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REVISED Mutation patterns of resistance genes for macrolides, aminoglycosides, and rifampicin in non-tuberculous mycobacteria isolates from Kenya [version 5; peer review: 2 approved, 1 not approved]

Previously titled: Mutation patterns of resistance genes for macrolides, aminoglycosides, and rifampicin in nontuberculous mycobacteria isolates from Kenya

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Abstract

Background: Non-tuberculous mycobacteria (NTM) treatment constitutes a macrolide-based antibiotic regimen in combination with aminoglycosides for Rapid-Growing Mycobacteria (RGM), and rifampicin for Slow-Growing Mycobacteria (SGM). Mutations in the anti-NTM drug target regions promote NTM evolution to mutant strains that are insusceptible to NTM drugs leading to treatment failure. We, therefore, described the mutation patterns of anti-NTM drug target genes including *rrl*, *rrs*, and *rpoB* in NTM isolates from Kenya.

Methods: We carried out a cross-sectional study that included 122 NTM obtained from the sputum of symptomatic tuberculosis-negative patients in Kenya. All 122 NTM underwent targeted sequencing of the rrl gene. The 54 RGM were also sequenced for *rrs*, and the 68 SGM were sequenced for *rpoB* genes using ABI 3730XL analyzer. The obtained sequences were aligned to their wild-type reference
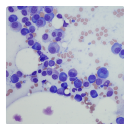
sequences for each gene using Geneious then mutations were
identified. Pearson chi-square at a 95% confidence interval tested the
association of NTM to mutation patterns for each gene.
Results: NTM harboring mutations associated with resistance to at
least one of the antibiotics used in the macrolide-based therapy were
23% (28/122). Of these NTM, 10.4% (12/122) had mutations in the *rrl*
gene with 58.3% (7/12) comprising RGM and 41.7% (5/12) being SGM.
Mutation at position 2058 (A2058G, A2058C, A2058T) of the *rrl* gene
was seen for 83.3% (10/12) of NTM, while 16.6% (2/12) harbored a
A2059G mutation. Of the 54 RGM included for *rrs* characterization,
11.1% (6/54) exhibited mutations at position 1408(A1408G), while
14.7% (10/68) of the SGM had mutations in the *rpoB* gene at positions
S531W, S531L, S531Y, F506L, E509H with *M.gastri* having multiple
mutations at positions D516V, H526D and, S531F.
Conclusion: We demonstrated a significant level of mutations
associated with drug resistance for macrolides, aminoglycosides, and
rifampicin in NTM isolated from symptomatic TB negative patients in
Kenya.

Keywords
Nontuberculous mycobacteria, slow-growing mycobacteria, rapid-
growing mycobacteria, macrolides, aminoglycosides, rifampicin

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This article is included in the Cell & Molecular
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> **REVISED** **Amendments from Version 4**
>
> Title: Inclusion of a hyphen in Non-tuberculous
> Abstract: Inclusion of a hyphen in Non-tuberculous Mycobacterium (NTM). Used upper case for the first letters of the acronym SGM and RGM. In the results section, the percentage are outside the brackets while proportions have been indicated in brackets. For mutations, nucleotide change has been indicated for each position.
> Introduction: Details of Lowenstein Jensen media for growth of mycobacteria has been included.
> Second paragraph, the duration of treatment of NTM infections has been indicated.
> indication that Rifampicin is recommended for first-line treatment of MTB alongside other drugs.
> Laboratory procedures:
> Sputum sample estimate volume for each participant has been provided.
> Details on FM microscopy readers and reviewers is now indicated.
> Results: The table with results indicating that the bulk of the samples were obtained from Lake Victoria, Coastal and Nairobi region has been mentioned.
> Discussion: rrl significant mutations are critically discussed and their relevance to macrolide drug resistance.
>
> **Any further responses from the reviewers can be found at the end of the article**

