

Original Research Article**Molecular Detection of *Mycobacterium Tuberculosis* Rifampicin Drug Resistance Using *rpoB* Gene Sequencing in Zimbabwe****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%) 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 detected were on codons 511, 516, 518, 526 and 533. 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]. Genetic resistance to this drug can arise due to mutations within an 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**

33 Archived Mtb isolates used in this study were collected from the National Microbiology Reference
34 Laboratory in Harare. The samples were isolated during the national TB survey that was carried out by
35 the Ministry of Health and Child Care in 2014. They were collected throughout Zimbabwe and were
36 isolated from human sputum in people with symptoms consistent with TB disease. The sputum samples
37 collected from each participant were processed for TB culture and inoculated onto solid media, Lowensen
38 Jensen (LJ) media (Becton Dickson Microbiology Systems, USA) and liquid media, Mycobacterial Growth
39 Indicator Tube (MGIT) (Becton Dickson Microbiology Systems, USA). LJ culture tubes were incubated at
40 37°C while MGIT tubes were incubated in the BACTEC MGIT 960 machine and growth monitored. Once
41 growth was observed on LJ and the BACTEC MGIT 960 Flagged positive, samples were stained with
42 Ziehl Neelsen stain. If acid fast bacilli were present, the isolates were tested for presence of Mtb bacteria
43 using a rapid identification kit for Mtb, the MPT64 Ag kit (Standard Diagnostics, Korea) according to
44 manufacturer's recommendations. Positive results indicated presence of Mtb. (Sample analysis not
referenced)

45 46 **2.2 Extraction of DNA from Mtb isolates**

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48 Genomic DNA was extracted from 1ml of the bacteria grown in liquid media. The bacterial culture was
49 centrifuged for 15 min at 10 000g, supernatant discarded and 100 ul sample lysis buffer (Hain
50 Lifescience, Germany) added to the bacterial pellet. The pellet was re-suspended and incubated at 95°C
51 in a heat-block for 5 minutes. A sample neutralization buffer (Hain Lifescience, Germany) (100 ul) was
52 added and the mixture briefly vortexed. The extract was centrifuged for 5 mins at 10 000g and
53 supernatant (DNA extract) transferred into new tube. The DNA extract was stored at -20°C until
54 polymerase chain reaction of the *rpoB* gene. (Method of extraction not referenced)

55 56 **2.3 Amplification of *rpoB* gene and DNA sequencing**

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58 The *rpoB* gene of the bacterial isolates was amplified using polymerase chain reaction. The
59 Mycobacterium-specific forward primer, 5'- GCT GAT CCA RAA CCA GAT CCG -3', and reverse primer,
60 5'- CTC GAT GAA CCC GAA CGG GT -3' were used for polymerase chain reaction amplification of the
61 gene. Each polymerase chain reaction contained 31.8 ul distilled water, 0.2 ul Taq polymerase buffer
62 (5U/ul stock), 3 ul MgCl (25 mM stock), 1 ul dNTPs (10 mM stock), 2 ul *rpoB* forward primer (10 uM
63 stock), 2 ul *rpoB* reverse primer (10 uM stock), 5 ul 10X PCR Buffer and 5 ul of template DNA. The
64 following cycling program was used for the amplification: initial denaturation of 5 minutes at 95°C followed
65 by 40 amplification cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, and
66 ending with a final extension step of 10 minutes at 72°C. PCR products (10 ul) were analyzed using 2.5%
67 agarose gel electrophoresis. Samples that showed good amplification were sent for sequencing at Inqaba
68 Biotechnical Industries, South Africa.

69 70 **2.4 Analysis of the 81 bp *rpoB* DNA sequences**

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72 DNA sequences were analyzed using Geneious Basic program (Biomatters, USA). The 81bp region of all
73 samples was extracted from the sequences. All the sequences were aligned and translated to show
74 mutations in the *rpoB* protein. (Method of extraction not referenced). Statistical package for data analysis
was not mentioned.

75 76 **3. RESULTS**

77 78 **3.1 Mtb isolates and *rpoB* gene amplification**

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

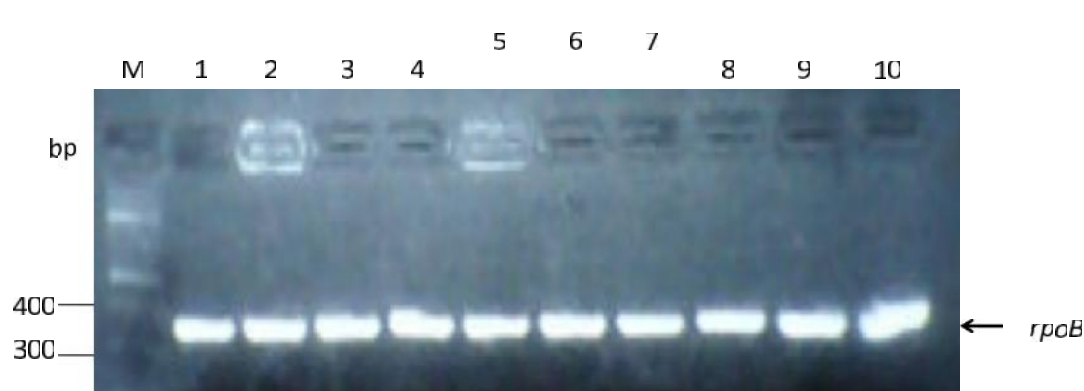


Figure. 1. Representative gel showing the *rpoB* amplicons. Lane M had the DNA marker. Lanes 1-10 showed *rpoB* amplicons from 10 samples. The expected *rpoB* amplicon was 392 bp.

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%) (Figure 2). The amino acid sequence of the *rpoB* gene was the same as that of the wild-type *Mtb* (Figure 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 (Figure 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 (Figure 2, Table 1). The codons with several mutations were 516 and 526 (Figure 2, Table 1). Mutations in other codons (511, 518 and 533) were also detected, but their prevalence was very low (Figure 2, Table 1). Missense mutations were not detected in this study.

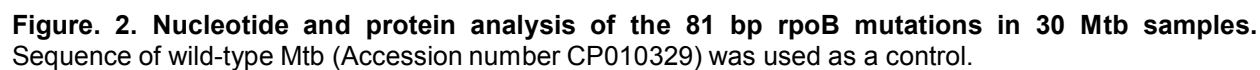


Table 1: Summary of the frequency of *rpoB* gene mutations in the 30 Mtb isolates

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%)

4. DISCUSSION

The rise in drug resistance to most human pathogens is becoming a global health challenge [5]. 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 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 (Figure 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 [6]. In a Nigerian study using GeneXpert, 22.2% of the Mtb isolates were rifampicin-resistant [7]. 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 [6]. 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 [8]. Other minor point mutations found in this study were also previously detected in Zimbabwe [6]. 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 [6-10]. Overall, in our study, it seems that the prevalence of the rifampicin drug resistance mutations in Zimbabwe is on the rise. Thus, national surveillance of drug resistance in Mtb should 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.

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