with coinfections and detect respiratory tract fungal and bacterial pathogens. Sputum samples, totaling 253 that were positive or negative for tuberculosis using the GeneXpert were used. The sputum samples were further analyzed using microbiological standard protocols, including culture, Gram-staining, and biochemical tests. The isolated pathogens were subjected to genomic DNA extraction using Zymo research quick-DNA fungal/bacterial miniprep kit. PCR amplification was carried out using universal primers that targeted 16S rRNA. The PCR products were purified and sent for sequencing, and the sequence results were checked on BLAST at NCBI. Phylogenetic trees were constructed using the Maximum Likelihood method in the MEGA 11 tool. All bacterial isolates were tested against antibacterial and antifungal agents to determine their resistance profiles. The study showed that the overall prevalence of tuberculosis was 6%, with the highest rate being observed in females (3.8%) and the lowest in males (2.8%). Among the isolated bacteria, *K. pneumoniae* was the most prevalent (35%) while *Pseudomonas aeroginosa* was the least prevalent (2%). *Candida albicans* was the most isolated fungus (31%) while Cryptococcus spp was the least commonly isolated fungus (4%). Ceftazidime and Meropenem were the most effective antibiotics for bacterial isolates (100% sensitivity), followed by Azithromycin (70%). However, Ciprofloxacin and Azitreonam (80% each) were highly resisted, followed by Gentamicin and Ceftriaxone (50%) and Ofloxacin (30%). The study estimated that 42.6% of male patients and 57.4% of female patients had bacterial co-infections with TB. Logistic regression analysis performed during the study revealed a significant association ($\chi 2$= 5.40, P=0.0455) between the prevalence of the pulmonary bacterial isolation rate and the gender of the patients. It would be helpful to investigate the occurrence of suspected co-infections alongside tuberculosis and analyze the molecular traits of the pathogens involved. Such research could aid in the use of molecular detection to diagnose these microbial infections in individuals. By determining the antimicrobial profile, physicians will be able to diagnose and treat these infections more accurately.

Keywords: *Bacterial infection, molecular detection, antimicrobial, resistance*

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Introduction

Coinfection is defined as the simultaneous presence of two or more infections, which may increase the severity and duration of one or both Progress of the diseases and prolonged antibiotic treatment (Ejo et al., 2021). Immunocompromised patients are susceptible

to many opportunistic infections (Hosseini et al., 2020). When the host defense is decreased, fatal acquired immune deficiency syndrome (AIDS) and the patients develop opportunistic infections (Please et al., 2021). Since opportunistic mycoses are a serious threat to such patients, it is anticipated that these infections may break out in epidemic proportions under suitable circumstances. In comparison to HIV non-infected individuals, the human immunodeficiency virus (HIV) infected individuals are more than 10 times more susceptible to developing tuberculosis (TB) (Pasipanodya et al., 2015)

Nigeria is ranked among the 10 countries that accounted for 77% of the global gap in TB case detection and notification in 2016 (Madikizela et al., 2014). It is reported that Nigeria contributes about 8% of the 4.3 million TB cases missed globally. Consequently, in 2019, WHO initiated 'community-informant' methods to find missing TB cases in Nigerian communities (WHO, 2019) (Ugwu et al., 2021). The recent development of culture-independent methods of microbial identification has enabled the study of microbial communities on mucosal surfaces of the human body, referred to as "microbiota." The Nigerian National Tuberculosis Control Program is based on the internationally recommended WHO Stop TB strategy and provides for free investigations for diagnosis as well as free quality drugs for treatment. Patients suspected of having TB are usually asked to submit three early morning sputum for PCRs. Diagnosis is either based on sputum positive Gene-xpert or clinical and radiological judgment when the sputum result is negative.

The Human Microbiome Project (HMP) and other similar large-scale sequencing projects worldwide have characterized the distinct microbial communities that have adapted to the unique environmental niches within the human, such as the gut, skin, airways, genitourinary tract, and oral cavity (Knight et al., 2017).

The gut microbiome has been shown to play an integral role in shaping the immune system starting early in life, with continued influence on priming the nature and robustness of immune responses throughout one's lifetime. The respiratory tract also harbors distinct communities of microbes, with multiple discrete ecological niches (e.g., nasal cavity, oropharynx, upper airways) that vary in terms of temperature, pH, oxygen tension, mucus production, and other factors (Hanada et al., 2018).

