Molecular differentiation of rifampicin and isoniazid drug resistant *Mycobacterium tuberculosis* complex isolated from selected parts of Zambia

O. Kapona[1], E. Kaimoyo[2]*, C. Katongo[2]
[1]Zambia National Public Health Institute, Ministry of Health, Lusaka, Zambia
[2]University of Zambia, School of Natural Sciences, Biological Sciences Department, Zambia

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ABSTRACT

The most prevalent *Mycobacterium* species in the *Mycobacterium tuberculosis* complex and frequency of mutations in the genes conferring resistance to rifampicin and isoniazid in Zambia have until now remained unknown. This study sought to differentiate the isolates of *Mycobacterium tuberculosis* complex (MTBC) using genetic regions *cfp32*, RD9 and RD12 and determine the prevalence of resistance-associated mutations in two specific resistance marker genes (*rpoB* and *katG*) and the *inhA* promoter region of *M. tuberculosis* isolated at the University Teaching Hospital Tuberculosis Laboratory between January 2013 and June 2014. Genomic DNA was extracted from 40 isolates and amplified by multiplex PCR for regions *cfp32*, RD9 and RD12 and single PCRs for three drug-resistance conferring loci (*katG, rpoB* and *inhA*). All isolates were positive for the three regions typical of *M. tuberculosis*. Seventy-five percent had the S315T mutation in *katG* gene and one had mutations in the *inhA* promoter and *rpoB* in addition to the *katG* 315 mutation. The S450L (48.7%) and H445Y (20.5%) mutations were the most frequently observed mutations in the *rpoB* gene while rifampicin mono-resistance was observed in 2.6% of the rifampicin-resistant isolates. Molecular diagnostic tests based on detecting these predominant mutations could be useful for the rapid detection of multi drug resistant tuberculosis in Eastern, Lusaka, Western parts of Zambia and TB patients in these regions can be treated with the standard first line tuberculosis drugs.

1 Introduction

Tuberculosis (TB), an air-borne disease caused by an acid-fast staining bacterial pathogen, *Mycobacterium tuberculosis* has continued to be a serious global health problem. In 2017, the World Health Organization (WHO) estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis* (MTB). Nine million new cases of active TB and 2-3 million deaths were also reported to occur annually, and out of these 26% were estimated to occur in Africa (Global TB report, 2012). Despite its status as a treatable disease, TB has high morbidity and mortality rates (Global TB report, 2005; Muchemwa et al., 2017)) and in Zambia some of the factors that adversely affect treatment outcomes including exposure to HIV, smoking and occupational hazards have been analyzed (Nanzaluka et al., 2019). In 2014, the first ever national tuberculosis survey in Zambia reported 638 incident cases per 100 000 adult population (MOH, 2015). Worldwide, 4.1% of new cases and 19% of previously treated cases were estimated to have multidrug resistant tuberculosis (MDR-TB), defined as resistance to at least rifampicin (RIF) and isoniazid (INH), as reported in 2017 (Global TB report, 2017). In

*Corresponding author: *E-mail address:* emailekaimoyo@unza.zm (E. Kaimoyo)

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Zambia, 1.8% and 2.3% MDR-TB among new and previously treated cases respectively (Mulenga et al., 2010) have been reported.

The pathogens that cause tuberculosis in humans and other animals belong to the *Mycobacterium tuberculosis* complex (MTBC). The complex consists of a group of genetically closely-related species of the genus *Mycobacterium*. Members of this group include *M. tuberculosis, M. bovis, M. africanum, M. microti, M. caprae, M. pinnipedii* and *M. canetti* (Niemann et al., 2002). Although MTBC species are genetically closely-related, they differ in certain characteristics, including phenotypes, pathogenicity and host and geographic distribution with *M. tuberculosis* being the most common species in Europe and America, while *M. africanum* is mostly prevalent in African TB patients (Parsons et al., 2002).

Differentiation of the MTBC into individual species and subspecies is important not only for epidemiological reasons but also for accurate diagnosis and adequate treatment of each patient. Significant efforts have gone towards early and accurate diagnostic methods as a means to improve treatment outcomes (Mwanza et al., 2017). The use of pathogen genotyping assays and technologies including Xpert for detection of TB pathogen strains resistant to first-line drugs is increasingly being viewed as one of the game-changing options in the arsenal of strategies to control the disease (Mwanza et al., 2017) and while results appear encouraging, additional work remains to be done to optimize these technologies. The need for early and accurate diagnosis of TB is made more urgent when the fact that some of the species in the MTBC are naturally resistant to anti-TB drugs is taken into consideration. A case in point is that of *M. bovis* which is naturally resistant to pyrazinamide (PZ) (Niemann et al., 2000). Zambia has a low incidence rate of MDR-TB among newly-diagnosed cases than among previously treated cases. Moreover studies in Zambia (Bwalya et al., 2018) have also observed that half of TB patients with INH and RIF resistant TB also have PZ-resistance. A drug resistance survey conducted by Zambia AIDS Related Tuberculosis (ZAMBART) research team reported 1.8% and 2.3% MDR-TB among new and previously treated cases respectively (Mulenga et al., 2010; Kapata et al., 2013). Still, the low prevalence of MDR-TB is a serious concern because of the huge cost and debilitating side effects associated with its treatment. Additionally, its transmission via aerosols is the same as that of drug susceptible *M. tuberculosis*.

