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Article

Multi Drug Resistant and Heteroresistant *Mycobacterium tuberculosis* Isolates Variants from Patients in Rural Areas in Eastern Cape

Lindiwe M. Faye [1,*], Mojisola C. Hosu [1], Yanga Mbiza [2], Nomzi Lubuzo [2], Ncomeka Sineke [1], Thulani Gumede [3], Carine Bokop [4], Ntandazo Dlatu [5], Bonnie Ifedi [2], Sandeep Vasaikar [1] and Teke Apalata [1]

[1] Department of Laboratory Medicine and Pathology, Walter Sisulu University and National Health Laboratory Services (NHLS), Private Bag X5117, Mthatha 5099, Eastern Cape, South Africa. fayelindiwe@yahoo.com; mojisolahosu@gmail.com; ncomekasineke3@gmail.com; sandeepvasaikar@yahoo.com; ruffinapalata@gmail.com

[2] National Health Laboratory Services (NHLS), Mthatha 5099, Eastern Cape, South Africa; Lubuzonomzi@gmail.com; mbinzayanga@gmail.com; Bonnieifaedi@gmail.com

[3] Nelson Mandela Academic Clinical Research Unit (NeMACRU), Mthatha, 5099, Eastern Cape, South Africa; thulani.elliot@gmail.com

[4] Division of Medical Microbiology, Department of Laboratory Medicine & Pathology, Faculty of Health Sciences, Walter Sisulu University, Mthatha 5099, Eastern Cape, South Africa; Africa; carinebokopf@gmail.com

[5] Department of Public Health, Faculty of Health Sciences, Walter Sisulu University, Private Bag X1, Mthatha 5117, Eastern Cape, South Africa; dlatun@gmail.com

* Correspondence: fayelindiwe@yahoo.com; Tel.: +27-47-502-1995

Abstract: Multidrug-resistant tuberculosis emerged as a serious challenge to tuberculosis management and control. In the Eastern Cape, the Beijing variants are prevalent and a driving force of multidrug-resistant tuberculosis; hence, we investigated the distribution of gene mutations in Beijing strains compared to non-Beijing strains. Multidrug-resistant tuberculosis and heteroresistant isolates were identified in 412 sputum cultures by drug susceptibility testing. The isolates were analyzed for mutations in three genes associated with resistance to antituberculosis first-line drugs: *katG* and i*nhA* promoters for isoniazid and *rpoB* for rifampicin. All isolates were genotyped by spoligotyping. There were more males than females and a more economically active age group in the study. The most prevalent mutations in *rpoB* resistance were in S531L, *katG* in S315Tb, and *inhA* in c-15tb. Heteroresistance was found in 18 isolates. Beijing variants were predominant. Most of the heteroresistant isolates were INH, with heteroresistance occurring more in the *inhA* gene mutation region c-15tb. Beijing and LAM variants were found more frequently in INH heteroresistant isolates. Mutations in *katG* S315Tb and *rpoB* S531L were higher in Beijing variants. The Beijing family is a major contributor to the epidemiological picture and accounts for most of the multidrug-resistant tuberculosis in the study area.

Keywords: keyword multidrug-resistant tuberculosis; gene mutations; heteroresistance; Beijing variants

1. Introduction

Tuberculosis (TB) is a preventable and treatable infectious disease and is a global human health threat. In 2019 alone, an estimated 360,000 South Africans became ill with TB, and 58,000 people were estimated to have died from the disease [1]. South Africa is known to have a high level of clonal drug-resistant tuberculosis (DR-TB) transmission and it is one of the DR-TB-burdened countries in Africa [2]. DR-TB emerged as a major risk to global TB control. The increased rate of co-infection of TB-HIV is another factor aggravating the control of multi drug resistant tuberculosis (MDR-TB) [3]. Out of

