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Research Article

Keywords: Tuberculosis, drug-resistant tuberculosis, multidrug-resistant tuberculosis, phenotypic drug susceptibility testing, Mycobacterium tuberculosis, isoniazid resistance, Zambia.

Posted Date: November 13th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11282726/v1

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Additional Declarations: No competing interests reported.

Phenotypic Drug Resistance Patterns among *Mycobacterium tuberculosis* Isolates at the National Tuberculosis Reference Laboratory, Zambia, 2021–2025

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Abstract

Background

Drug-resistant tuberculosis (DR-TB) remains an important challenge to TB control, and characterization of phenotypic resistance patterns is essential for surveillance and appropriate treatment. However, routine laboratory data on resistance to individual first-line anti-TB drugs and specific resistance patterns among isolates referred to the National Tuberculosis Reference Laboratory (NTRL), Zambia, have not been recently described. This study characterized phenotypic resistance patterns and factors associated with multidrug-resistant tuberculosis (MDR-TB) among *Mycobacterium tuberculosis* isolates processed at the NTRL.

Methods

A retrospective laboratory-based observational study was conducted using routinely collected NTRL laboratory data between 1 January 2021 and 31 December 2025. Culture-confirmed *M. tuberculosis* isolates from patients with phenotypic drug susceptibility testing (DST) results for isoniazid, rifampicin, streptomycin and ethambutol were included. Resistance patterns were classified as fully susceptible, monoresistant, polyresistant non-MDR, or MDR-TB. Logistic regression was used to assess factors associated with MDR-TB. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) were estimated. The adjusted model included age, sex, HIV status and previous treatment.

Results

A total of 172 *M. tuberculosis* isolates from 172 patients were included. Overall, 76 (44.19%) isolates were resistant to at least one drug. Resistance was highest for isoniazid (64/172, 37.21%; 95% CI: 30.25–44.75), followed by rifampicin (40/172, 23.26%; 95% CI: 17.49–30.22), streptomycin (22/172, 12.79%; 95% CI: 8.54–18.73) and ethambutol (18/172, 10.47%; 95% CI: 6.66–16.06). 96 (55.81%) isolates were fully susceptible, 31 (18.02%) showed monoresistance, 13 (7.56%) showed polyresistance without MDR-TB, and 32 (18.60%) were MDR-TB. Previous second-line treatment was independently associated with MDR-TB (aOR 5.29, 95% CI: 1.77–

15.87; P=0.003). HIV status, sex and age were not significantly associated with MDR-TB.

Conclusion

A substantial proportion of *M. tuberculosis* isolates referred to the NTRL showed resistance to first-line anti-TB drugs, with isoniazid resistance being the most common and MDR-TB identified in 18.60% of isolates. Previous second-line treatment was independently associated with MDR-TB. Continued phenotypic DST and routine laboratory surveillance are important for characterizing resistance patterns and supporting the management of DR-TB in Zambia.

Keywords

Tuberculosis; drug-resistant tuberculosis; multidrug-resistant tuberculosis; phenotypic drug susceptibility testing; *Mycobacterium tuberculosis*; isoniazid resistance; Zambia.

1 Introduction

Tuberculosis (TB) remains a major global public health problem and continues to cause substantial morbidity and mortality despite the availability of effective diagnostic and treatment strategies [1]. In 2024, an estimated 10.7 million people developed TB globally, including approximately 1.2 million children, and TB caused an estimated 1.23 million deaths among HIV-negative people and 150,000 deaths among people living with HIV [1]. Drug-resistant tuberculosis (DR-TB), particularly rifampicin-resistant tuberculosis (RR-TB) and multidrug-resistant tuberculosis (MDR-TB), remains a major challenge to TB control because resistance restricts effective treatment options and requires more complex treatment regimens [1–3]. MDR-TB is defined as TB caused by *Mycobacterium tuberculosis* resistant to at least rifampicin and isoniazid, the two most important first-line anti-TB drugs [3]. WHO estimated that 390,000 people developed MDR/RR-TB globally in 2024 (95% uncertainty interval, 360,000–430,000) [1]. However, only 164,545 people with RR-TB were reported as having started treatment in 2024, corresponding to approximately 40% of the estimated number who developed RR-TB [4].

Drug susceptibility testing (DST) is an essential component of the laboratory investigation of DR-TB because it identifies susceptibility or resistance to individual anti-TB drugs and supports selection of an effective treatment regimen [3]. Phenotypic DST determines resistance by assessing the growth of viable *M. tuberculosis* in the presence of defined concentrations of anti-TB drugs [5]. Culture-based phenotypic DST can be performed using solid or liquid culture systems, with liquid culture generally providing faster detection of mycobacterial growth than conventional solid culture. Phenotypic testing also provides a direct assessment of the organism's growth response to the drug tested and can therefore identify resistance patterns beyond those targeted by individual molecular assays [5, 6].

