

The occurrence and frequency of genomic mutations that mediate Isoniazid and Rifampicin resistance in *Mycobacterium tuberculosis* isolates from untreated pulmonary Tuberculosis cases in urban Blantyre, Malawi

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Abstract

Background

The emergence and spread of drug-resistant Tuberculosis (TB) is a major public health threat. TB resistance originates in the course of treatment due to genomic mutations in *Mycobacterium tuberculosis* (MTB). An increase in new cases with drug-resistant TB could be an indicator of high levels of circulating resistant strains. This study was conducted to determine the occurrence and frequency of genomic mutations that mediate Isoniazid (INH) and Rifampicin (RIF) resistance among isolates from untreated TB cases in urban Blantyre, Malawi.

Methods

A cross-sectional retrospective study was conducted on a panel of 141 (n=141) MTB clinical isolates recovered between June 2010 and January 2012 from $\geq 2+$ Ziehl-Neelsen smear positive new pulmonary-TB patients with no history of treatment. Frozen isolates were revived using the BACTEC MGIT detection system. DNA was extracted using GenoLyse DNA extraction kit and detection of genomic mutations was carried out using the GenoType MTBDRplus Ver 2.0 assay.

Results

Out of the 141 isolates studied, 3 (2.1%) were found carrying mutations in the *katG* gene that confer resistance to Isoniazid (INH). No mutations were detected in the *inhA* promoter region gene that confer weak INH resistance or in the *rpoB* gene that confer Rifampicin resistance. All *katG* mutant genes had a S315T1 single point mutation, a genomic alteration that mediates high INH resistance.

Conclusion

The *katG* mutant gene conferring resistance to INH was the only genomic mutation observed among the isolates studied and the frequency of occurrence was low. Our findings suggest low levels of circulating drug-resistant MTB strains in urban Blantyre, Malawi.

Introduction

Tuberculosis (TB) is an important global infectious disease that kills more than 1.2 million people annually¹⁻³. This is largely attributed to HIV co-infection^{4,5} and most important, the emergence of drug-resistant TB⁶⁻⁸. Isoniazid (INH) and Rifampicin (RIF) forms the backbone of the first-line TB treatment⁹. TB that is resistant to both drugs (referred to as Multidrug-resistant TB, MDR-TB) is usually associated with poor prognosis and increased mortality^{6,8}. More than 600, 000 cases of MDR-TB are reported annually³. Genomic mutations in *Mycobacterium tuberculosis* (MTB) mediate resistance¹⁰⁻¹². More than 96% of RIF resistant MTB possess a genetic mutation in the *rpoB* gene at codon positions 531, 526 and 516 located within the 81-bp core region (Rifampicin Resistance Determining Region, RRDR) spanning codons 507-533¹³⁻¹⁷. The molecular basis of INH resistance involves mutations in more than one gene with the most mutations occurring in the *katG* gene on codon 315 (>70%) and within the promoter region of *inhA* gene (15-35%)^{10,18}. Mutations in the *katG* gene are relatively more common and usually the first to occur¹⁸⁻²⁰. It is estimated that more than 90% of RIF-resistant MTB isolates are also resistant to INH, making RIF resistance a surrogate marker for MDR-TB diagnosis^{10,21-24}.

Active surveillance and screening for drug-resistant TB, early isolation and management of confirmed cases are key steps in the fight against the spread of drug-resistant TB^{1,2}. TB remains a serious health problem in Malawi^{25,26}. However, low levels of drug-resistant MTB have been reported. A 2012 phenotypic culture-based large-scale survey in several districts of Malawi indicated MDR-TB levels of <0.4% among new TB cases and 4.8% among previously treated patients (J. Mpunga, personal communication, August, 2012). While phenotypic studies may still provide useful data, they are time-consuming and may be misinterpreted in cases where susceptible MTB and resistant MTB co-exist in the same patient (Hetero-resistance)²⁷⁻²⁹. There is currently no evidence of the existence of transferable genetic elements (such as plasmids) between strains of MTB^{10,18}. Thus, indicating that drug resistance in MTB is solely mediated by chromosomal mutations and that the presence of such mutations in MTB strains from treatment naïve TB patients would indicate a direct person-to-person transmission³⁰. In this study, we screened MTB strains isolated from treatment naïve pulmonary-TB (PTB) patients living in urban Blantyre, Malawi, to determine the occurrence and frequency of genomic mutations that confer resistance to

INH and RIF using the GenoTypeMTBDRplus Version 2.0. The GenoTypeMTBDRplus is a molecular assay designed to detect common genetic mutations associated with resistance to RIF and INH^{11,23,31,32}.

