



Molecular Detection of *Mycobacterium tuberculosis* Rifampicin Drug Resistance Using rpoB Gene Sequencing in Zimbabwe

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Authors' contributions

This work was carried out in collaboration between all authors. Author NC designed the study. Author EM isolated the bacteria and performed DNA extractions. Authors NC and WM performed RpoB gene amplification and the bioinformatics analysis of the results. Author NC wrote the protocol and the first draft of the manuscript. All authors contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To detect Mtb rifampicin drug resistance mutations in Zimbabwe by rpoB gene sequencing.

Study Design: This was a retrospective study.

Place of Study: The study was conducted in Zimbabwe in 2015 using archived Mtb isolates previously isolated throughout Zimbabwe during a national TB Survey.

Methodology: Archived Mtb isolates at the National Microbiology Reference Laboratory were retrieved. Genomic DNA from the isolates was extracted by the boiling method. The 81 bp region of rpoB gene that contains rifampicin drug resistance mutations was amplified by polymerase chain reaction (PCR). The PCR products were sequenced and sequences analyzed to determine mutations in the rpoB protein.

Results: RpoB mutations in 30 Mtb isolates were analyzed. Out of the 30 Mtb isolates, 19 (63.3%)

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had at least one codon mutation in the *rpoB* gene that resulted in a change of amino acid. The commonest mutation was on codons 531 (S>L), with a prevalence of 20%. Other mutations were detected at codons 511 (L>P) (3.3%), 516 (D>Y) (10.0%), 516 (D>V) (3.3%), 518 (N>H) (3.3%), 526 (H>D) (10.0%), 526 (H>L) (3.3%), 526 (H>C) (3.3%), 526 (H>Y) (3.3%) and 533 (L>P) (6.6%). The other 11 *Mtb* isolates (36.7%) did not have any mutations in the gene coding for drug resistance.

Conclusion: Genetic mutations that code for rifampicin drug resistance are prevalent in *Mtb* isolates from Zimbabwe. Further studies need to be instituted to ascertain the scope and threat of the *Mtb* drug resistance.

Keywords: *Mycobacterium tuberculosis*; rifampicin; drug resistance; mutations.

1. INTRODUCTION

Infection with *Mycobacterium tuberculosis* (*Mtb*), the pathogen that causes tuberculosis (TB) remains a global health problem. According to the latest global TB report, infection with *Mtb* killed about 1.5 million people in 2014 and these included 890 000 men, 480 000 women and 140 000 children [1]. The emergence and rise of multi-drug resistance TB (MDR-TB), especially in Sub-Saharan Africa, has worsened the public health threat of TB. It has been estimated that 123 000 patients with MDR -TB or rifampicin-resistant tuberculosis (RR-TB) were notified globally in 2014 and about three quarters of these lived in South Africa, China, India or European region [1]. The challenge of fighting MDR-TB in most countries remains daunting. Many countries in Africa still lack surveillance systems to detect and monitor MDR-TB. This makes the scope of the problem poorly known in most countries as no national surveys are ever made to gather data on MDR-TB. Rifampicin is one of the main drugs used for the treatment of *Mtb* infection [2]. The drug's mode of action is by binding to the β -subunit of the RNA polymerase of the bacterium, thereby inhibiting the elongation of the messenger RNA [3]. Resistance to this drug by *Mtb* may be due to mutations within the 81-base pair region of the *rpoB* gene (codon 507-533) [4]. Thus, it is critical to employ rapid and reliable methods such as DNA sequencing for the detection of drug-resistant *RpoB* genotypes of *Mtb*. The aim of this study was to detect *Mtb* rifampicin drug resistance mutations in Zimbabwe by sequencing the 81 bp region of *rpoB* gene.

2. MATERIALS AND METHODS

2.1 Archived *Mtb* Samples

Archived *Mtb* isolates used in this study were collected from the National Microbiology

Reference Laboratory in Harare. The samples were isolated during the national TB survey that was carried out by the Ministry of Health and Child Care in 2014. They were collected throughout Zimbabwe and were isolated from human sputum in people with symptoms consistent with TB disease. The sputum samples collected from each participant were processed for TB culture and inoculated onto solid media, Lowensen Jensen (LJ) media (Becton Dickson Microbiology Systems, USA) and liquid media, Mycobacterial Growth Indicator Tube (MGIT) (Becton Dickson Microbiology Systems, USA). LJ culture tubes were incubated at 37°C while MGIT tubes were incubated in the BACTEC MGIT 960 machine and growth monitored. Once growth was observed on LJ and the BACTEC MGIT 960 Flagged positive, samples were stained with Ziehl Neelsen stain. If acid fast bacilli were present, the isolates were tested for presence of *Mtb* bacteria using a rapid identification kit for *Mtb*, the MPT64 Ag kit (Standard Diagnostics, Korea) according to manufacturer's recommendations. Positive results indicated presence of *Mtb*.