Materials and Methods

Ethical Consideration and Consent Participation

Ethical approval was sought and obtained from the review boards of Bauchi State University, Gadau, Bauchi State Ministry of Health and the Abubakar Tafawa Balewa University Teaching Hospital Bauchi, Nigeria. Prior to sample collection, participants were thoroughly informed about the study's objectives and procedures. We obtained written informed consent and assent from all participants and their caregivers, and participation was entirely voluntary. All information collected for this study was treated as confidential and used solely for research purposes.

Study Site

The research was carried out at the TB, Microbiology and Molecular genetics and infectious diseases research laboratories of the Abubakar Tafawa Balewa University Teaching Hospital Bauchi, Bauchi state, which is one of the standard tertiary medical institutions owned by the Federal Government of Nigeria, providing all kinds of medical services. It is located at Hospital Road, Bauchi, Bauchi state Nigeria. Bauchi (earlier Yakoba) is a city in the northeast Nigeria, the administrative center of Bauchi State, in the Bauchi Local Government Area within the state. It is located on the northern edge of Jos, Plateau state, at an elevation of 616m. The Local Government Area covers an area of 3,687km^2 and had the population of 493,810 in 2006.

Study Design

This research was cross-sectional with descriptive approach. Information about the study participant's status concerning the presence or absence of *tuberculosis* and probable microbial coinfections. Then the participants were categorized into groups to be able to compute chi-square and unadjusted as well as adjusted odds ratios. The study's main outcome (TB status) and the suspected microbial coinfections are not rare. MDR Tuberculosis are also a chronic health condition. The design has also been found to be ideal as a measure of prevalence. The time frame of the study is medium.

Study Population

The target population of this research consisted of clients with or without TB testing for TB at the Abubakar Tafawa Balewa University Teaching Hospital, Bauchi. The **Inclusion Criteria**

Include,
GeneXpert confirmed TB patient on drugs and willingness to participate, whereas the **Exclusion Criteria**
include unwilling to participate. Data about the study variables was collected from the study population and analyzed.

Sample Size Determination

Using SCHWARTZ formula for sample size, For this study.
Where, n = Sample size
t = Value based on 95% confidence level (1.96)
p = Sample proportion (from a similar prevalence study; 66.7%, (Zhao et al., 2021)
i = Margin of error (8%; 0.08).

- Therefore, Sample size was calculated as;

$n= t^2 (p (p-1)/i^2$
$n= 1.96^2 (0.667 (1-0.667)/0.08^2$
n = 3.84 (0.667(-0.657)/0.0064
n =3.84(0.429)/0.0064
n=1.647/0.0064= 257.4 (adjusted to 253).

Samples/Data Collection

An early morning expectorated sputum samples were collected in sterile containers from all participating patients included in the study. Any sample that was thin, watery and with no purulent matter was considered unsuitable for further processing. All unsuitable specimens were not included. Samples were divided into two portions: one for the geneXpert and the other for the microbiological analysis (V Ramana et al., 2013).

Detection of TB via GeneXpert
The automated computer-based molecular Gene Expert machine (CBNAAT, Cepheid, USA) with cartridges was used for confirmation of TB tests as well as molecular characterization of *Mycobacterium tuberculosis* (Maka et al., 2020).

Bacterial isolation
The sputum specimens were inoculated onto Chocolate agar with 10% sheep blood and MacConkey agar plates. After overnight growth of 37 ℃ for 24 h in microaerophilic condition (candle jar), bacterial colonies with distinct morphology were picked from the plate and subsequently cultured as single isolate on muller Hilton/nutrient agar (Asare et al., 2022).

Bacterial identification
Any significant bacterial growth was further identified according to standard procedure (Shailaja et al., 2004). Baseline Gram stain was carried out on all isolates first before biochemical tests. Isolated bacterial strains were subjected to various biochemical tests specific for these isolated bacteria. These tests facilitated the identification and characterization of the isolated strains. Biochemical tests used in this study were: catalase test, coagulase test, TSI test, hemolysis in blood/chocolate agar plate (BAP), hydrolysis (He et al., 2017).