Introduction

Non-tuberculous Mycobacteria (NTM) are a group of about 170 Mycobacteria species that do not include *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Falkinham, 2017; Koh *et al.*, 2006; Peixoto *et al.*, 2020; Sam *et al.*, 2020; Simons *et al.*, 2011). In mycobacterial culture using Lowestein-Jensen (LJ) media which is a solid selective media for mycobacteria, NTM varies in their growth characteristics, with Rapid Growing Mycobacteria (RGM) forming visible colonies within seven days of incubation and Slow-Growing Mycobacteria (SGM) taking up to fourty days. SGM include *Mycobacterium avium complex* (MAC), *Mycobacterium chimaera*, *Mycobacterium kansasii*, *Mycobacterium malmoense*, and *Mycobacterium xenopi*, whereas RGM comprise species from the *Mycobacterium abscessus* and *Mycobacterium fortuitum complexes* (Alffenaar *et al.*, 2021). In human infections, identifying NTM is crucial for determining clinically relevant species and the best treatment plan (Chalmers *et al.*, 2019; Mwangi *et al.*, 2022a).

NTM treatment consists of a macrolide-based antibiotic regimen which include clarithromycin or azithromycin, combined with other selected antibiotics which are given for 12 to 18 months until culture negativity is achieved. (Falkinham, 2018). In addition to macrolides, the antibiotic of choice is largely determined by the infecting NTM, its growth rate, and the intricacy of the mycolic acid cell wall (Goswami *et al.*, 2016). Rifampicin and ethambutol are also used in SGM treatment, while aminoglycosides, cefoxitin, imipenem, or tigecycline are used in RGM treatment (Brown-Elliott *et al.*, 2012; Saxena *et al.*, 2021). Due to the slow growth of NTM compared to other bacterial infections, antimicrobial combination therapy is strongly recommended in NTM treatment to avoid the development of drug resistance (Falkinham, 2018; Pharmd *et al.*, 2019). The antibiotic regimen of patients with pre-existing lung diseases including asthma, chronic obstructive pulmonary disease, cystic fibrosis, and bronchiectasis or with immunodeficiencies are the same as for patients without these comorbidities (Koh, 2017).

Anti-mycobacterial drugs attach to their binding sites with a high affinity, preventing the target gene product from functioning normally (Alffenaar *et al.*, 2021). Changes in the structure of the target regions caused by mutations, on the other hand, interfere with the medications' ability to attach to them, resulting in antibiotic resistance. As a result, determining the antibiotic resistance profile of NTM is critical for determining an effective treatment strategy for a specific NTM infection (Goswami *et al.*, 2016; Nasiri *et al.*, 2017).

Acquired resistance to anti-NTM drugs develops due to mutations in the NTM drug target regions, subsequently promoting NTM evolution to mutant strains that are insusceptible to anti-NTM drugs (Huh *et al.*, 2019; Munita & Arias, 2016; Nasiri *et al.*, 2017; Pharmd *et al.*, 2019; Saxena *et al.*, 2021). Prolonged exposure to NTM antibiotics, as seen in the lengthy NTM regimen, sub-optimal administration of anti-NTM drugs, as seen in patients who do not adhere to the regimen or who are lost to follow up, and incorrect prescription for NTM infection due to NTM misdiagnosis all promote mutation in the drug target regions (Gopalaswamy *et al.*, 2020; Munita & Arias, 2016; Zhou *et al.*, 2020).

Macrolides inhibit protein synthesis by binding to the peptide exit tunnel of ribosomes, hence preventing the growing peptide chain from exiting the peptidyl transferase center of the ribosome (Hansen *et al.*, 2002). Mutation of the *rrl* gene at positions A2058 and A2059 accounts for 80-100% of the macrolide resistance in NTM. Macrolide resistance can also be conferred by the *erm* (41) gene which encodes a ribosomal methyltransferase that methylates the *rrl* thus blocking the drug-binding site of the macrolide (Bastian *et al.*, 2011; Huh *et al.*, 2019).

Aminoglycosides inhibit protein synthesis by binding the bacterial 30S ribosomal subunit interfering with bacterial protein translation and leading to cell death (Saxena *et al.*, 2021). Drug resistance to aminoglycosides is associated with modification in the *rrs* gene mainly observed as point mutation at position 1408 (Brown-Elliott *et al.*, 2013). In addition, mutations at positions 1406, 1409, and 1491 have been shown to confer resistance to aminoglycosides in some NTM (Olivier *et al.*, 2017).