the estimated 465,000 people worldwide who developed rifampicin-resistant (RR-TB) and MDR-TB, only 150,359 persons were enrolled for treatment [4,5]. An effective method to limit the spread of MDR-TB, prevent the emergence of new resistance and development of extensively drug-resistant TB (XDR-TB) is early diagnosis of patients using accurate drug susceptibility testing (DST) of *M. tuberculosis* in clinical specimens and culture isolates to first-line medications [4–7]. The *katG* and *inhA* mutations give rise to high-level and low-level INH resistance respectively. Recent research has revealed that different mutations in *M. tuberculosis* can confer varying levels of phenotypic resistance to anti-TB medications [8–10]. Consequently, the aggregation of mutations at several positions has a comprehensive effect on drug resistance [11]. Heteroresistant tuberculosis (HR-TB) is defined as tuberculosis where there is a coexistence of susceptible and resistant organisms to anti-tuberculosis drugs in the same patient. Heteroresistance is considered a preliminary stage to full resistance or low levels of drug-resistant TB [12]. This may result from mixed infection, whereby resistant and susceptible strains infect the same individual, or from a single clone changing from a susceptible to a resistant strain as a result of genetic mutation under antibiotic stress [13]. The population structure of RR-TB isolates in South Africa is dominated by Beijing and Euro-American (LAM, T, S, and X) strains which can be explained by the historical movement of strains as South Africa was located in a geographically central position in the historical trade route between East and West for hundreds of years [14]. Karmaka et al [15] reported that the Beijing variants is more transmissible than other families.

Several studies have been published on the prevalence of mutations causing MDR in *M. tuberculosis* in Eastern Cape but this is the first study to provide a distribution and frequencies of gene mutations of MDR-TB and HR -TB among Beijing and non-Beijing variants in rural Eastern Cape.

2. Materials and Methods

Isolates obtained from 412 sputum samples confirmed patients with tuberculosis in National health laboratory services, TB Laboratory, Nelson Mandela Academic Hospital were tested for detection of mutations conferring resistance to anti-TB drugs. This was carried out using GenoType MTBDRplus VER 2.0 and thereafter were spoligotyped. The demographics information of patients was recorded from the laboratory requisition forms.

GenoType MTBDRplus version 2.0 [16] is a DNA strip-based method designed for simultaneous detection of the most important *rpoB* mutations, which confer RIF resistance and *katG* and *inhA* mutations, which confer high-level and low-level INH resistance, respectively [16,17] was used in this study. Three procedures namely DNA extraction, multiplex amplification with biotinylated primers, and reverse hybridization, were performed following the manufacturer's instructions [18]. DNA extraction was performed using a Genolyse kit [18] and following method as prescribed by [16]. Multiplex amplification was carried out in different cycles at different temperatures followed by reverse hybridization using an automated hybridization system, Auto-Lipa 48 system (Innogenetics) following the manufacturer's instructions [18]. The tested strips were pasted on evaluation sheets provided with the kit in the designated fields by aligning the conjugate control and amplification control bands with the respective lines on the sheet. With the aid of GenoLyse package inserts, the mutation bands were interpreted; the absence of a wild-type band and the presence of a mutant band for a specific gene on the strip implied resistance.

To determine spoligotypes of *M. tuberculosis* isolates, spoligotyping was done on 412 isolates. The samples were heat-killed and subjected to DNA extraction. Spoligotyping was performed using microbeads from TB-SPOL Kit (Beamedex®, Orsay, France) and the fluorescence intensity was measured using Luminex 200® (Austin, TX) following manufacturer's instructions. The hybridization patterns were translated into binary and octal formats. The generated binary codes of the isolates were entered into the SITVIT2 database of the Pasteur Institute of Guadeloupe and assigned specific shared international spoligotype signatures (SIT) [19]. Quality control was ensured by using *M. tuberculosis* H37Rv as a positive control.

3. Results

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A total of 412 clinical isolates were analyzed. There were 394 (95,6%) MDR-TB and 18 (4,4%) heteroresistant isolates. Of these isolate patterns 406 (98,5%) isolates matched a pre-existing SIT in the SITVIT2 database, while 6 (1,6%) isolates were not in SIT in the SITVIT2. The majority, 244 (59,2%), were from males, while 168 (40.8%) were from females. The age range was 2 years to 86 years.

3.1. Demographics

Males had more HR isolates compared to females. The economically active age group was more prevalent in the study, with more HR strains (Table 1).