Manual Extraction of Genomic DNA (Spin column protocol)
A 100g (wet weight) of bacterial growth colonies that have been resuspended in 200 µl of nuclease-free water were added to a ZR Bashing Bead TM Lysis Tube (0.5 mm) followed by the addition of 750µl Bashing Bead TM Buffer to the tube. This was vortexed for 5minutes and then centrifuged in a microcentrifuge (Centrifuge 5427 R, Eppendorf, North America) at 10,000 x *g* for 1 minute. Genomic Lysis Buffer and DNA Pre-Wash Buffer were used to remove inpurities. Then, g-DNA Wash Buffer was added and centrifuge at 10,000 x *g* for 1 minute. Zymo-Spin TM IICR Column was transferred to a clean 1.5 ml microcentrifuge tube and 35 µl of DNA Elution Buffer was added directly to the column matrix. It was then incubated at 50^0C (HB120-S, China) for 1min and centrifuged at 10,000 x *g* for 30 seconds to elute the DNA. Ultra-pure DNA was then ready for use. The extracted DNA was quantified using NanoDrop One Microvolume UV-Vis's spectrophotometer (Thermo Fisher scientific company, USA).

Conventional PCR Amplification
The universal primers designed from a Uncultured bacterium clone KSR-CFL19 16S ribosomal RNA gene (Accession number; KR612067.1) were used to amplify the 16S rRNA gene. The sequence of the primer: F (5′-AGAGTTTGATCCTGGCTCAG -3′) and R (5′-ACGGCTACCTTGTTACGACTT -3′). PCR amplifications of 16S rDNA genes were carried out in 50 µL of total reaction volumes containing of 25 µL of OneTaq QuicK-Load 2x Master Mix with standard buffer, (SM0486S New England Biolabs Inc, England), 1 µL of DNA template (100-200 ng), 1 µL of 10 µM each primer (forward and reverse) and nuclease free water to make up the volume. Negative template controls (NTC) were included in the 16S rRNA PCR reactions containing all mastermix reaction components except template DNA for PCR efficiency and contamination detection. The 16S rRNA genes were amplified on an Alpha Thermal cycler (Cole-Parmer Ltd, product code AC196, UK) using an initial polymerase activation step at 94℃ for 30 sec, followed by initial denaturation at 94℃ for 15 sec, annealing at 56℃ for 30 sec, and extension at 68℃ for 30 sec of 35 cycles, ending with a final extension at 68℃ for 3 min for the 16S rRNA gene (Tilahun et al., 2024).

Agarose Gel Electrophoresis (AGE) and visualization
Preparation of Genomic DNA and PCR products' separations using AGE and TAE buffer was used during the electrophoresis. 0.8% agarose gel was used for PCR product by adding 0.16g of agarose powder to 20mls of 1X TAE respectively. The agarose-TAE solution was heated to dissolve the agarose powder, was then cooled, and poured into a casting tray after addition of 0.8µL amount of diluted ethidium bromide (Glentham LIFE SCIENCES, GT9211) (10mg/ml) with the specific comb edge already in place that, once the gel solution has cooled down and solidified at room temperature and in a dark system, it created a gel slab with a row of wells at the top. For visualization, one microliter (1µL) of Loading dye (New England Biolabs Inc, England) was added to 1µL microliter or 200ng of the extracted genomic DNA samples and 4 µL of PCR products were used and with nuclease-free water to top to 10 µL per gel well. The DNA samples were carefully loaded into the gel wells accordingly. An appropriate DNA size markers (Quick-Load Purple 1 kb DNA ladder, SN0552S New England Biolabs Inc, England) were loaded along with experimental samples. The gel was run until the dye has migrated to an appropriate distance. The gel electrophoresis was set for forty (40) minutes. Afterwards, the gel was visualized under UV light through gel documentation of Invitrogen iBright CL750 imaging system (Thermo Fisher Scientific, Singapore). The genomic DNA was checked using DNA marker of up to 40ng/10.0kb. And gel images were captured and recorded simultaneously (Abdullah et al., 2023).

Sequencing
The amplified 16S sequenced data were theoretically confirmed through homology search using BLAST (Basic Local Alignment Search Tool) software analysis in NCBI (National Center for Biotechnology Information) Genbank data base available at http://www.ncbi.nlm.nih.gov/ (Assefa et al., 2022).