Zambia is ranked among the world's top ten countries with a high burden of tuberculosis caused by *Mycobacterium tuberculosis*. The TB disease burden has been shown to be quite significant especially in older adults. This could be due to inadequate sanitation and compromised immune system (Coffman et al., 2017; Negin et al., 2015).Treatment for new cases of TB consists of a 6-month fixed-dose combination therapy of four first-line drugs: INH, RIF, ethambutol (EMB) and PZ combined in a regime consisting of the first 2–months using a combination of INH/RIF/PZ/EMB and the last 4 months using INH/RIF (WHO, 2010). The disease is almost always curable if patients are given sufficient uninterrupted therapy, Directly Observed Treatment Course (DOTS) the internationally recommended strategy for TB control. However, despite its being treatable, tuberculosis has proved to be difficult to eliminate, with an increase in the number of drug-resistant cases reported. Global efforts to control the TB pandemic have been undermined by the emergence and spread of isolates that are resistant to the commonly used first-line anti-TB drugs (WHO, 2003). INH and RIF are the most important first-line anti-TB drugs, and development of resistance to these drugs results in high rates of treatment failure and death (Espinal et al., 2000).

While significant amounts of insights have been gained on MDR-TB from studies elsewhere (Bakonyte et al., 2003; Haas et al., 1997; Ramaswamy and Musser, 1998; Lee et al., 2003) a lot remains to be done towards the understanding of MDR-TB in Zambia. At the molecular level the variation in TB isolates at the gyrA/B locus in Zambian TB isolates was characterized (Mitarai et al., 2005). Mutations that have been reported to be associated with resistance to fluoroquinolones such as pyrazinamide (Hameed et al., 2018) were isolated in a study conducted in Zambia (Mitarai et al., 2005). Changes in amino acid sequences were observed at residue 74 (A74T) and at residue 88 where glycine was substituted by threonine in some of the isolates. Significant and notable contributions have also been made towards the understanding of TB pathology in the country with

focus on aspects of the disease including early and accurate diagnostic methods (Mwanza et al., 2017, 2018) and prevalence of the disease among patients infected with the HIV (Muchemwa et al., 2017). In another study (Muchemwa et al., 2017), the prevalence of TB bacteraemia in a cohort of HIV-infected adults was elucidated in the country.

The current study describes the common MTB species as well as prevalence of resistance-associated mutations in two specific resistance marker genes (*rpoB, katG*) and the *inhA* promoter region of *M. tuberculosis* in isolates from Lusaka, Eastern and Western parts of Zambia. The importance of analyzing the types of mutations in the loci associated with resistance to first-line drugs has been recognized as a means to contribute to the fight against TB in Zambia (Muchemwa et al., 2017). The frequency of the mutations in these isolates was compared with the frequencies of mutations observed in related studies elsewhere.

2 Materials and Methods

2.1 Study site

The study was conducted at the University Teaching Hospital (UTH)'s Tuberculosis (TB) Laboratory in Lusaka which is the TB Reference Laboratory for the Southern region of Zambia. It is a referral TB culture facility for Lusaka, Eastern and Western Provinces of the country and provides TB diagnostic services for UTH. The laboratory also offers External Quality Assurance Scheme for TB smear microscopy to diagnostic sites in the above provinces and is also enrolled with the South African National Institute for Communicable Diseases in TB smear microscopy External Quality Assessment (EQA) and Centre for Disease Control and Prevention (CDC) in GeneXpert EQA. The laboratory is internationally accredited under the Southern African Development Community Accreditation Services (SADCAS).

2.2 Study population

The study did not involve direct contact with human patient participants. Samples comprised of *Mycobacterium tuberculosis* isolates from patients that had exhibited resistance to INH and RIF through phenotypic drug sensitivity testing conducted at the UTH TB Laboratory during routine TB screening.

2.3 Sampling, inclusion and exclusion criteria

Samples were selected from all isolates deemed to qualify as MTBC that were resistant to first-line TB drugs archived at UTH TB Laboratory between January 2013 and June 2014. A purposive sampling method was used to select RIF and INH resistant isolates from the UTH collection previously tested for drug resistance using an automated Mycobacteria Growth Indicator Tube (MGIT). The purposive sampling method was chosen so as to include all RIF and/or INH resistant isolates to determine the type and frequency of mutations in the isolates. A total of 40 isolates from individual human sputum were included in this study. Mycobacterium other than tuberculosis (MOTT) isolates as well as MTBC samples resistant to streptomycin and ethambutol were excluded from the study. Patients infected with MOTT are generally not treated with the standard TB drugs and resistance to streptomycin and ethambutol does not qualify as MDR-TB (Global TB report, 2017; Niemann et al., 2000).