2.2 Extraction of DNA from *Mtb* Isolates

Genomic DNA was extracted from 1ml of the bacteria grown in liquid media as previously described [5]. Briefly, the bacterial culture was centrifuged for 15 min at 10 000 g, supernatant discarded and 100 μ l sample lysis buffer (Hain Lifescience, Germany) added to the bacterial pellet. The pellet was re-suspended and incubated at 95°C in a heat-block for 5 minutes. A sample neutralization buffer (Hain Life science, Germany) (100 μ l) was added and the mixture briefly vortexed. The extract was centrifuged for 5 mins at 10 000 g and supernatant (DNA extract) transferred into new tube. The DNA extract was stored at -20°C until polymerase chain reaction of the *rpoB* gene.

2.3 Amplification of *rpoB* Gene and DNA Sequencing

The *rpoB* gene of the bacterial isolates was amplified using polymerase chain reaction. The Mycobacterium-specific forward primer, 5'- GCT GAT CCA RAA CCA GAT CCG -3', and reverse primer, 5'- CTC GAT GAA CCC GAA CGG GT - 3' were used for polymerase chain reaction amplification of the gene. Each polymerase chain reaction contained 31.8 ul distilled water, 0.2 ul Taq polymerase buffer (5U/ul stock), 3 ul MgCl (25 mM stock), 1 ul dNTPs (10 mM stock), 2 ul *rpoB* forward primer (10 uM stock), 2 ul *rpoB* reverse primer (10 uM stock), 5 ul 10X PCR Buffer and 5 ul of template DNA. The following cycling program was used for the amplification: initial denaturation of 5 minutes at 95°C followed by 40 amplification cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, and ending with a final extension step of 10 minutes at 72°C. PCR products (10 ul) were analyzed using 2.5% agarose gel electrophoresis. Samples that showed good amplification were sent for sequencing at Inqaba Biotechnical Industries, South Africa.

2.4 Analysis of the 81 bp *rpoB* DNA Sequences

DNA sequences were analyzed using Geneious Basic program (Biomatters, USA) [6]. The 81bp region of all samples was extracted from the sequences as described in the Geneious user manual. All the sequences were aligned and translated to show mutations in the *rpoB* protein as described in the Geneious user manual. Counting of *rpoB* mutations and calculating their frequencies was done manually. So no special statistical package for data analysis was used.

3. RESULTS

3.1 Mtb Isolates and *rpoB* Gene Amplification

A total of 30 Mtb isolates were retrieved at the National Microbiology Reference Laboratory and analyzed in this study. They were originally collected from different parts of Zimbabwe during the national TB survey of 2014 that was done by the Ministry of Health and Child Care. DNA was successfully extracted from the culture samples. PCR of the *rpoB* was also successful for all the 30 samples. Analysis of PCR products showed the presence of the expected *rpoB* band of about 392 bp (Fig. 1).

3.2 Rifampicin Resistance Mutations in Mtb

Analysis of the 30 sequences of the 81 bp *rpoB* gene showed that mutations coding for rifampicin were absent in 11 samples (36.7%) (Fig. 2). The amino acid sequence of the *rpoB* gene was the same as that of the wild-type Mtb (Fig. 2). Nineteen samples (63.3%) out of the 30 had at least one codon mutation in the *rpoB* gene that encoded for the drug resistance (Fig. 2). The predominant mutations were on codons 516 (D>Y and D>V), 526 (H>D, H>L, H>C and H>Y) and 531 (S>L). The most prevalent mutation was found on codon 531 (Fig. 2, Table 1). The codons with several mutations were 516 and 526 (Fig. 2, Table 1). Mutations in other codons (511, 518 and 533) were also detected, but their prevalence was very low (Fig. 2, Table 1). Missense mutations were not detected in this study.