Rifampicin is a key drug in treating mycobacterial diseases. It is the recommended first line drug for treatment of *M. tuberculosis* alongside other drugs (WHO, 2014). It inhibits the synthesis of Ribonucleic acid (RNA) by binding to the ß-subunit of the RNA polymerase that is encoded by *rpoB* gene. Most rifampicin-resistant mycobacteria have mutations in an 81-bp rifampicin resistance determining region (RRDR) within the *rpoB* gene. Mutations in this region account for 95% of rifampicin resistance in mycobacteria. The commonly observed mutations within the RRDR of *rpoB* are often seen at codons 526, and 531 (Li *et al.*, 2016). *M. kansasii* has also shown mutation conferring resistance to rifampicin at codons 513 and 516, while *MAC* also shows resistance to rifampicin with mutations outside the RRDR at codon 626 (K626T) (Ramasoota *et al.*, 2006).

Detection of drug resistance in NTM can be carried out by drug susceptibility testing (DST) through broth microdilution, (Litvinov *et al.*, 2018; Park *et al.*, 2020), sequencing for mutations in the *rrl, rrs* and *rpoB* genes (Brown-Elliott *et al.*, 2012; Huh *et al.*, 2019; Saxena *et al.*, 2021), or by using GenoType NTM-DR test (Hain, Lifescience, Nehren, Germany) which is a line probe assay (LPA) that detects resistance to macrolides and aminoglycosides (Bouzinbi *et al.*, 2020).

We, therefore, investigated drug resistance in NTM by describing the mutation patterns in *rrl*, *rrs*, and *rpoB* genes for macrolides, aminoglycosides and rifampicin respectively, in NTM isolated from symptomatic TB-negative patients from Kenya.

Methods
We carried out a cross-sectional study that included 122 NTM identified by mycobacterial culture and *hsp65* targeted sequencing from the sputum of symptomatic TB-negative patients (Mwangi *et al.*, 2022a). The patients had provided approximately 5 ml of sputum to the National Tuberculosis Reference Laboratory (NTRL) in Kenya between January to November 2020.

In this study, the 122 NTM including 54 RGM and 68 SGM underwent targeted sequencing of the *rrl* gene. The RGM were also sequenced for *rrs*, and the SGM were sequenced for *rpoB* genes using ABI 3730XL analyzer (Applied Biosystems, Foster City, California, USA).

Laboratory procedures
Sample processing, mycobacterial culture and growth identification
The sputum samples were decontaminated using the N-acetyl-L-cysteine 2% NaOH (NALC-NaOH) procedure, then inoculated into Mycobacteria Growth Indicator Tube (MGIT) and Lowestein-Jenseen (LJ) medias, incubated at 37°C and monitored for growth for up to eight weeks respectively. At the same time, sputum smears were prepared, air dried, heat fixed then fluorochrome stained with auramine O where mycobacteria appeared as bright yellow fluorescent rods when viewed under a light emitting diodes (LED) microscope. Fluorescent microscopy was performed by NTRL laboratory technologist then smears were reviewed by the section head before reporting.

The culture growth in MGIT and LJ underwent the Mtb identification testing using the SD Bioline TB Ag MPT64 assay (capilia) (Standard Diagnostics, Yongin-si, Gyeonggi-do, Republic of Korea) and capilia positive samples were excluded from the study. The capilia negative samples underwent ZN microscopy with presence of AFB indicating a possible NTM.

DNA extraction
Mycobacterial DNA was extracted from 500 μL of re-suspended colonies using using **Geno**Lyse® (*Hain* Lifescience, Nehren, Germany) according to the manufacturer's instructions. Briefly, 100 ul of lysis buffer (A-LYS) buffer was added to each cryovial containing the resuspended colonies and incubated for five minutes at 95°C after which 100 ul neutralization buffer (A-NB) was added and centrifugation done at 5000G for ten minutes. The supernatant was transferred to a newly labelled cryovial awaiting PCR.