Materials And Methods

Study isolates

All MTB isolates (n = 159) used in this study were obtained from the frozen culture collections of Dr. Derek Sloan from the Malawi Liverpool Wellcome Trust. Isolates were recovered from treatment naïve patients with PTB, living in urban Blantyre Malawi, between June 2010 and January 2012 and were identified using Ziehl-Neelsen (Z-N) microscopy at Queen Elizabeth Central Hospital (Q.E.C.H) Laboratory in Blantyre, Malawi. Specimens with 2+ or 3+ Z-N result were cultured in Mycobacterial growth indicator tubes (MGIT) on a BACTEC MGIT automated Mycobacterial detection system (Becton Dickson, New Jersey, USA) and were stored in cryovials at -80°C in 30% glycerol. A fixed sample size of 159 MTB isolates was the biggest available.

Ethical Issues

Ethical approval for this study was obtained from the University of Malawi, College of Medicine Research Ethics Committee (COMREC). All MTB clinical isolates were numerically coded and delinked from patient identifiers and no patient information was accessible. Therefore, informed consent was not required for this study.

Reviving frozen Mycobacterium tuberculosis isolates

During this study, all frozen isolates were revived and analyzed at Malawi Liverpool Wellcome Trust/College of Medicine (MLWT/CoM) TB Research Laboratory at the College of Medicine, Blantyre, Malawi. All specimen handling and manipulations were performed in a Class II safety cabinet in a Bio-safety level-2 (BSL-2) laboratory. During revival process, isolates were thawed at room temperature for one hour and then a 0.5 ml aliquot of the sample was inoculated into MGIT, prepared for each isolate by adding 0.8 ml oleic acid-albumin-dextrose-catalase (OADC)-PANTA (Becton Dickinson, New Jersey, USA). The tubes were then incubated at 37°C for 6 - 7 days in an automated BACTEC MGIT960 (Becton Dickinson, New Jersey, USA) device with continuous monitoring and the time to positivity (TTP) was recorded. The isolates were further sub-cultured on a nonselective blood agar (BA) media (Trypticase soy agar enriched with 5% sheep blood) for 24 - 48 hours to rule out contamination and were later stained using the Ziehl Neelsen (Z-N) technique to confirm the presence of characteristic coding of alcohol acid fasting Bacilli (AAFB). Out of 159 MTB isolates, 18 (11%) were lost to contamination and a total of 141 isolates were successfully revived (Table 1).

DNA extraction

DNA extraction was done using GenoLyse DNA extraction Kit Ver 1.0 (Hain Lifesciences, Nehren, German) according to the manufacturer’s protocol. A total of 1000 µl of MGIT liquid bacterial culture was transferred into a 1.5 ml tube and centrifuged for 15 min at 10,000 Xg. The pellet was resuspended in 100 µl of Genolyse and incubated for 5 min at 95°C. Subsequently, 100 µl of neutralization buffer was added to the lysate which was vortexed, and centrifuged for 5 min at full speed. A total of 5 µl supernatant with extracted DNA was obtained.

Table 1: Revived *M. tuberculosis* isolates (n=159).

	Number of <i>M. tuberculosis</i> isolates	%
Number revived	159	
Pure growth on BA	141	77.47
Pure growth with positive Z-N staining	141	100
Lost to contamination	18	11.32

BA, Blood Agar; Z-N, Ziehl Neelsen

Table 2: Detected genomic mutations that confer INH and RIF resistance using (n=141) (see Fig.1 for Interpretation)

TB drugs	Gene	Number of isolates	GenoTypeMTBDRplus Strip Interpretation	Mutation detected/ Amino acid change	% mutation detected
INH	<i>katG</i>	3	∇ <i>katG</i> WT1 Δ <i>katG</i> MUT1 ∇ <i>katG</i> MUT2	S315T1genomic mutation detected/ AGC→ACC (Serine→Threonine)	2.1
		138	Δ <i>katG</i> WT1 ∇ in all <i>katG</i> MUT	No Mutation detected	
	<i>inhA</i>	141	Δ in all <i>inhA</i> WT ∇ in all <i>inhA</i> MUT	No Mutation detected	0
RIF	<i>rpoB</i>	141	Δ in all <i>rpoB</i> WT ∇ in all <i>rpoB</i> MUT	No Mutation detected	0

∇, absence of band; Δ, presence of band; MUT, mutant; WT, wild-type.