The BACTEC MGIT 960 system is a liquid culture platform used for mycobacterial isolation and phenotypic DST [7]. The system detects mycobacterial growth in liquid culture and, for DST, compares growth in drug-containing tubes with that in a drug-free control to classify an isolate as susceptible or resistant to the drugs tested [7]. Phenotypic DST therefore remains an important approach for characterizing resistance among viable *M. tuberculosis* isolates, particularly when culture isolates are

available for further testing [8]. Its value is complementary to rapid molecular testing, which can identify resistance-associated genetic markers but does not provide the same breadth of phenotypic information for all anti-TB drugs [6].

Zambia remains among the countries with a high burden of MDR/RR-TB [9]. WHO includes Zambia among the 30 countries classified as having a high MDR/RR-TB burden for the 2021–2025 period [10]. In 2024, Zambia achieved at least 80% coverage for rifampicin-resistance testing among people with bacteriologically confirmed TB, placing the country among the high MDR/RR-TB burden countries with high coverage of resistance testing [1]. Nevertheless, molecular detection of rifampicin resistance does not fully characterize resistance to other anti-TB drugs, making complementary DST important for isolates requiring extended resistance characterization [6].

Previous studies from Zambia have demonstrated substantial heterogeneity in phenotypic and genotypic drug resistance. A study of 132 bacteriologically confirmed TB isolates collected from Southern and Eastern provinces during 2020–2021 found resistance to at least one anti-TB drug in 23.5% of isolates and MDR-TB in 9.8% of patients [11]. Previously treated patients were 40 times more likely to have drug-resistant isolates in that study, although the study was limited to two provinces and a relatively small sample [11]. Another study of 274 clinical *M. tuberculosis* isolates from Lusaka collected between 2013 and 2017 identified 134 MDR-TB isolates and 126 pan-susceptible isolates, and demonstrated variation in MDR-TB according to bacterial genotype [12]. These findings provide evidence of substantial resistance among isolates studied in Zambia but do not describe contemporary phenotypic resistance patterns among isolates referred to the National Tuberculosis Reference Laboratory (NRTL).

Phenotypic testing has also identified resistance to individual drugs that may not be captured by rifampicin and isoniazid testing alone. In a study of 131 *M. tuberculosis* isolates from Lusaka, phenotypic pyrazinamide susceptibility testing using BACTEC MGIT 960 identified pyrazinamide resistance in 32 isolates; among 35 MDR isolates, 21 (58.3%) were resistant to pyrazinamide [13]. The study also demonstrated discordance between phenotypic pyrazinamide resistance and individual genetic mutations, supporting the role of phenotypic testing in resistance characterization [13].

Despite these studies, contemporary data describing the full phenotypic resistance profile to the four first-line drugs including isoniazid, rifampicin, streptomycin and ethambutol among sputum specimens referred to the NTRL remain limited. Existing Zambian studies have largely focused on specific geographical areas, selected patient populations, molecular resistance markers or particular resistance groups [9, 11, 14]. A laboratory-based analysis of routine NTRL phenotypic DST data can therefore complement molecular surveillance by describing resistance to individual drugs, resistance combinations and MDR-TB across the provinces served by the laboratory [15]. Therefore, this study retrospectively characterized phenotypic drug-resistance patterns among sputum specimens processed at the NTRL between January 2021 and December 2025.

2 Methods

2.1 Study design and setting

This was a retrospective laboratory-based observational study that used routinely collected laboratory data from the NTRL, Zambia. The study included *M. tuberculosis* isolates recovered from sputum specimens processed for DST at the NTRL between 1 January 2021 and 31 December 2025. The study characterized phenotypic resistance patterns to first-line anti-TB drugs among culture-confirmed *M. tuberculosis* isolates during the study period. The study was conducted at the NTRL in Lusaka, Zambia. The NTRL is a specialized tuberculosis reference laboratory within the national tuberculosis laboratory network and provides mycobacterial culture and DST services [9]**.** During the study period, the laboratory received sputum specimens referred from health facilities in five provinces: Central, Eastern, Muchinga, Northern and Southern. These specimens were referred for specialized tuberculosis investigations, including mycobacterial culture and DST. For this study, the analysis was restricted to first-line phenotypic DST results for rifampicin, isoniazid, streptomycin and ethambutol. DST was performed based on the tests requested by the referring health facility on the laboratory request form.