2.4 Handling and storage of isolates

All work with pathogenic *Mycobacterium tuberculosis* was performed in a Biosafety Level 3 laboratory at the UTH. The pathogenic *Mycobacterium tuberculosis* used in this study were MTBC isolates from routine Mycobacteria detection and recovery stored in Mycobacteria Growth Indicator Tube (MGIT) at $-80°C$. The MGIT used for the detection and recovery of Mycobacteria contained $7ml$ of modified Middlebrook 7h9 broth

base supplemented with nutrients (glycerol, oleic acid, albumin and dextrose). A mixture of five antibiotics (BBL MGIT PANTA) was reconstituted in $15000\,\mu l$ was deionised distilled water to give final concentrations of the drugs as follows: polymyxin B (0.4 units/μl), amphotericin B ($0.04\,\mu g/\mu l$), nalidixic acid ($0.16\,\mu g/\mu l$), trimethoprim ($0.04\,\mu g/\mu l$, and azlocillin ($0.04\,\mu g/\mu l$). $500\,\mu l$ of the reconstituted antibiotic mixture was added to each MGIT medium (Lee et al., 2003).

2.5 Genomic DNA extraction

Approximately $5\,ml$ of each pathogen isolate was transferred from MGIT to a labelled 50ml Falcon tube (Thermo Fisher Scientific, Waltham, MA, USA). Samples were centrifuged at $3,000\,g$ in a Tomy LX-141 refrigerated microcentrifuge (Tomy Kogyo-co. Tokyo Japan) for 15 minutes at $4^{\circ}C$. $500\,\mu l$ each of the centrifuged bacterial pathogen supernatant was transferred from the bottom of the Falcon tubes into a 2 millilitres microcentrifuge tube. DNA was extracted from the cell pellet by heating at $95^{\circ}C$ for 20 minutes. The supernatant of each isolate containing DNA was transferred to a fresh labelled microcentrifuge tube. The concentration and integrity of the extracted DNA were assessed using a nanodrop spectrophotometer and by agarose gel electrophoresis.

2.6 Mycobacterium tuberculosis Complex (MTBC) differentiation by multiplex PCR

To differentiate the isolates included in the study, pathogen genomic DNA was analyzed by multiplex PCR with primer pairs designed to amplify three genetic regions *cfp32*(Rv0577F5'-ATGCCCAAGAGAAGCGAATACAGGCAA-3';Rv0577R5'-CTATTGCTGCGGTGCGGGCTTCAA-3'),RD9(Rv2073cF5'-TCGCCGCTGCCAGATGAG-3'; Rv2073cR 5'-TTTGGGAGCCGCCGGTGGTGATGA-3'),and RD12(Rv3120F5'-GTCGGCGATAGACCATGAGTCCGTCTCCAT-3'; Rv3120R5'-GCGAAAAGTGGGCGGATGCCAG-3'). These have been authenticated in multiple distinct *Mycobacterium tuberculosis* Complex (MTBC) strains in previous studies and have been used as gold standard genetic markers for MTC species. Additionally, these have been used for phylogenetic lineage prediction and classification of MTBC (Huard et al., 2003, 2010; Nakajima et al., 2012).To conduct the PCR, $2\,\mu l$ of the extracted genomic DNA was transferred to each separate PCR tube. PCR reaction mixes were prepared by transferring $1\,\mu l$ of $MgCl_2$ (25mM), $1\,\mu l$ dNTP mix (10mM), $4\,\mu l$ 5X Go Taq buffer green, $2\,\mu l$ betain (5M), $2.6\,\mu l$ primers mixes for *cfp32,RD9*, and *RD12* in the ratio 2:1:1 of both forward and reverse primers respectively. Molecular grade nuclease free water ($7.4\,\mu l$) was added bringing the final reaction volume to $20\,\mu l$. The PCR thermal cycle profiles were preceded by an initial template denaturation at $96^{\circ}C$ for 5 minutes followed by 35 cycles of 30 seconds denaturation at $96^{\circ}C$, 30 seconds, annealing at $60^{\circ}C$, for 60 seconds and extension at $72^{\circ}C$. A final extension at $72^{\circ}C$ for 5 minutes was included in the amplification step. All subsequent PCRs were conducted using similar thermal cycling conditions. The PCRs were performed on a Biometra T-Gradient thermos block 230V 50-60Hz (Biomedizinische Analytik GmbH. Göttingen Germany). For quality control, *M. tuberculosis* H37Rv was used as a positive control and molecular grade water as a negative control in every PCR run. PCR products were analysed on 1 % horizontal agarose gel electrophoresis in tris acetate EDTA (TAE) buffer stained with 0.5 picograms/ml ethidium bromide.