Antibiotic Sensitivity Testing
Each of the isolated bacterial and fungal strains was tested against various antimicrobial agents using agar disc diffusion method. Briefly, for bacteria, an overnight grown bacteria were first adjusted to 0.5 McFarland turbidity standards (~1.0×10 colony forming units or cfu ml^{-1}). The culture (100 µl) was spread over Muller Hilton agar plate. The plates were left at room temperature for 15 min. The pre-determined antibiotic disks were dispensed onto the seeded plates by a sterile pair of forceps and gently pressed to ensure complete contact with the agar at 15 mm away from the edge of the plate and 25 mm away from each other. The plates incubated at 37℃ for 18 to 24 hours. The diameters of the zones of inhibition were measured and interpreted in accordance with

standards approved by the Clinical and Laboratory Standard Institute (Richter et al., n.d.). The bacterial antibiotic discs used include Amoxacillin (25ug), Azitreonam (10ug), Gentamicin (30ug), Trimethoprim sulphamethoxazole (30ug), ceftriaxone (30ug), ceftazidime (30ug), cefotaxime (5ug), Meropenem (30ug), Azithromycin (30ug), Ciprofloxacin (10ug), Levofloxacin (10ug), Ofloxacin (30ug) (Ekuma et al., 2023).

The percentage of isolates susceptible to each antibiotic was also calculated using the Clinical and Laboratory Standards Institute breakpoints for antimicrobial susceptibility of the isolates (Tunney et al., 2008).

Statistical Analysis

The data analysis was conducted using the SPSS version 27.0 software package. The presence and strength of the association between male and female and the outcome variable were assessed using crude odds ratios with 95% confidence intervals (Beceiro et al., 2013).

Results

The overall prevalence of tuberculosis in this study was 6%. The highest prevalence was observed in females (3.8%), while the lowest prevalence was recorded in males (2.8%). Upon statistical analysis, a significant difference ($\chi 2$=0.47, P=0.4953) was found between males and females as shown in Table **1**.

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Table 1: Prevalence of Tuberculosis according to gender

Factors		TB Status				
	Level	Positive	Negative	Total	c^2	P-value
Gender	Male	7 (2.8%) 46.7	82 (32.4%)	89	0.47	0.4953
	Female	8 (3.2%) 53.3	156 (61.6)	164		
	Total	15 (6%) 100	238 (94%)	253		

Bacteriological profiles of culture-positive patients.

The bacterial pathogens were identified based on colony appearance (gram reaction, and biochemical tests, some of the biochemical tests used to identify bacterial pathogens included catalase, coagulase, indole, citrate utilization, urease, oxidase, Triple Sugar Iron (TSI), Mannitol Fermentation, and production of Hydrogen sulphide.

The prevalence of the bacterial pathogens is shown in **Table 2**.

Table 2: Frequency of Occurrence of Bacterial Pathogens

Bacterial Isolates	**Frequency**	**Prevalence (%)**
K. Pneumoniae	38	35
K.oxytoca	27	25
Enterobacter spp	15	13.90
Staphylococcus aureus	16	14.80
Streptococcus spp	8	7.40
Pseudomonas aeroginosa	2	1.90
Micrococcus spp	2	1.90
No bacterial growth	145	---
Total	**108**	**100**

Most of the bacterial isolates in this study were Gram-negative bacteria, predominantly from the bacterial family Enterobacteriaceae. *Klebsiella pneumoniae* was the most frequently occurring species, accounting for 35% of the isolates, followed by *Klebsiella oxytoca* (25%) and Enterobacter spp (13.90%) with a total of fifteen (15) species isolated. *Pseudomonas aeruginosa* accounted for only 1.9% of the total Gram-negative bacteria, with just 2 isolates. Gram-positive organisms isolated included *Staphylococcus aureus* (14%) and *Streptococcus* spp (7%).

Prevalence of bacterial pathogens according to gender

To determine the prevalence of bacterial co-infections among TB patients, this study was conducted. The results showed that the study participants had bacterial co-infections, with a male-to-female ratio of 42.6% and 57.4% respectively. The statistical analysis also indicated a significant association between the prevalence of pulmonary bacterial isolation rate and the gender of patients, as shown in **Table 3**.

Table 3: Occurrence of Bacterial Pathogens according to Gender

		Prevalence of Bacterial Pathogens				
	Level	Positive	Negative	Total	c^2	P-value
Gender	Male	46(42.6%)	43 (29.7%)	89	4.00	0.0455
	Female	62 (57.4%)	102 (61.4%)	164		
	Total	108 (100%)	145 (70.3%)	253		

It is common for individuals to have both tuberculosis (TB) and bacterial infections. However, there are instances when TB patients develop a secondary bacterial infection which can delay or unspecify diagnosis and lead to inadequate treatment. Tubercular-bacterial coinfection should be considered, especially if TB occurs in unusual pulmonary or extrapulmonary locations (Arora et al., 2015).