2.2 Study population and eligibility

The study population comprised culture-confirmed *M. tuberculosis* isolates processed for phenotypic DST at the NTRL between 1 January 2021 and 31 December 2025. All culture-confirmed *M. tuberculosis* isolates processed during the study period with phenotypic DST results for isoniazid, rifampicin, streptomycin and ethambutol were included in the analysis. The laboratory records provided information on year of testing, province of origin, diagnostic category, treatment history, previous treatment, HIV status, age, sex and phenotypic DST results. Each isolate represented a unique patient; therefore, no repeated isolates from the same patient were included. HIV status was categorized as positive, negative or unknown, and isolates with unknown HIV status were retained in the analysis. A total of 172 culture-confirmed *M. tuberculosis* isolates were included in the study.

2.3 Data sources and collection

Data were retrospectively extracted from the NTRL Laboratory Information System (LIS; DISA V4.22.03) and corresponding laboratory registers (CDL-TB-F-119). Electronic LIS records and laboratory documentation for culture-confirmed *M. tuberculosis* isolates processed for phenotypic DST between 1 January 2021 and 31 December 2025 were reviewed. Data quality was assessed through completeness and consistency checks, with discrepancies resolved against the original laboratory records.

2.4 Laboratory procedures and culture outcomes

Sputum specimens were processed using routine NTRL procedures. Specimens were decontaminated with 4% N-acetyl-L-cysteine–sodium hydroxide according to CDL-TB-TECH-003, inoculated into MGIT tube medium and cultured using the BACTEC™ MGIT™ 960 system according to CDL-TB-TECH-005. MGIT-positive specimens underwent Ziehl–Neelsen microscopy for acid-fast bacilli detection according to CDL-TB-TECH-007. *Mycobacterium tuberculosis* Complex (MTBC) and non-tuberculous mycobacteria (NTM) were differentiated using the Capilia TB-Neo Chromatographic Immunoassay according to CDL-TB-TECH-008. For isolates for which first-line DST was requested by the referring facility, Capilia-positive isolates were subjected to

phenotypic DST according to CDL-TB-TECH-010. First-line DST assessed susceptibility to rifampicin, isoniazid, streptomycin and ethambutol. Results for each drug were classified as susceptible or resistant

2.5 Statistical analysis

Data were analysed using Stata/SE version 14.2. Categorical variables were summarized using frequencies and percentages, while age was summarized using the median and interquartile range (IQR). The proportions of isolates resistant to rifampicin, isoniazid, streptomycin and ethambutol were calculated with corresponding 95% confidence intervals (CIs). Resistance patterns were classified based on the individual DST results. Fully susceptible was defined as susceptibility to all four drugs tested. Monoresistance was defined as resistance to one drug only. Polyresistance, non-MDR was defined as resistance to two or more drugs without simultaneous resistance to both rifampicin and isoniazid. MDR-TB was defined as phenotypic resistance to both rifampicin and isoniazid, consistent with the World Health Organization definition [3]. These categories were mutually exclusive.

Characteristics of isolates with and without MDR-TB were compared using Pearson's chi-square test for categorical variables and the Wilcoxon rank-sum test for age. Logistic regression was used to assess factors associated with MDR-TB. Crude odds ratios (ORs) with 95% CIs were estimated separately for age, sex, HIV status, previous treatment, year, province and treatment history. The multivariable model included age, sex, HIV status and previous treatment based on clinical relevance and model parsimony given the number of MDR-TB events. Adjusted odds ratios (aORs) with 95% CIs were reported. Statistical significance was assessed using two-sided P values, with $P<0.05$ considered statistically significant.

3 Results

3.1 Characteristics of *Mycobacterium tuberculosis* isolates

A total of 172 *Mycobacterium tuberculosis* clinical isolates were included in the analysis. The median age of the participants was 34 years (IQR, 28–41.5). Most isolates were from Southern Province (59/172, 34.3%), followed by Eastern Province

(45/172, 26.2%) and Central Province (44/172, 25.6%); Muchinga and Northern provinces contributed 13/172 (7.6%) and 11/172 (6.4%), respectively. By diagnostic category, 111/172 (64.5%) isolates were from patients classified as having a diagnosis, while 61/172 (35.5%) were from follow-up patients. Among the treatment-history categories, relapse was the most common (71/172, 41.3%), followed by new cases (49/172, 28.5%), failure (19/172, 11.0%), uncertain history (17/172, 9.9%), lost to follow-up (12/172, 7.0%), and DR-TB contact (4/172, 2.3%). For HIV status, 76/172 (44.2%) were HIV-positive, 67/172 (39.0%) were HIV-negative, and 29/172 (16.9%) had unknown HIV status. By year, the number of isolates was highest in 2025 (60/172, 34.9%), closely followed by 2021 (59/172, 34.3%), while 2022 contributed the fewest isolates (3/172, 1.7%). Overall, 125/172 (72.7%) isolates were from males, compared with 47/172 (27.3%) from females (**Table 1**).