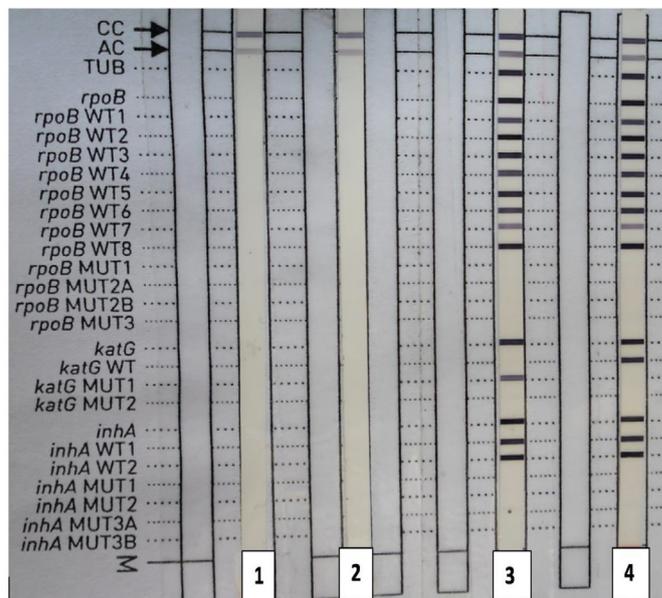


Figure 1: Shows developed GenoTypeMTBDRplus 2.0 strips (Hain Lifesciences, German) attached on an interpretation worksheet. Lane A: negative control containing amplification mix A and B. Lane G: Negative control containing Genolyse DNA extraction kit. Lane 10: *katG* (MUT 1) genomic mutation indicating a S315T1 point mutation. No mutations in both *rpoB* and *inhA* genes. Lane 9: Absence of genomic mutations in all the genes.

Molecular assay

The GenoTypeMTBDRplus Ver 2.0 (Hain Lifesciences, Nehren, Germany) was used to detect MTB drug-resistant

related chromosomal point-mutations within the *katG*, *inhA* promoter, and *rpoB* genes. The GenoTypeMTBDRplus employs a multiplex PCR during which *katG*, *inhA*, *rpoB* genes are amplified using biotin-labeled primers and the resulting amplicons are hybridized to DNA probes bound to a membrane. During PCR, a total amplification volume of 50 µl comprising of 10 µl amplification mix-A, 35 µl amplification mix-B and 5 µl template DNA was prepared according to the manufacturer's recommendations.³³ The amplification protocol was optimized and consisted of 15 min initial denaturation at 95°C, 10 cycles; 95°C for 30 sec and 65°C for 2 min, and then 20 cycles; 95°C for 25 sec, 50°C for 40 sec, and 70°C for 40 sec, and the final cycle at 70°C for 8 min.

Reverse hybridization of biotin-labeled amplicons to membrane-bound probes on the strip was performed manually at 45°C using a shaking water bath and this consisted of the addition of conjugate and substrate to detect visible band patterns as recommended by the manufacturer. After several washing steps, the membrane-strips were allowed to dry. The membrane-bound DNA included the following probes: one *katG* wildtype (WT) probe; two *katG* mutant (MUT) probes (S315T1 and S315T2); four *rpoB* MUT probes (D516V, H526Y, H526D, and S531L); two *inhA* WT probes and four *inhA* MUT probes (C15T, A16G, T8C, and T8A) (Figure 1). All results were interpreted according to the manufacturer's instructions. The absence of mutation was defined as hybridization (indicated by the presence of a colored band) to all the WT probes and no hybridization to the MUT probes. The presence of mutation was defined as the absence of hybridization on any one of the WT probe, with or without hybridization on the corresponding MUT probe (Figure 1).

Data analysis

Simple data summaries and descriptive statistics including proportions and percentages were used to present the occurrence and frequency of genomic mutations conferring resistance to rifampicin or isoniazid or to both drugs among the studied MTB isolates.