2.7 Sequencing of the *rpoB* and *KatG* genes and the *InhA* promoter region

To determine variation among pathogen isolates with reference to gene sequences associated with drug resistance, three resistance marker genes were amplified in three separate PCR using the primers TB *rpoB* F and TB *rpoB* R;TB *katG* F and TB *katG* R;TB *inhA* F and TB *inhA* R(Table 1) for *rpoB, katG*, and *inhA* genes respectively. The target region were mutations occur in the *rpoB* locus is between 1276 and 1353, *katG* (823-1140) and *inhA* (-50 to-1) as illustrated in table 1. PCR products were extracted from the 1% agarose gel by cutting out the bands on the gel with a blade under UV and transferring the gel pieces into individual Eppendorf tubes which were left in a freezer overnight. Eppendorf tubes with frozen pieces of gel were centrifuged at $4^{\circ}C$ at 15,000 rpm for 10 minutes. Supernatants were transferred to fresh tubes and the concentration of the amplicon DNA

was determined using a BioDrop μLITEUV-Vis spectrophotometer (SERVA Electrophoresis GmbH. Heidelberg Germany) with an inbuilt computer system.

The amplicon DNA was sequenced using the respective primers by Big Dye Terminator v3.1 cycle sequencing kit on the ABI Prism 3500xlGenetic Analyser (Life Technologies Corp).

Table 1: Primers for amplification and sequencing of RIF and INH drug resistance associated genes.

Locus	Primer	Nucleotide sequence (5' - 3')	Target region (position)	Expected PCR product size (bp)
rpoB	TB *rpoB* F	CAGGACGTGGAGGCGATCAC	1276 - 1353	278
	TB *rpoB* R	GAGCCGATCAGACCGATGTTGG		
katG	TB *katG* F	ATGGCCATGAACGACGTCGAAAC	823 - 1140	392
	TB *katG* R	CGCAGCGAGAGGTCAGTGGCCAG		
InhA	TB *inhA* F	TCACACCGACAAACGTCACGAGC	-50 to -1	231
	TB *inhA* R	AGCCAGCCGCTGTGCGATCGCCA		

2.8 Data management and analysis

The data collected on each individual isolate were recorded in a laboratory worksheet and analysed in Microsoft Excel. The place/town of origin was extracted from the laboratory database as the types of gene mutations vary depending on the geographical location (Morgan et al., 2005; Ling et al., 2008). The type of specimen was also considered to ascertain possibility of disseminated drug resistant TB. MTBC differentiation was achieved based on the pattern observed in the results of the agarose gel electrophoresis. Mutations in the 3 genes were determined by aligning the resulting sequences of each gene with the wild-type sequences of MTB H37Rv control strain using Bio-edit software version 7.03. After the alignment, single nucleotide polymorphisms were highlighted and used to determine the type of gene mutations prevalent in the study population. The identified mutations were then compared with those identified in other countries using the TBDReaMDB databases (Sandgren et al., 2009).

2.9 Ethical considerations

Permission was sought from the University Teaching Hospitals Tuberculosis Laboratory through the Head of Pathology and Microbiology Department's office to use the archived samples for this study. Ethical clearance for the study was obtained from the University of Zambia Biomedical Research Committee (UNZABREC) I.R.B No. 00001131, F.W.A. No. 00000338 under approval reference number 010-08-16. Authorization to conduct research was granted by the National Health Research Authority of Zambia. Since this was a laboratory-based study in which only pathogenic bacteria were handled there was no direct contact with participants. Laboratory serial numbers and town of residence for the study participants were only used to identify the sources of the pathogen isolates. No personal identification details of any patient who provided the sample was collected in this study.

3 Results

3.1 All isolates included in this study belonged to the *Mycobacterium tuberculosis* group

The geographical distribution of the MTBC species varies widely among continents of the world. *M. tuberculosis* is the most prevalent human TB pathogen in Europe and America, whereas *M. africanum* is widely distributed among African patients (Brosch et al., 2002). Although the MTBC species are highly similar to each other at DNA level, MTBC members differ widely in terms of host tropism, phenotype and pathogenicity (Brandau and Bohle, 2001). Differentiation of MTBC is thus important not only for epidemiological purposes and adequate treatment of each patient (Heep et al., 2001) but also because design of molecular drug susceptibility tests is dependent on the type and frequency of mutations occurring in a given population (Morgan et al., 2005). Additionally, and their performance varies by geographic location, depending on the prevalent Isolates of MTB, the type and frequency of drug resistance-conferring mutations in the population (Zhang et al., 2009; Ramaswamy et al., 2004). To determine the *Mycobacterium* genus or genera that were prevalent in the three provinces from which the sputum samples were collected, three genetic loci that have been used in previous studies to differentiate the various species within the *Mycobacterium* genus were targeted for PCR amplification using three primer pairs as follows: Rv0577F and Rv0577R targeting the *Cfp32* locus, Rv2073cF and Rv2073cR for RD9 and, Rv3120F and Rv3120R targeting the RD12 locus (Morgan et al., 2005).