Table 1. Characteristics of *Mycobacterium tuberculosis* clinical isolates included in the study, 2021–2025

Characteristic	Category	n (%)
Age, years	Median (IQR)	**34 (28–41.5)**
Province	Central	44 (25.58)
	Eastern	45 (26.16)
	Muchinga	13 (7.56)
	Northern	11 (6.40)
	Southern	59 (34.30)
Category	Diagnosis	111 (64.53)
	Follow-up	61 (35.47)
History	DR contact	4 (2.33)
	Failure	19 (11.05)
	Lost to follow-up	12 (6.98)
	New	49 (28.49)
	Relapse	71 (41.28)
	Uncertain	17 (9.88)
HIV status	Negative	67 (38.95)
	Positive	76 (44.19)
	Unknown	29 (16.86)

Year	2021	59 (34.30)
	2022	3 (1.74)
	2023	15 (8.72)
	2024	35 (20.35)
	2025	60 (34.88)
Sex	Female	47 (27.33)
	Male	125 (72.67)
Total		**172 (100.00)**

3.2 Phenotypic resistance to individual anti-TB drugs

Among the 172 *M. tuberculosis* isolates, 64 (37.2%) were resistant to isoniazid (95% CI, 30.2–44.7%), while 40 (23.3%) were resistant to rifampicin (95% CI, 17.5–30.2%). Resistance to streptomycin was detected in 22 (12.8%) isolates (95% CI, 8.5–18.7%), and resistance to ethambutol in 18 (10.5%) isolates (95% CI, 6.7–16.1%). The corresponding proportions susceptible to the four drugs were 62.8%, 76.7%, 87.2%, and 89.5%, respectively (**Table 2**).

Table 2. Phenotypic resistance to individual anti-tuberculosis drugs among *Mycobacterium tuberculosis* clinical isolates

Drug	Resistant, n (%)	95% CI	Susceptible, n (%)
Isoniazid	64 (37.21)	30.25–44.75	108 (62.79)
Rifampicin	40 (23.26)	17.49–30.22	132 (76.74)
Streptomycin	22 (12.79)	8.54–18.73	150 (87.21)
Ethambutol	18 (10.47)	6.66–16.06	154 (89.53)
Total	**172 (100.00)**		**172 (100.00)**

3.3 Overall and specific phenotypic resistance patterns

Among the 172 *M. tuberculosis* isolates, 96 (55.8%) were fully susceptible to the four drugs tested (95% CI, 48.2–63.1%). Monoresistance was identified in 31 (18.0%) isolates (95% CI, 12.9–24.6%), while 13 (7.6%) showed polyresistance without MDR-TB (95% CI, 4.4–12.6%). MDR-TB was identified in 32 (18.6%) isolates (95% CI, 13.4–25.2%) (**Table 3**).

Table 3. Overall phenotypic resistance patterns among *Mycobacterium tuberculosis* clinical isolates

Resistance pattern	n (%)	95% CI
Fully susceptible	96 (55.81)	48.24–63.12
Monoresistance	31 (18.02)	12.93–24.56
Polyresistance, non-MDR	13 (7.56)	4.41–12.64
MDR-TB	32 (18.60)	13.43–25.20
Total	**172 (100.00)**	

The most common resistance combination was isoniazid plus rifampicin, identified in 20/172 (11.6%) isolates. isoniazid monoresistance was observed in 19/172 (11.0%) isolates, followed by rifampicin monoresistance in 8/172 (4.7%). Among isolates with resistance to three drugs, 6/172 (3.5%) had resistance to isoniazid, streptomycin and ethambutol, while 4/172 (2.3%) had resistance to isoniazid, rifampicin and ethambutol and 3/172 (1.7%) had resistance to isoniazid, rifampicin and streptomycin. Resistance to all four drugs occurred in 5/172 (2.9%) isolates (**Table 4**).