Molecular Detection

For the molecular identification of some pathogens, this study selected the most prevalent pathogenic bacteria confirmed via biochemical analysis and proceeded to molecular confirmation. Therefore, the 16S rRNA gene was used to identify bacterial pathogens resulting in a single band. The PCR products of the 16S rRNA gene were electrophorised resulting to a specific band size across (1600bp) as shown on figure. The 16S rRNA gene sequence of quality conrol checked/passed bacterium was compared to reference sequences in NCBI using BLAST to determine the specific bacterial species.

Antibiotic Susceptibility Pattern of Pathogenic Bacteria Isolated from the sputum samples

This study focused on bacterial pathogens isolated from sputum samples of patients attending Abubakar Tafawa Balewa University Teaching Hospital. The antibiotic sensitivity profile of these pathogens was also evaluated using the disc diffusion method against 12 antibiotic agents. The interpretive profile criteria for each bacterial isolate; selected ten (10) of the most prevalent from both the gram positive and negative group (*K. Pneumoniae (3), K.oxytoca (2), Staphylococcus aureus (2), Enterobacter spp(1), Streptococcus spp (1), and Pseudomonas aeroginosa (1)*) to the respective commercial antibiotic concentration were recorded as susceptible (S) with zone of inhibition diameter of > 18-24mm, intermediate (I) with zone of inhibition diameter of >15-20mm, and resistant (R) with zone of inhibition diameter of <14-17mm, the diameter of the isolate's inhibited growth was also measured to the nearest whole millimeter (mm) as described in **Table 4**.

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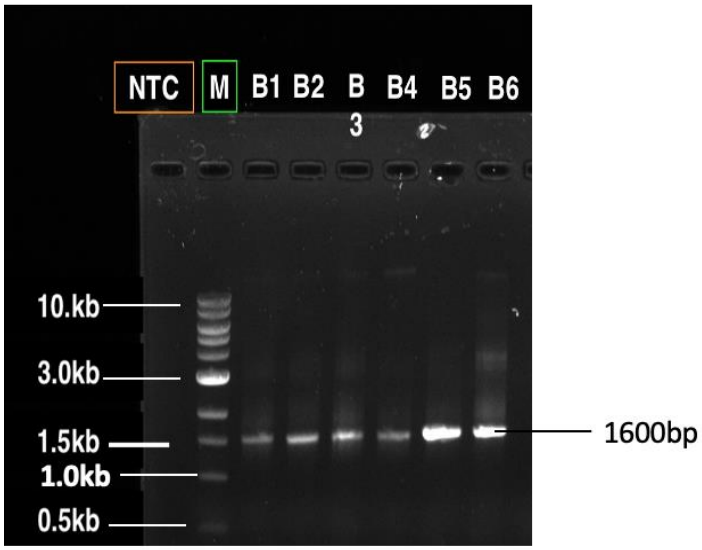

Figure 1: Agarose gel electrophoresis of amplified PCR products of 16S rRNA gene using universal primer. M-represents marker, NTC-represents negative control, lanes 3-8 represent bacterial isolates.

Table 4: Antibiotic susceptibility profile of bacterial isolates from sputum samples

	Antibiotics (Gram Positive & Negative)	Code	Conc.	Diameter of zone of inhibition in mm (as per the manufacturer guidelines)		
				≥ **Sensitive%**	**Intermediate%**	≤ **Resistant%**
1	Amoxicillin	AM	25µg	5(50%)	3(30%)	2(20%)
2	Aztreonam	ATM	10 µg	0(0%)	2(20%)	8 (80%)
3	Gentamicin	CN	30 µg	4(40%)	1(10%)	5(50%)
4	Trimethoprim sulphamethoxazole	CXM	30 µg	0 (0%)	2(20%)	8(80%)
5	Ceftriaxone	CRO	30 µg	5(50%)	0(0%)	5(50%)
6	Ceftazidime	CAZ	30 µg	10(100%)	0(0%)	0(0%)
7	Cefotaxime	CTM	5 µg	8(80%)	2(20%)	0(0%)
8	Meropenem	MEM	30 µg	10(100%)	0(0%)	0(0%)
9	Azithromycin	AZ	30 µg	7(70%)	1(10%)	2(20%)
10	Ciprofloxacin	CIP	10 µg	0 (0%)	2(20%)	8 (80%)
11	Levofloxacin	LEV	10 µg	8 (80%)	0(0%)	2(20%)
12	Ofloxacin	OFL	30 µg	3(30%)	4(40%)	3(30%)