Table 2: Primers for amplification and sequencing of RIF and INH drug resistance associated genes.

Species	***cfp32*, 786bp**	**RD9, 600bp**	**RD12, 404bp**
M. tuberculosis	Band Present	Band Present	Band Present
M. africanum or other MTBC[1]	Band Present	Band Absent	Band Present
M. bovis or *M. caprae*	Band Present	Band Absent	Band Absent
other bacteria	Band Absent	Band Absent	Band Absent

Three fragments were observed in the multiplex PCR amplification of all the 40 isolates and were estimated to be 786bp, 600bp and 404bp long as shown in Figure 1 for seven randomly selected isolates 2, 7, 8,15,16,18 and 21. While the 786bp fragment indicative of the *cfp32* region is present in all *Mycobacteria* genera, the RD9 is only present in *M. tuberculosis* but absent from *M. africanum, M. bovis, M. caprae* and other MTBC. RD12 on the other hand is present in *M. tuberculosis* and *M. africanum* but absent in *M. bovis* and *M caprae*. Based on the results in Figure 1 and those presented in Table 2 from previous studies, it was inferred that all of the isolates that were included in this study were *Mycobacterium tuberculosis* species only and not any other species that have been identified to belong to the complex.

[1]MTBC other than MTB, *M. caprae* or *M. bovis*

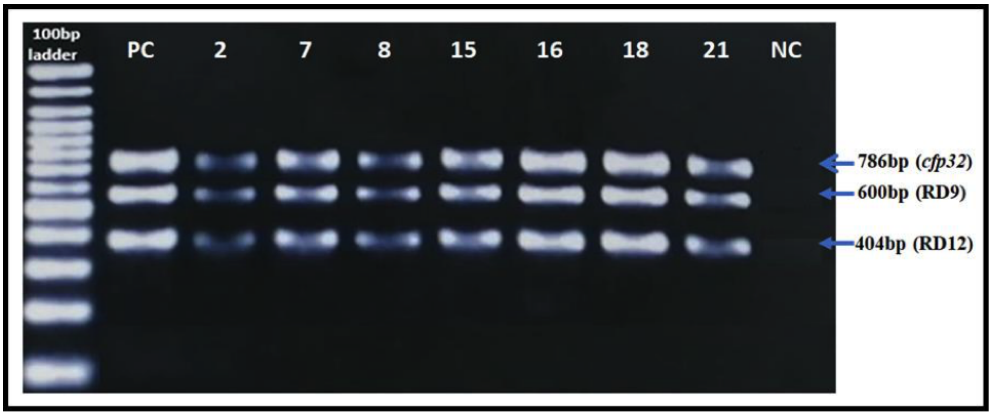

Figure 1: Agarose gel electrophoresis analysis of PCR amplification of regions of differentiation of seven randomly selected bacterial genomic DNA extracted from the MTBC isolates L=100bp molecular weight marker; Lane labeled positive control using genomic DNA from *Mycobacterium tuberculosis* strain H37RV; Lane labeled NC is negative control in which all reagents were added in the PCR except template DNA which was replaced with molecular grade nuclease-free water;All the forty isolates included in this study showed similar band migration profile.The experiment was repeated three times with similar results being observed.

3.2 All the three loci associated with *M. tuberculosis* resistance to INH and RIF were intact in all the 40 isolates

After having established that the isolates included in the study were all *Mycobacterium tuberculosis* isolates, next was to determine if any of the two genes and one regulatory sequence associated with resistance to first-line drugs, namely *katG, rpoB* and *inhA* were intact and not deleted by mutation events. The *katG* gene encodes catalase-peroxidase and point mutations in this gene results in altered enzyme structure which consequently decreases conversion of INH to a biologically active form (Bayraktar et al., 2011). Point mutations in the *katG* have been demonstrated to cause resistance in M. tuberculosis to INH. The *rpoB* codes for an RNA polymerases and point mutations in the locus have been associated with resistance to RIF. The targeted sequences within the three loci were amplified in three separate PCRs using three primer pairs presented in Table 1.

The results of the PCR demonstrated that all the amplified genes were intact and had fragment sizes of 278bp (*rpoB*), 231 bp (*inhA*) and 392 bp (*katG*). As shown in Fig 2, all isolates were positive for katG locus giving a fragment in each case of 392 bp, (Fig 2A), rpoB 278 bp (Fig 2B) and inhA231 bp (Fig 2C).