Table 4. Specific phenotypic resistance combinations among *Mycobacterium tuberculosis* clinical isolates

Resistance combination	n (%)
Susceptible to all four drugs	96 (55.81)
Isoniazid only	19 (11.05)
Rifampicin only	8 (4.65)
Streptomycin only	3 (1.74)
Ethambutol only	1 (0.58)
Isoniazid + Rifampicin	20 (11.63)
Isoniazid + Streptomycin	5 (2.91)
Isoniazid + Ethambutol	2 (1.16)
Isoniazid + Rifampicin + Streptomycin	3 (1.74)
Isoniazid + Rifampicin + Ethambutol	4 (2.33)
Isoniazid + Streptomycin + Ethambutol	6 (3.49)
Isoniazid + Rifampicin + Streptomycin + Ethambutol	5 (2.91)

Total	**172 (100.00)**

3.4 Factors associated with MDR-TB

Among the 172 *Mycobacterium tuberculosis* isolates, 140 (81.4%) were non-MDR and 32 (18.6%) were MDR-TB. By province, the proportion of MDR-TB ranged from 15.6% in Eastern Province to 36.4% in Northern Province, although the overall difference between provinces was not statistically significant (P=0.583). By year, MDR-TB accounted for 8.5% of isolates in 2021, 0% in 2022, 26.7% in 2023, 17.1% in 2024 and 28.3% in 2025 (P=0.058). Among females, 11/47 (23.4%) isolates were MDR-TB compared with 21/125 (16.8%) among males (P=0.321). By HIV status, MDR-TB was identified in 18/76 (23.7%) HIV-positive, 8/67 (11.9%) HIV-negative, and 6/29 (20.7%) isolates with unknown HIV status (P=0.188). By treatment history, MDR-TB was identified in 7/19 (36.8%) isolates from patients with treatment failure, 3/12 (25.0%) among those lost to follow-up, 9/49 (18.4%) among new cases, 9/71 (12.7%) among relapse cases, and 4/17 (23.5%) among those with an uncertain history; none of the four isolates from DR-TB contacts were MDR-TB. The overall difference by treatment history was not statistically significant (P=0.197). Previous treatment was the only characteristic with a statistically significant difference between MDR-TB and non-MDR isolates (P=0.002). MDR-TB was identified in 9/20 (45.0%) isolates with previous second-line treatment, compared with 11/51 (21.6%) among those with no previous treatment specified and 12/101 (11.9%) among those with previous first-line treatment. The median age was 32 years (IQR, 28–40) among MDR-TB isolates and 35 years (IQR, 28–42) among non-MDR isolates. The age distributions did not differ significantly between the two groups (P=0.401) (**Table 5**).

Table 5. Characteristics of *Mycobacterium tuberculosis* isolates by MDR-TB status

Characteristic	Category	Non-MDR, n (%)	MDR-TB, n (%)	P value
Province	Central	36 (81.82)	8 (18.18)	0.583
	Eastern	38 (84.44)	7 (15.56)	
	Muchinga	10 (76.92)	3 (23.08)	
	Northern	7 (63.64)	4 (36.36)	

	Southern	49 (83.05)	10 (16.95)	
Year	2021	54 (91.53)	5 (8.47)	0.058
	2022	3 (100.00)	0 (0.00)	
	2023	11 (73.33)	4 (26.67)	
	2024	29 (82.86)	6 (17.14)	
	2025	43 (71.67)	17 (28.33)	
Sex	Female	36 (76.60)	11 (23.40)	0.321
	Male	104 (83.20)	21 (16.80)	
HIV status	Negative	59 (88.06)	8 (11.94)	0.188
	Positive	58 (76.32)	18 (23.68)	
	Unknown	23 (79.31)	6 (20.69)	
History	DR contact	4 (100.00)	0 (0.00)	0.197
	Failure	12 (63.16)	7 (36.84)	
	Lost to follow-up	9 (75.00)	3 (25.00)	
	New	40 (81.63)	9 (18.37)	
	Relapse	62 (87.32)	9 (12.68)	
	Uncertain	13 (76.47)	4 (23.53)	
Previous treatment	First line	89 (88.12)	12 (11.88)	0.002
	None specified	40 (78.43)	11 (21.57)	
	Second line	11 (55.00)	9 (45.00)	
Age, years	Median (IQR)	35 (28–42)	32 (28–40)	0.401

In crude analysis, previous second-line treatment was associated with higher odds of MDR-TB compared with previous first-line treatment (OR, 6.07; 95% CI, 2.09–17.65; P=0.001). This association remained after adjustment for HIV status, sex and age (aOR, 5.29; 95% CI, 1.77–15.87; P=0.003). Isolates from patients with no previous treatment specified had higher odds of MDR-TB than those with previous first-line treatment, although the association was not statistically significant after adjustment (aOR, 2.14; 95% CI, 0.85–5.43; P=0.108). HIV status, sex and age were not significantly associated with MDR-TB in the adjusted model. Compared with HIV-negative status, the adjusted odds were 1.91 (95% CI, 0.73–4.98; P=0.187) among HIV-positive patients and 1.64 (95% CI, 0.50–5.41; P=0.418) among those with unknown HIV status. Male sex was not significantly associated with MDR-TB

compared with female sex (aOR, 0.68; 95% CI, 0.28–1.62; P=0.382). Age was also not significantly associated with MDR-TB (aOR, 0.99 per year; 95% CI, 0.96–1.02; P=0.528). The overall multivariable model was statistically significant (likelihood-ratio χ^2=14.15; P=0.028) (**Table 6**).