Table 1. Characteristics of study subjects and their association with MDR and HR resistance among isolates.

Patient characteristics	Total (n)	MDR-TB (n)	HR-TB (n)
Sex			
Male	244	231	13
Female	168	163	5
Age category, years			
≤25	103	100	3
26–45	207	194	13
46 – 60	63	62	1
≥ 61	40	38	2

3.2. Resistance profile

All samples were confirmed to be multi-drug-resistant or heteroresistant by the presence of resistance-associated mutations and wild types. Of the 412 DR-TB isolates, there were 394 (95,6%) MDR isolates and 18 (4.4%) heteroresistant isolates.

In the *rpoB* gene, the S531L region had the highest number of mutations; *katG*, the S315Tb region, that confers a high level of drug resistance, had the highest number of mutations, and *inhA*, the c-15tb region, had the highest number of mutations (Table 2).

Table 2. Number of isolates per region of mutation in *rpoB*, *katG* and *inhA* genes.

rpoB		***KatG***		***inhA***	
Region of mutation	Number of isolates	Region of mutation	Number of isolates	Region of mutation	Number of isolates
D516V	124	S315Tb	338	c-15tb	40
S531L	216	Missing	74	t-8c	4
H526Y	3			t-8a	2
H526D	3			Missing	366
H526D & S531L	2				
D516V & S531L	1				
Missing	64				

Most of the HR isolates were INH-HR isolates, with HR occurring more in the *inhA* gene in mutation region c-15tb. The Beijing variants were found more in INH-HR isolates, and the LAM variants was also more prevalent in INH-HR isolates than RIF-HR isolates (Table 3).

Table 3. Variants and heteroresistant genes Isolates.

LPA score of HR isolates	Region of HR	Variant
rpoB WT-MUT/katG MUT/inhA WT	rpoB D516V	LAM
rpoB WT/ katG WT/ inhA WT-MUT	*inhA* c-15tb	T
rpoB WT- MUT/ katG WT/ inhA WT- MUT	*rpoB* H526Y and *inhA* c-15tb	T
rpoB MUT/ katG WT- MUT/ inhA WT	*katG* S315Tb	X

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rpoB MUT/ katG MUT/ inhA WT-MUT	*inhA* c-15tb	LAM
rpoB WT-MUT/ katG MUT/ inhA MUT	*rpoB* H526D	LAM
rpoB WT/katG WT/inhA WT-MUT	*inhA* c-15tb	Beijing
rpoB MUT/katG WT/inhA WT-MUT	*inhA* c-15tb	Beijing
rpoB WT/ katG WT/ inhA WT-MUT	*inhA* c-15tb	X
rpoB WT-MUT/ katG MUT/ inhA WT-MUT	*rpoB* S531L *inhA* c-15tb	X
rpoB WT-MUT/ katG WT-MUT/ inhA MUT	*rpoB* S531L *katG* S315Tb	T
rpoB MUT/ katG WT-MUT/ inhA WT	*katG* S315Tb	Beijing
rpoB MUT/ katG WT/ inhA WT-MUT	*inhA* c-15tb	Beijing
rpoB MUT/ katG WT- MUT/ inhA WT	*katG* S315Tb	LAM
rpoB MUT/ katG WT-MUT/ inhA WT-MUT	*katG* S315Tb and *inhA* c-15tb	T
rpoB WT/ katG WT/ inhA WT-MUT	*inhA* c-15tb	X
rpoB WT/katG WT-MUT/inhA WT	*katG* S315Tb	LAM
rpoB MUT/ katG WT-MUT/ inhA WT-MUT	*katG* S315Tb and *inhA* c-15tb	Beijing

3.3. Genetic diversity

Beijing family was the most prevalent varient among eight varients identified (Table 4)

Table 4. Prevalence of variants.