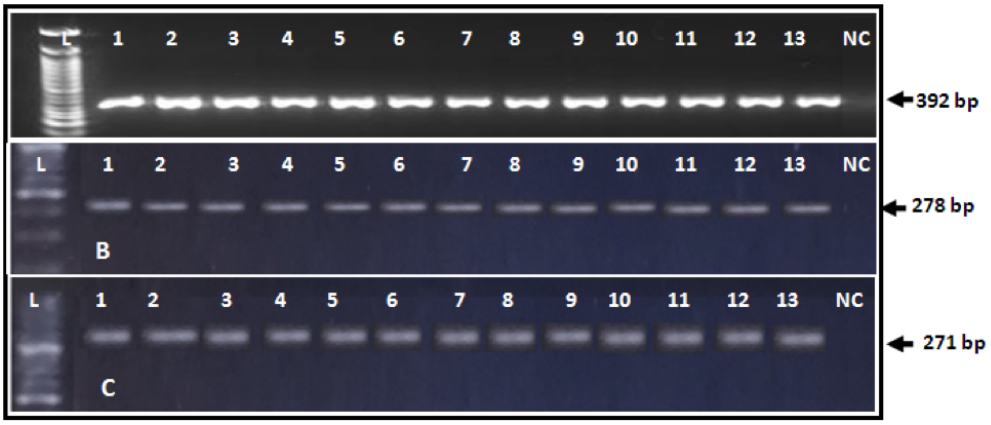

Figure 2: Gel electrophoresis analysis of *katG* gene (A), *rpoB* (B) and *inhA* (C) loci. PCR products were analyzed on 1% agarose and visualized with 0.01% ethidium bromide. Primers were designed to produce a 392 bp fragment (for *katG*), 278 bp (for *rpoB*), and 271 bp (for *inhA*); NC, negative control. Only thirteen of the 40 reaction products are shown and no other bands were observed in all the three individual PC reactions. Experiments were repeated three times with similar results being observed.

3.3 Isolates showed unique as well as common mutations in the loci that confer resistance to RIF and INH

Having established that all *M. tuberculosis* isolates included in this study had the three loci that have been shown to confer resistance to INH and RIF as a result of mutations, we set out to determine the nature of substitution mutations that might be prevalent in these loci. Haas et al. (1997) and Ramaswamy et al. (2004) established that a mutation at S315T was very prevalent in isolates from Lithuania and S450L was prevalent in the *rpoB* gene from isolates from Mexico. To determine the variation among pathogen isolates with reference to gene sequences associated with drug resistance, the 40 clinical isolates of MTBC from sputum resistant to INH and/or RIF were analysed by sequencing of the loci amplified by PCR. DNA was isolated from the agarose gel and sequenced using ABI3500 gene analyzer and the results were analyzed by MEGA7.3 and Bio-Edit version 7.03.

DNA sequences were aligned by MUSCLE in MEGA 7 software and sequence variations analysed. Several significant mutations that have been hypothesized to be directly linked to drug resistance were discovered in the sequence alignment results. Majority of the isolates [75% (30/40)] had *katG* mutations at codon 315, 7.5% (3/40) had the *katG* mutation alone while 2.5% (1/40) had mutations in the *inhA* promoter and *rpoB* in addition to the *katG* 315 mutation. *KatG* S315T mutation and the -10CT *inhA* mutation accounted for only 2.6% (1/39) of the INH-resistant isolates. Thirty-nine out of the forty isolates analysed in the *rpoB* locus had various types of substitution mutations with the commonest substitution mutation being S450L (48.7%), followed by H445T (20.5%) as demonstrated in table 3. In one phenotypically-RIF resistant isolate, no substitution or deletion mutations in the *rpoB* gene were observed. In the current study, mutations not previously documented were observed at codons 535 were CAA coding for glutamine was changed to GAA coding for glutamic acid (Q535E) and 545 were CTG coding for leucine was changed to CCG coding for proline (L545P). One out of 39 (2.6%) RIF resistant isolates demonstrated resistance to rifampicin alone.

Table 3: Frequency of mutations in *rpoB* gene region associated with RIF resistance detected by sequence alignment.

Location of Mutation	Nucleotide changes	Amino acid changes	No. (%) of Isolates N=39
Codon 450	TCG-TTG	Ser - Leu (S450L)	19 (48.7)
Codon 445	CAC-TAC	His - Tyr (H445Y)	8 (20.5)
Codon 516	GAC-GTC	Asp -Val (D516V)	5 (12.8)
Codon 545	CTG-CCG	Leu - Pro (L545P)	2 (5.1)
Codon 535	CAA-GAA	Gln - Glu (Q535E)	2 (5.1)
Codon 524	TTC-CTC	Phe - Leu (F524L)	1 (2.5)

The most frequent *rpoB* mutation was a transition mutation occurring at codon 450 in which serine was substituted for leucine (S450L). The second most frequent mutation occurred at codon 445, also a transition mutation in which histidine was substituted for tyrosine (H445Y) as demonstrated in Figure 3.