Table 6. Crude and adjusted associations between selected characteristics and MDR-TB among *Mycobacterium tuberculosis* clinical isolates

Characteristic	**Category**	**Crude OR**	**95% CI**	**P**	**Adjusted OR**	**95% CI**	**P**
Age, years	Per 1-year increase	0.99	0.96–1.02	0.470	0.99	0.96–1.02	0.528
Sex	Female	Ref	—	—	Ref	—	—
	Male	0.66	0.29–1.50	0.323	0.68	0.28–1.62	0.382
HIV	Negative	Ref	—	—	Ref	—	—
	Positive	2.29	0.92–5.68	0.074	1.91	0.73–4.98	0.187
	Unknown	1.92	0.60–6.16	0.270	1.64	0.50–5.41	0.418
Previous treatment	First line	Ref	—	—	Ref	—	—
	None specified	2.04	0.83–5.01	0.120	2.14	0.85–5.43	0.108
	Second line	6.07	2.09–17.65	0.001	5.29	1.77–15.87	0.003

4 Discussion

This study described phenotypic resistance patterns to four first-line anti-TB drugs among 172 culture-confirmed *M. tuberculosis* isolates processed at the NTRL between 2021 and 2025. Nearly half of the isolates (44.2%) showed resistance to at least one drug, while 18.6% were MDR-TB. Isoniazid resistance was the most frequent individual resistance phenotype (37.2%), followed by rifampicin (23.3%), streptomycin (12.8%) and ethambutol (10.5%). These findings indicate a substantial burden of first-

line drug resistance among isolates referred to the NTRL for phenotypic DST. However, the findings should not be interpreted as population-level prevalence estimates for Zambia because the study population comprised isolates referred to the NTRL and was therefore likely enriched for patients with clinical or programmatic indications for DST.

The proportion of MDR-TB observed in this study was higher than estimates reported from previous nationally representative Zambian drug-resistance surveys. The 2019 national survey reported MDR-TB in 1.1% of patients by phenotypic DST and 0.5% by line-probe assay, while rifampicin resistance and isoniazid resistance were 3.0% and 6.2%, respectively [16]. The earlier 2008 national survey reported MDR-TB prevalence of 1.1% by culture-based DST and 1.6% by line-probe assay [17]. In contrast, an NTRL study based on GenoType MTBDRplus records from 2019–2020 reported MDR-TB in 21.2% of isolates, with 35% resistant to rifampicin and/or isoniazid [9]. The 18.6% MDR-TB observed in the present study was therefore similar to the earlier NTRL-based estimate but considerably higher than national survey estimates [16, 17]. This difference is likely to reflect the different study populations and referral pathways rather than a direct increase in national MDR-TB prevalence. National surveys are designed to estimate resistance among representative TB populations, whereas reference-laboratory datasets contain a selected group of patients whose specimens are referred for specialized investigations[18].

The overall resistance profile was also higher than that reported in a phenotypic DST study conducted in Southern and Eastern Provinces of Zambia between 2020 and 2021. That study found resistance to at least one first-line drug in 23.5% of 132 patients and MDR-TB in 9.8%, compared with 44.2% and 18.6%, respectively, in the present study [11]. The difference may partly reflect the broader geographic coverage of the present study, which included five provinces, as well as differences in referral criteria and patient treatment history. The present study also included a large proportion of previously treated patients, particularly those with relapse and treatment-related histories, who may have been more likely to undergo culture and phenotypic DST. Therefore, direct comparison of the percentages should be made cautiously.

Isoniazid resistance was the most frequent individual resistance phenotype in the present study, occurring in 37.2% of isolates, while 11.1% had isoniazid

monoresistance. The predominance of isoniazid resistance is consistent with previous evidence from Zambia and other settings. A Zambian phenotypic DST study from 2020–2021 reported isoniazid resistance as the most common resistance phenotype, although at a substantially lower level of 9.8% [11]**.** Similarly, the 2019 national drug-resistance survey found isoniazid resistance in 6.2% of patients [16]. The higher proportion observed in the present NTRL dataset may reflect enrichment for patients with previous TB treatment or suspected drug resistance. Isoniazid resistance is particularly important because isoniazid is a core first-line drug and is also widely used for TB preventive treatment; undetected isoniazid resistance can compromise treatment effectiveness and may contribute to selection of additional resistance when treatment is not appropriately adjusted [19–22].