Variant	Number of isolates n (%)
Beijing	184 (44,6%)
LAM	82 (19,9%)
X	48 (11,7%)
T	34 (8,3%)
S	31 (7,5%)
EAI	16 (3.9%)
H	6 (1.5%)
CAS	5 (1.2%)
UNKNOWN	6 (1,5%)

There were 184 (44,6%) Beijing, 82 (19,9%) LAM, 140 (34,0%) other variants, and 6 (1,5%) unknown variants. Mutations were observed more frequently in region S531L in all strains (Table 5)

Table 5. Distribution and Frequency of rpoB gene Mutations Among Beijing and Non-Beijing Isolates.

rpoB mutation region	Beijing (n= 184)	LAM (n = 82)	Other (n =140)	Unknown (n=6)
D516V	60	21	42	1
S531L	105	40	66	5
H526Y	1	1	1	0
H526D	2	1	0	0
H526D & S531L	1	0	1	0
D516V & S531L	0	0	1	0
Missing mutation	15	19	30	0

We found that the most frequent mutations of katG and inhA occurred at the S315Tb and c-15tb mutation region respectively in Beijing, LAM, and non-Beijing isolates (Table 6).

Table 6. Distribution of different mutations conferring INH resistance among Beijing and non-Beijing genotype.

katG mutation region	Beijing (n = 184)	LAM (n = 82)	Other (n =140)	Unknown (n = 6)
S315Tb	152	65	115	6

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Missing	32	17	25	0
***inhA* mutation region**	**Beijing (n = 184)**	**LAM (n = 82)**	**Other (n = 140)**	**Unknown (n = 6)**
c-15tb	20	4	16	0
t-8c	1	3	0	0
t-8a	1	1	0	0
Missing	162	74	124	6

4. Discussion

The effectiveness of TB treatment is significantly impacted by drug resistance; therefore, effective management of DR-TB depends on the accurate identification of resistance to inform optimal treatment [20]. Due to the high prevalence of TB in South Africa, it is crucial to keep researching the population structure of circulating *M. tuberculosis* strains in order to identify and comprehend their frequency and distribution. Information on the relationship between gene alterations and the many strains circulating is lacking in our study setting, particularly for the Beijing variant, which is the most common in the East Cape of South Africa and known to be linked to treatment resistance.

Comparable to the findings of [21], the prevalence of males was higher in men than in women (Table 1). This could be due to the social mixing of men, which increases contact rates with TB patients and TB transmission [22]. Men socialize more than women do, and social interaction changes the patterns of infection for infectious diseases. Men are considered to have an advantage over women when it comes to accessing medical services, which has led to an increase in the rate at which men are being diagnosed with TB [20,23], this could be another reason of having more males than females.

The prevalence of Beijing variants (Table 4) which are known to be more contagious than other families, are being explained by the effects of gender-related social mixing patterns on the spread of *M. tuberculosis* [24] so as HR in males, which confirms the link between socializing in this area and tuberculosis in men. The economically active age group also had prevalence of HR strains, this poses a serious challenge in this setting as transmissibility of these strains can frequently increase due to the social activity of this group. RIF and INH, the two most potent bactericidal medicines for treating TB, are currently the cornerstone of therapy.

Rifampicin and isoniazid resistance are identified using the molecular markers *rpoB* and *katG*, respectively. The prevalence of mutations in rpoB gene in this study was higher than the other genes (*katG* and *inhA* genes). Resistance to RIF is almost entirely coupled (>97%) to mutations within an 81-bp region of the *rpoB* gene, called the RIF resistance-determining region (RRDR) [25]. Our study revealed that INH had more mutation in *katG* gene compared to inhA gene. The katG is associated with high levels of isoniazid resistance. The *inhA* promoter is associated with a low-level drug resistance which indicates a possible benefit of a drug either with standard or increased dosing from a different drug with the same class [26]. Knowing this is crucial because if the treating clinicians are aware of which patients have the *inhA* promoter mutation and a significant portion of the patients have isolates with that mutation, there may be a big number of patients who could benefit from high-dose INH [27]. It is crucial that clinicians from this study area are aware of this knowledge since it will reduce the amount of ineffective ethionamide use in these patients, additionally, this gives a warning that in this study setting the treating clinicians needs to monitor closely treatment progress of patients in INH drug.