Discussion

The overall prevalence of TB in the study population was 6%, with females (3.8%) showing a higher prevalence compared to males (2.8%). Additionally, females accounted for 81.4% of cases co-infected with TB and HIV, a finding that was statistically significant (χ^2=5.40, P=0.020).

These findings align with earlier studies that have often reported a higher burden of TB and TB/HIV co-infections among females in certain settings (Ekuma et al., 2023). Previous research indicates that socio-cultural and economic factors, such as limited access to healthcare, increased exposure due to caregiving roles, and stigma, may contribute to this disparity in low- and middle-income countries (LMICs). Furthermore, studies have highlighted that women living with HIV are more susceptible to TB due to immunosuppression caused by the virus (Sherchan & Humagain, 2020).

While earlier studies have documented the gender-based differences in TB/HIV prevalence, the statistical validation in this study strengthens the evidence. However, it also highlights the need to address gender-specific barriers in TB and HIV care in regions like Bauchi State.

The study reported the prevalence of bacterial isolates in TB-suspected patients' sputum samples, with *Klebsiella pneumoniae* (35%) and *Klebsiella oxytoca* (25%) being the most common pathogens. Other bacteria like *Staphylococcus aureus* (14%), Enterobacter spp. (13%), and *Pseudomonas aeruginosa* (2%) were also detected.

Previous studies have similarly noted that secondary bacterial infections, particularly from Gram-negative bacteria such as Klebsiella spp., are common in TB patients (Mulemena, n.d.). These infections often complicate treatment outcomes and increase morbidity and mortality rates. Additionally, the presence of co-infections, particularly in HIV/TB patients, highlights the interplay between weakened immunity and pathogen susceptibility.

By identifying the predominant bacterial species and their prevalence rates, this study adds to the growing body of evidence on TB co-infections in LMICs. Moreover, the findings can guide targeted interventions and antimicrobial stewardship efforts.

The study highlights AMR as a major concern, with Ceftazidime and Meropenem demonstrating 100% sensitivity, while Aztreonam, Trimethoprim-Sulfamethoxazole, and Ciprofloxacin showed high resistance rates (80%). This AMR pattern poses a significant threat to effective disease management.

The findings are consistent with global trends in AMR, particularly in LMICs, where the misuse and overuse of antibiotics are rampant (Kayta et al., 2022). Reports such as the Global Antimicrobial Resistance and Use Surveillance System (GLASS) have indicated rising resistance to commonly used antibiotics in many countries, which aligns with the resistance observed in this study. The observed sensitivity to carbapenems like Meropenem mirrors similar trends in other studies, where carbapenems remain effective but are often a last-resort treatment (Birhanu et al., 2024).

While AMR patterns are well-documented globally, region-specific data in LMICs are often scarce (Alelign et al., 2022). This study addresses that gap by providing localized resistance data for pathogens in Bauchi State, offering critical information for public health strategies and antibiotic prescription policies.

The study also found significant gender differences in bacterial co-infections among TB patients, with females showing a higher prevalence (57.4%) compared to males (42.6%). Statistical analysis confirmed a significant association (χ^2=5.40, P=0.0455).

Gender disparities in co-infection prevalence have been noted in earlier studies, but explanations often vary (Miriti, n.d.). In some contexts, biological factors (e.g., hormonal influences on

immune responses) and social determinants (e.g., access to healthcare) have been cited (Cm et al., 2013). This study corroborates previous findings while emphasizing the importance of gender-specific considerations in disease management.

By statistically validating gender disparities in bacterial co-infection rates, this study reinforces the need to address gender-based differences in TB and bacterial infection management.

Conclusion

This study successfully builds on prior research while addressing important gaps in the understanding of TB and its co-infections in Bauchi State. The findings have critical implications for public health, including the need for gender-sensitive healthcare policies, improved AMR surveillance, and targeted interventions for TB and co-infections. Future studies should aim to explore the socio-economic and environmental factors contributing to these findings to develop holistic and sustainable solutions.

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