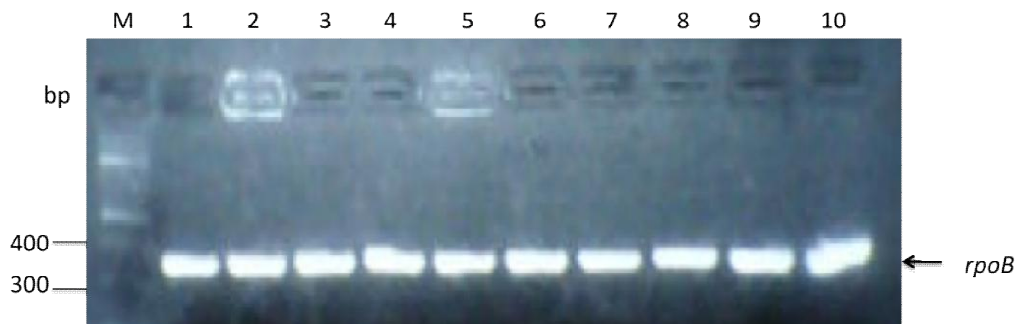


Fig. 1. Representative gel showing the *rpoB* amplicons

Lane M had the DNA marker. Lanes 1-10 showed *rpoB* amplicons from 10 samples. The expected *rpoB* amplicon was 392 bp.