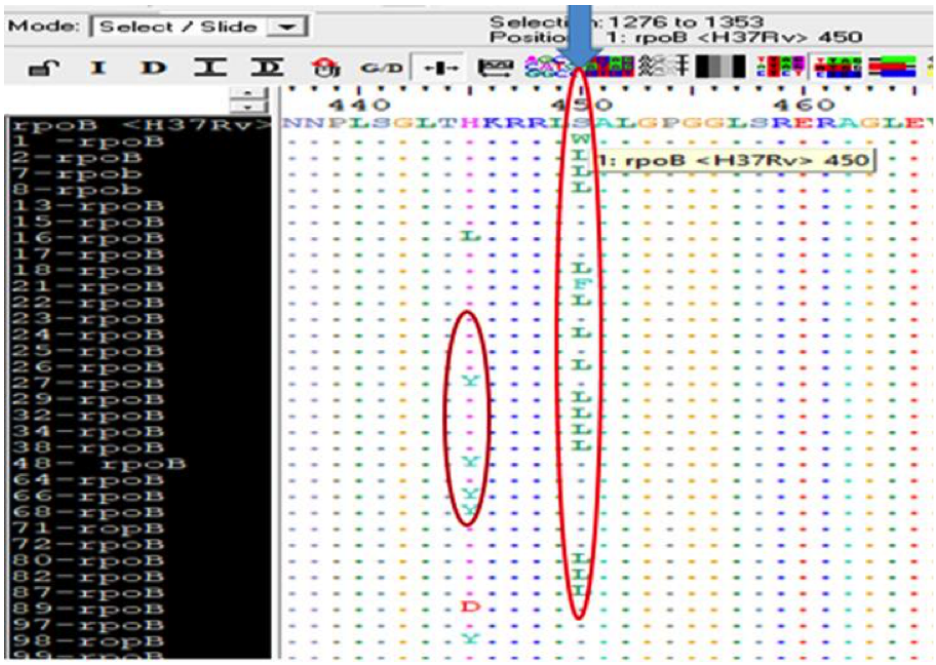

Figure 3: Alignment of *rpoB* Sequence from selected isolates with the H37RV control strain showing the most observed mutations. The dots (.) represented positions at which all amino acids were similar while amino acid changes are shown by standard amino acid symbols.

The most frequent *katG* mutation was a transversion mutation at codon 315 in which serine was substituted for threonine (S315T), Figure 4.

Figure 4: Alignment of the *KatG* sequences from selected isolates showing the most frequent mutation.

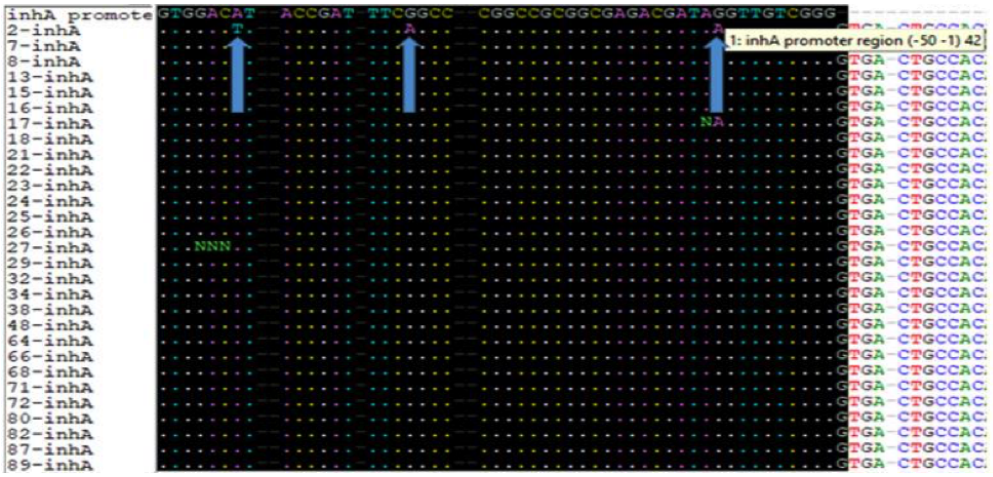

Figure 5: Alignment of the *inhA* promoter sequences from selected isolates showing the most frequent mutation.

4 Discussion

The current study is the first to document the prevalent MTBC species, type and frequency of mutations associated with RIF and INH resistance at the University Teaching Hospitals (UTH) Tuberculosis (TB) Laboratory. Our results will augment those of recent studies on molecular TB drug resistance conducted in Zambia which focused on mutations associated with resistance in fluoroquinolone such as PZ (Bwalya et al., 2018; Mitarai et al., 2005). Results from these earlier studies concluded that screening for PZ resistance in patients with MDR-TB is necessary as approximately half of MDR-TB patients also have PZ resistance.