The rise of RIF-R is a critical public health issue because RIF resistance is a surrogate marker for MDR-TB [28]. This study identified *M. tuberculosis* as a frequent carrier of mutations in the *rpoB* gene, particularly in the rifampicin resistance determining region (RRDR) S531. These results concur with studies by [29,30], and they could be attributed to the spread of an established clone. Furthermore, there are instances of S531L mutations occurring often in various South African provinces, demonstrating the prevalence of these mutations in the nation [31] and other parts of the world [32,33]. INH resistance is frequently connected to the primary genetic changes in MDR-TB, notably

at S315T [26], suggesting that INH is unlikely to be clinically beneficial. Patients from this region may soon need INH to be given in higher doses as HR is the start of full resistance to a medicine.

The INH had more HR incidence in the *inhA* gene, which is related with low level resistance. According to [34,35], HR has a role in the failure of TB treatment outcomes. The region of *inhA* known as c-15tb, which is also the region that is known as the marker of low-level resistance, showed a higher prevalence of HR. Patients with INH-HR TB can use low dose isoniazid but with caution and have their treatment outcomes closely monitored. The foundation of the first-line TB treatment consists of the drugs INH and RIF [36]. MDR-TB, which is resistant to both RIF and INH, typically has a poor prognosis for therapy and a higher fatality rate. It can be difficult to identify drug-resistant isolates in clinical samples when drug-susceptible and drug-resistant isolates coexist [34,35], which can lead to the masking of drug-resistant isolates by drug-susceptible ones [37]. It is thought that this process, known as HR is one of the key steps in the development of drug resistance [38]. When resistant and susceptible strains of an infection infect a person at the same time, or when a single clone undergoes genetic mutation under the influence of antibiotics, HR may result [13].

Beijing variants in this study were found more in INH-HR isolates, LAM variants were also predominating in INH-HR than RIF-HR isolates. GeneXpert can detect rifampicin resistance only when >50 per cent of MTB strains in the samples are resistant, and LPA can detect rifampicin resistance when ≥5 per cent of MTB strains in the samples are resistant [39]. In comparison to non-Beijing variants, Beijing variants are frequently shown to be more drug resistant [40]. The Beijing variant of *M. tuberculosis*, which was initially discovered in Beijing, China, in 1995 and is now mostly found throughout the world [41] and is the most common lineage in this area [42]. According to reports, the Beijing variant of *M. tuberculosis* is more lethal, pathogenic, and transmitting more quickly than other variants [15]. It also exhibits increased drug resistance, particularly MDR-TB characteristics, and has a higher fatality rate [43]. Beijing's genetic family is the global TB epidemic, *M. tuberculosis* is one of the most common genotypes [44], and in some places, like Beijing, it is the most common lineage among MDR-TB strains. The Beijing variants were more widespread than other variants in this study, which may be related to its recognized presence in the nearby provinces of the Eastern Cape namely KwaZulu Natal and Western Cape [15,45]. This variant family has been identified in studies from other regions of South Africa, such as Limpopo, the Western Cape, and Mpumalanga [45,46], where some residents of the rural Eastern Cape relocate in search of employment. In South Africa, it's typical for people to commute between rural and urban areas in pursuit of work; this movement acts as a bridge for the transmission of pathogens across large distances [47].

5. Conclusions

This first report on understanding *M. tuberculosis* variants association with gene mutations of MDR-TB and heteroresistant cases in rural Eastern Cape and serves as a foundation for future research into the association between gene alterations and the Beijing family, which is more common in this study setting. The future of INH use in this region is in jeopardy due to increased *katG* gene mutations and increased HR in *inhA*, which suggest that INH at low doses may no longer help treat patients. Since HR is an important stage in the development of full drug resistance in isolates in our investigation, its influence on treatment progression needs to be watched carefully.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethics and Biosafety Committee of the Faculty of Health Sciences of Walter Sisulu University (Ref. No. 026/2019) and Eastern Cape Department of Health (Ref No EC_201904_011).

Informed Consent Statement: Not applicable. This study used routine samples received in National Health Laboratory Services (NHLS) TB laboratory.

Data Availability Statement: Data can be requested from the corresponding author.

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