Results

A total of 141 MTB isolates recovered from treatment naïve pulmonary TB patients were screened to determine the occurrence and frequency of genomic mutations within the *katG*, *inhA* promoter region and *rpoB* genes. Out of the 141 MTB isolates studied, gene mutation was only confirmed for the *katG* gene. No mutations in *inhA* promoter region and *rpoB* gene were detected in any of the isolates (Table 2). Since no MTB isolate carried both INH and RIF resistant genes, no MDR-MTB were detected. As for the frequency of *katG* genetic mutation, only 3 out of the 141 MTB (2.1%) possessed a *katG* mutant gene. All *katG* mutations were S315T1, a AGC-to-ACC (Ser → Thr) point mutation (Table 2). All of the *katG* mutations detected at wild-type probes were also observed at the corresponding mutant probes.

Discussion

The emergence of drug-resistant TB is a major threat to TB treatment and eradication. The consequences of the rise of TB drug-resistance in Malawi, a country with a high burden of HIV and poverty, would be extremely devastating. Active screening for drug-resistant TB and early management of cases is therefore important. The geographical distribution of genomic mutations, that are associated with TB drug-

resistance, may vary considerably, even within the same country³⁴⁻³⁶. Such data would be useful clinically and epidemiologically. In this study, we found a low number of TB drug-resistance related genomic mutations among MTB isolates recovered from untreated PTB patients in urban Blantyre, Malawi. Mutations were detected only in the *katG* gene and the frequency of occurrence was very low (2.1%). All *katG* mutations detected were S315T1 (AGC – ACC), a serine to threonine single point mutation that mediates high INH resistance^{10,18,20}. In this study, no MTB isolates were found harboring mutations in the *rpoB* gene that mediate RIF resistance. Whilst RIF resistance due to *rpoB* mutations has widely been reported in other epidemiological studies,^{37,38} such were not the findings of our study. Similarly, we found no MTB isolates harboring mutations within the *inhA* promoter region. Unlike *katG* and *rpoB* genomic mutations that are common, *inhA* mutations are very rare and are usually associated with low-level INH resistance^{18,39,40}. Our findings, therefore, suggest that most MTB resistant strains circulating in urban Blantyre, Malawi, carry the *katG* S315T1 mutation. High prevalence of INH resistance, attributed to *katG* gene S315T1 mutations has been reported to account for a high proportion of INH resistance in high-TB-prevalence countries^{29,41}. Another putative *katG* gene mutation (S315T2), a AGC (Ser) - ACA (Thr) point mutation that mediates INH resistance, has been sporadically reported in other studies^{42,43}. However, this mutation is very uncommon and was not observed in this study. Our findings generally agree with other epidemiological reports that indicate *katG* gene mutation as relatively more common than the other two^{44,45}. No isolates carrying both *katG* and *rpoB* gene mutations were detected during this study, suggesting a very low incidence of MDR-TB in Blantyre, Malawi.

South Africa (SA) is ranked second as the country with the highest drug-resistant TB cases in the world^{22,46,47}. Extensively drug-resistant TB (XDR-TB; MDR plus resistance to injectable second-line TB drugs) and pan drug-resistant TB (PDR-TB; resistant to all available TB drugs) have been increasingly reported in SA⁴⁸⁻⁵⁰. This has generated concern worldwide, especially in the public health sector of neighboring countries including Malawi^{51,52} because of high risk of imported drug-resistant MTB strains^{53,54}. Therefore, constant monitoring of resistance related genomic mutations in Malawi is relevant especially in persons with presumptive TB returning from SA.

Study Limitations

Our study had some limitations. First, as a PCR based technique, the GenoTypeMTBDRplus Version 2.0 is designed to detect previously described chromosomal mutations of known nucleotide sequences^{29,33}. Therefore, rare and novel mutations within or outside the described genes are not detected^{11,55}. Second, the data collected and analysed in this study is not a representation of the whole community of Blantyre but rather the urban area only and is limited to one hospital (i.e., QECH) and should therefore cautiously be generalized. Finally, this was a sub-study and a fixed sample size of bacterial isolates used may not be statistically adequate.

Conclusion

We observed a very low number of genomic mutations that confer resistance among the study isolates, suggesting low levels of circulating MTB resistant strains in urban Blantyre,

Malawi. The characterization and distribution of specific types of genomic mutations that mediate resistance in MTB could be important for optimization of the diagnosis, treatment, and prevention of drug-resistant TB. Low levels of circulating drug-resistant MTB isolates evidently indicate remarkable efforts by the National TB control program in the fight against TB in Malawi.

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