Our results indicate that *M. tuberculosis* and no other MTBC species is the only prevalent species in Lusaka, Western and Eastern provinces of Zambia. These findings agree with those of a similar study conducted in Turkey

which demonstrated that the most prevalent MTBC species among clinical isolates was *M. tuberculosis* (94.1%) followed by *M. bovis* (mostly causing disease in cattle) which accounted for 4.3% of the isolates (Aslan et al., 2008). Our findings also demonstrate that mutations at position 315 of the *katG* gene are associated with INH resistance in 75% of the *Mycobacterium tuberculosis* species circulating in Lusaka, Western and Eastern Provinces of Zambia. The high percentage of mutations at position 315 of the *katG* gene (75%, $n = 40$) demonstrates the importance of this codon in the development of resistance to INH in *Mycobacteria tuberculosis* complex (MTBC). The most common mutation was the S315T. This mutation leads to poor binding of the drug to the enzyme catalase peroxidase which then limits drug activation and brings about INH resistance (Yu et al., 2009). These results on one hand agree with results of a similar study conducted in Lithuania that documented mutation rates at codon 315 of the *KatG* genes of 83.9% (Bakonyte et al., 2003). On the other hand, the findings from this study differ from those that were obtained from a related study in South Africa that documented a 60% mutation rate at codon 315 as well as a 60 percent mutation rate documented in southern Turkey, 65.4% in Australia, 66.2% in Poland and 41.3% in Spain (Haas et al., 1997; Aslan et al., 2008). All the mutations in the *katG* gene were characterised as single nucleotide changes. Differences in the prevalence of mutations in different countries could be explained by geographically based prevalence (Barnard et al., 2008) and mutation frequency differences associated with different sub lineages of *M. tuberculosis* within the East Asian lineage (Iwamoto et al., 2008). Another explanation is that there may be a higher selective pressure for *katG315* mutations in MDR isolates (Caws et al., 2006).

With regards *rpoB* mutations, our findings demonstrate that the most prevalent *rpoB* mutations in Zambian isolates were located at positions 450 (S450L) and 445 (H445T), accounting for 48.7% and 20.5% ($n = 39$) of rifampicin resistance in the MTB isolates respectively. These results compare with findings of Ramaswamy et al. (2004) who documented S450L and H445T as the most common mutations but do not compare with those from Aslan et al. (2008) who reported *rpoB* gene mutations at codon 531 as the most common mutations (41 - 60%) responsible for rifampicin resistance in most *Mycobacterium tuberculosis* species (Morgan et al., 2005; Ling et al., 2008). The next most frequent mutations affecting codons in this gene were at codon 516 (12.8%) which also compare with observations in the study by Aslan et al. (2008) which reported 7.3% to 16% mutation rate at codon 516. This study also detected mutations at codon 524 and mutations not previously reported were found at codons 535 and 545. This result demonstrates the varying geographical distribution of the *rpoB* mutations in distinct regions of different countries (Espinal et al., 2000; Ling et al., 2008). In one phenotypically RIF resistant isolate (2.6%) no nucleotide changes were detected in the *rpoB* gene. Resistance could therefore, be due to mutations occurring in the gene region not screened in study, probably *kasA* and *ahpC*. In the current study, one out of all 40 isolates analysed for RIF and INH resistance had a nucleotide change at position -10 in *InhA* regulator region, as well as at position -33 and -44 accounting for 5.1% resistance rate. This differs from results of several studies that have demonstrated that *inhA* regulator region mutations appeared with low frequency ranging between 10-25.8% of INH resistant isolates (Ramaswamy and Musser, 1998).

This study detected rifampicin mono resistance (RMR) in 2.6% ($n = 39$) of all the rifampicin resistant *Mycobacterium tuberculosis* isolates analysed. On one hand, these findings do not compare with the 4.8% RMR documented by Kapata et al. (2013). The discrepancy could be due to the difference in the time period during which the cases were analysed. On the other hand, the findings of this study compare with those that documented RMR frequency of 1.3% among new cases, and 3.2% among previously treated cases and those of other studies which have documented that resistance against RIF usually occurs together with other drugs, especially isoniazid, and for this reason rifampicin resistance is considered a surrogate marker for MDR TB (Barnard et al., 2008; Caws et al., 2006).

5 Conclusion

This study presents important findings on the type of species as well as frequency and different kinds of mutations occurring at various target loci concerned with rifampicin and isoniazid resistance in clinical isolates

resistant to RIF and INH in Lusaka, Western and Eastern Provinces of Zambia. The results of this study suggest that mutations differ depending on region and therefore determination of frequency of mutations associated with drug resistance in isolates from various regions is important for improving rapid and specific molecular diagnostic techniques. The most common combinations of mutations responsible for MDR TB in Lusaka, Western and Eastern provinces of Zambia are *katG* S315T and *rpoB* S450L. Molecular diagnostic tests based on detecting these predominant mutations could, therefore, be useful for the rapid detection of multi drug resistant tuberculosis in Zambia.

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