Rifampicin resistance was identified in 23.3% of isolates, and rifampicin-only resistance accounted for 4.7%. This was substantially higher than the prevalence reported at Ndola Teaching Hospital, where rifampicin-resistant tuberculosis increased from 1.53% in 2020 to 3.13% in 2021 and 4.6% in 2022 [14]. This difference may reflect differences in study setting, patient characteristics, referral patterns and the selection of isolates for phenotypic DST. Rifampicin resistance is important because it is a key marker used in the programmatic detection of MDR/RR-TB and is central to the selection of drug-resistant TB treatment regimens [3, 23–25]. At the same time, recent evidence from Zambia has highlighted that rifampicin resistance detected by rapid molecular testing may require confirmation. In a Lusaka cohort of patients started on MDR-TB treatment following rifampicin resistance detected by Xpert MTB/RIF, confirmatory testing identified rifampicin-susceptible TB in 28% of those who underwent additional testing [26]. This supports the continued value of phenotypic DST and complementary diagnostic approaches when investigating complex or discordant resistance profiles [27].

More than half of the isolates were fully susceptible to all four drugs, while 18.0% showed monoresistance and 7.6% showed polyresistance without MDR-TB. The predominance of susceptible isolates and the substantial proportion of monoresistance indicate that resistance in this dataset was not confined to MDR-TB. This pattern is relevant because reliance on rifampicin resistance alone may overlook clinically important non-rifampicin resistance. Recent programmatic data from Zambia

similarly demonstrated that a proportion of drug-resistant TB involved non-rifampicin resistance, including isoniazid monoresistance, supporting the use of diagnostic approaches capable of detecting resistance beyond rifampicin [14, 28]. The present findings therefore reinforce the value of comprehensive DST among isolates referred for specialized testing.

Previous treatment history showed the clearest association with MDR-TB. In the adjusted analysis, isolates from patients with a history of second-line treatment had approximately five-fold higher odds of MDR-TB than those with first-line treatment history (aOR 5.29, 95% CI 1.77–15.87; P=0.003). This finding is consistent with earlier Zambian evidence showing substantially greater drug resistance among previously treated patients. In the 2020–2021 Zambian study, previously treated patients had markedly higher odds of drug-resistant TB than patients without previous treatment (OR 40.3, 95% CI 11.1–146.5) [11]. The magnitude of association in that study was greater than that observed in the present analysis, but both findings indicate a strong relationship between treatment history and drug resistance. Previous treatment may reflect earlier exposure to anti-TB drugs, treatment failure, incomplete treatment or selection of resistant organisms [29]. However, the present study could not determine whether resistance was acquired during previous treatment or transmitted from an already resistant strain.

HIV status, sex and age were not significantly associated with MDR-TB in the adjusted analysis. HIV-positive isolates had higher odds of MDR-TB than HIV-negative isolates, but the association was not statistically significant (aOR 1.91, 95% CI 0.73–4.98; P=0.187). This finding is consistent with earlier Zambian evidence in which HIV status was not significantly associated with drug-resistant TB [11, 14, 30]. The lack of statistical significance should not be interpreted as evidence that HIV has no relationship with drug resistance. The relatively small number of isolates and the limited number of MDR-TB events may have reduced the precision of the estimates. Similarly, the absence of significant associations with age and sex may reflect limited statistical power rather than the absence of any underlying relationship.

The proportion of MDR-TB varied across the study years, from 8.5% in 2021 to 28.3% in 2025, with no MDR-TB isolate recorded in 2022. Although the overall comparison by year approached but did not reach statistical significance (P=0.058), the small

number of isolates in 2022 and the relatively small annual sample sizes limit interpretation of this pattern. The higher proportion observed in 2025 may reflect changes in referral patterns, clinical selection for DST, testing practices or the characteristics of patient's specimens referred to the NTRL rather than a population-level increase in MDR-TB. Therefore, these data should not be used to infer a national temporal trend without population-based surveillance data. WHO emphasizes that resistance estimates derived from routine surveillance and nationally representative surveys serve different purposes and that drug-resistance surveillance should be interpreted in relation to the underlying population and testing coverage [31].

The findings have implications for laboratory surveillance and management of drug-resistant TB in Zambia. The high proportion of resistance to isoniazid and rifampicin among NTRL-referred isolates, together with the substantial proportion of non-MDR resistance patterns, supports continued access to diagnostic methods that identify resistance beyond rifampicin. Zambia has achieved high coverage of rapid diagnostic testing and, in 2024, was among countries reporting at least 80% coverage of rifampicin-resistance testing among bacteriologically confirmed TB cases [1]. Continued strengthening of culture and phenotypic DST at the NTRL level remains important for resolving complex resistance profiles and detecting resistance to drugs not assessed by Xpert MTB/RIF Ultra [32]. The increasing availability of rapid tests for additional resistance markers may complement, rather than replace, reference-laboratory DST, particularly for surveillance and characterization of resistance patterns [18, 33, 34].