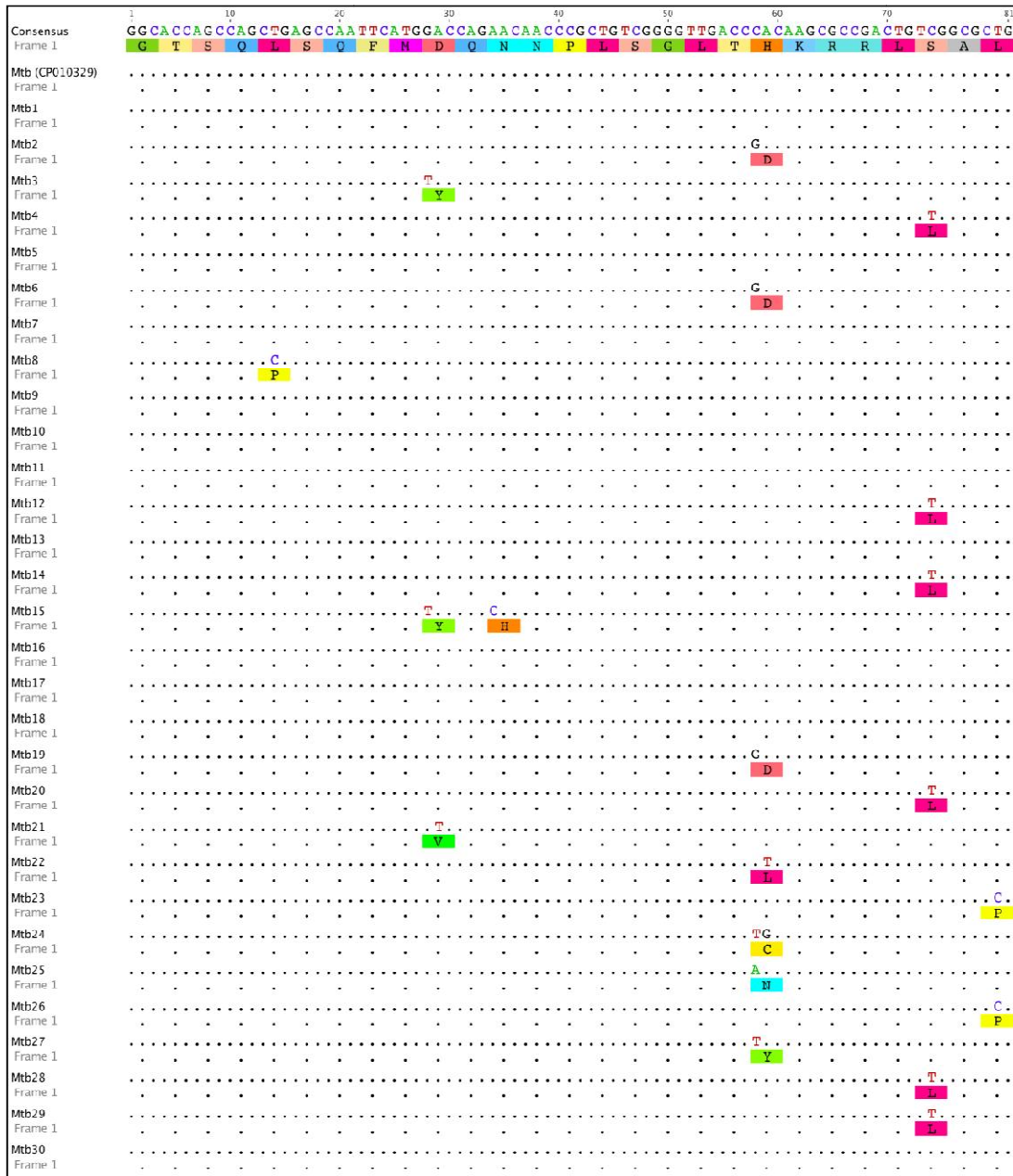


Fig. 2. Nucleotide and protein analysis of the 81 bp *rpoB* mutations in 30 Mtb samples
 Sequence of wild-type Mtb (Accession number CP010329) was used as a control

4. DISCUSSION

The rise in drug resistance to most human pathogens is becoming a global health challenge [7]. Pathogens such as Mtb are becoming more resistant to several drugs and this is becoming a threat to most parts of the world especially in Sub-Saharan Africa. The lack of Mtb drug resistance surveillance has worsened the threat.

In this study, we evaluated the prevalence of rifampicin drug resistance mutations in few Mtb isolates from Zimbabwe by sequencing the 81 bp drug-determining region of *rpoB* gene. The isolates we used had been collected throughout Zimbabwe during the National TB survey. Although the sample size (n = 30) was small, it was representative of the whole country. Rifampicin is a key drug for the treatment of

Table 1. Summary of the frequency of rpoB gene mutations in the 30 Mtb isolates

| Mutated codon no. | Normal codon (amino acid) | Mutated codon (amino acid) | Change of amino acid | Frequency of mutation (n=30) |
|-------------------|---------------------------|----------------------------|----------------------|------------------------------|
| 511 | CTG (L) | CCG (P) | YES | 1 (3.3%) |
| 516 | GAC (D) | TAC (Y) | YES | 3 (10.0%) |
| 516 | GAC (D) | GTC (V) | YES | 1 (3.3%) |
| 518 | AAC (N) | CAC (H) | YES | 1 (3.3%) |
| 526 | CAC (H) | GAC (D) | YES | 3 (10%) |
| 526 | CAC (H) | CTC (L) | YES | 1 (3.3%) |
| 526 | CAC (H) | TGC (C) | YES | 1 (3.3%) |
| 526 | CAC (H) | TAC (Y) | YES | 1 (3.3%) |
| 531 | TCG (S) | TTG (L) | YES | 6 (20.0%) |
| 533 | CTG (L) | CCG (P) | YES | 2 (6.6%) |

tuberculosis in Zimbabwe and other African countries. Therefore, genetic resistance to this drug by Mtb can result in very poor disease prognosis. In this study, a number of mutations in *rpoB* gene that are known to code for rifampicin drug resistance were detected (Fig. 2, Table 1). It was interesting to note that 63.3% of the samples had at least one mutation in the gene. This was relatively high prevalence of the mutations. Previous studies in Zimbabwe using GeneXpert showed that 30.8% of the isolates were rifampicin-resistant [8]. In a Nigerian study using GeneXpert, 22.2% of the Mtb isolates were rifampicin-resistant [9]. The most prevalent mutation in this study was also on codon 531. In a previous study in Harare using GeneXpert, the codon 531 mutation was also found to be the most frequent mutation [8]. The point mutation at codon 531) has also been found to be dominant in other countries. A study in Uganda showed that most of the Mtb isolates (10%) had this mutation and the same trends were found in Iran, Honduras and Belarus [10]. Other minor point mutations found in this study were also previously detected in Zimbabwe [8]. In most studies, mutations on codons 516, 526 and 531 have been found to be most commonly associated mutations with the majority of rifampicin resistance [8-12]. More studies are necessary to determine whether the prevalence of the rifampicin drug resistance mutations in Zimbabwe is on the rise. National surveillance on drug resistance in Mtb should also be instituted. Without a surveillance programme, it is difficult to monitor and control the threat of multi-drug resistance TB in Zimbabwe.

5. CONCLUSION

Genetic mutations that code for rifampicin drug resistance are prevalent in Mtb isolates from Zimbabwe. Further studies need to be done to

ascertain the true scope and threat of the Mtb drug resistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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