Strengths and limitations

This study had several strengths. First, it used routinely collected laboratory data from the NTRL, a specialized reference laboratory providing mycobacterial culture and DST services, and included isolates tested over a five-year period. Second, the study used phenotypic DST results for four first-line anti-TB drugs, allowing characterization of individual resistance and specific resistance combinations beyond MDR-TB. Third, the analysis distinguished monoresistance, polyresistance and MDR-TB, providing a more detailed description of the resistance patterns. Fourth, each isolate represented a unique patient, which minimized potential bias from repeated isolates. Finally, the

study examined treatment history and other patient characteristics in relation to MDR-TB using both crude and adjusted logistic regression.

The study also had limitations. First, the study was retrospective and relied on routinely collected laboratory records; therefore, some variables, particularly HIV status and treatment history, had incomplete information. Second, the study included only isolates referred to the NTRL for culture and phenotypic DST and therefore may have overrepresented patients with suspected or previously documented drug resistance. The findings should consequently not be interpreted as population-level estimates of drug resistance in Zambia. Third, the relatively small number of MDR-TB events limited the precision of the multivariable estimates and restricted the number of variables that could be included in the adjusted model. Some categories also contained few observations, resulting in wide confidence intervals. Fourth, the study was based on phenotypic DST results and did not include whole-genome sequencing or other molecular methods to characterize resistance-conferring mutations or distinguish acquired from transmitted resistance. Finally, the observational design precluded causal inference regarding the relationship between previous treatment and MDR-TB.

5 Conclusion

A substantial proportion of *M. tuberculosis* isolates referred to the NTRL showed phenotypic resistance to first-line anti-TB drugs, with isoniazid resistance being the most common and MDR-TB identified in 18.6% of isolates. Previous second-line treatment was independently associated with higher odds of MDR-TB. These findings highlight the importance of continued phenotypic DST and comprehensive resistance surveillance at the reference-laboratory level to characterize resistance patterns and support appropriate management of drug-resistant TB in Zambia.

Abbreviations

aOR:	Adjusted Odds Ratio
CI:	Confidence Interval
DR-TB:	Drug-Resistant Tuberculosis
DST:	Drug Susceptibility Testing

HIV:	Human Immunodeficiency Virus
IQR:	Interquartile Range
LIS:	Laboratory Information System
MDR/RR-TB:	Multidrug-Resistant/Rifampicin-Resistant Tuberculosis
MDR-TB:	Multidrug-Resistant Tuberculosis
MTB/RIF:	*Mycobacterium tuberculosis*/Rifampicin
MTBC:	*Mycobacterium tuberculosis* Complex
NTRL:	National Tuberculosis Reference Laboratory
OR:	Odds Ratio
RR-TB:	Rifampicin-Resistant Tuberculosis
TB:	Tuberculosis
WHO:	World Health Organization

Declarations

Ethics approval

The study used routinely collected, de-identified LIS data and involved no direct contact with patients. Ethical approval was obtained from the Livingstone Center for Prevention and Translational Science Research Ethics Committee (Ref: LCPTS/REC/012). The study was conducted in accordance with the principles of the Declaration of Helsinki. The requirement for informed consent was waived by the Livingstone Center for Prevention and Translational Science Research Ethics Committee Research Ethics Committee because the study involved retrospective analysis of de-identified routine laboratory data and did not pose any risk to participants.

Clinical trial number

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

Funding

No funding was received to assist with the preparation of this manuscript.

Authors' contributions

FP and JM conceived and designed the study and developed the methodology. FP and JM coordinated data collection and data management, performed the statistical analysis, and interpreted the findings. AM, MW, JW, BMM, WN, AS, JMu, WN, EKN, JS, JMz, KN, PM, and DKM contributed to data collection, data curation, laboratory work, and interpretation of the findings. FP led the drafting of the manuscript. AM, MW, JW, BMM, WN, AS, JMu, EKN, JS, JMz, KN, PM, and DKM contributed to manuscript review and revision. All authors critically reviewed the manuscript for important intellectual content, approved the final version, and agreed to be accountable for all aspects of the work.

Acknowledgements

We acknowledge the leadership and management at the National Tuberculosis Reference Laboratory in Lusaka, Zambia, for allowing us to process these data and disseminate the findings.

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