Conventional PCR, DNA gel electrophoresis and DNA purification
Conventional PCR targeting *rrl, rpoB and rrs* genes of NTM were conducted using the Horse-Power™ Taq DNA Polymerase MasterMix (Canvax, Córdoba, Spain) in a final reaction volume for each gene of 13 μl comprising 6.25 μl of

Table 1. Nucleotide sequence of primers for *rrl*, *rpoB* and *rrs* genes.

Gene	Primers	Size	Reference
rrl	F (5′-TTTAAGCCCCAGTAAACGGC-3′) R (5′-GTCCAGGTTGAGGGAACCTT-3′)	420 bp	(Park *et al.*, 2020)
rpoB	F (5′-GGCAAGGTCACCCCGAAGGG-3′) R (5′-AGCGGCTGCTGGGTGATCATC-3′)	723 bp	(Adékambi *et al.*, 2003)
rrs	F (5′-AAGGAGGTGATCCAGCCGCA-3′) R (5′-TCCCTTGTGGCCTGTGTGCA-3′)	341 bp	(Kim *et al.*, 2021)

2X Horse-Power™ Taq DNA Polymerase MasterMix, 2.5 μl DNA template, 0.25 μl of each of both forward and reverse primers (Table 1) at a final concentration of 10 pmoles, and 3.75 μl of nuclease-free water to make up the reaction volume. The PCR assays were carried out with a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA) (Table 1). Thermal cycling conditions for *rrl* were as follows: one cycle of 95°C for five minutes, 35 cycles of 95°C for one minute, 55°C for one minute, 72°C for one minute, and a final extension for ten minutes at 72°C. PCR for *rrs* was conducted as follows 95°C for five minutes, 35 cycles of 95°C for one minute, 60°C for one minute, 72°C for one minute, and a final extension for seven minutes at 72°C. PCR for the *rpoB* was conducted as follows: 95°C for five minutes, 35 cycles of 95°C for one minute, 58°C for one minute, 72°C for one minute, and a final extension for seven minutes at 72°C. Amplified products were confirmed on a 1% Agarose gel stained with 4.6 μl SYBR safe DNA stain (Invitrogen, Carlsbad, California, USA), and results were visualized with an UltraViolet gel viewer (Terra Universal, S. Raymond Ave, Fullerton, CA, USA). For the corresponding runs, a positive control containing drug-resistant NTM with mutations in the *rrl, rpoB* and *rrs* genes was used, as well as a negative control containing drug sensitive NTM without mutations in the drug target genes were included.

The PCR products were enzymatically purified using ExoSAP IT (Applied Biosystems, Foster City, California, USA). Purification conditions were done at 37°C for fifteen minutes followed by a second incubation at 80°C for fifteen minutes and a final cooling step at 4°C for five minutes.

rrl, rpoB and *rrs* genes sequencing
The purified amplicons were sequenced in the forward and reverse directions by Sanger sequencing using Big Dye™ Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) and the forward and reverse primers. The sequencing reaction for each gene was a 10 μl reaction comprising 1.25 μl of Big Dye Terminator, 3 μl of 5× Sequencing Buffer, 1 μl of 1 pmol of the sequencing primer, and 1.5 μl of the PCR product. The reaction volume was made up by adding 3.25 μl of nuclease-free water. The reaction proceeded through 96°C for 1 minute then 25 cycles of 96°C for 10 seconds, 50°C for five seconds, and 60°C for four minutes.

Purification of cycle-sequencing products was done using the BigDye X Terminator™ purification kit following the manufacturer's instructions (Applied Biosystems, Foster City, California, USA) and purified products were loaded onto the ABI 3730 genetic analyzer (Applied Biosystems, Foster City, California, USA) for capillary electrophoresis.

Data analysis
The obtained sequences were first assembled into contigs and the consensus sequences aligned to their wild-type reference sequences for each gene using Geneious version 11.0 (Biomatters Ltd, Auckland, New Zealand). Mutations in the drug resistance genes were identified visually. STATA version 14 (StataCorp, College Station, Texas, USA) was used to test the association of NTM species to mutation patterns using Pearson chi-square at 95% confidence interval.

Results
Our study established that 23% (28/122) of NTM harbored mutations associated with resistance to at least one of the antibiotics in the macrolide-based therapy. One isolate (C47) containing *M. abscessus* had mutations conferring resistance in both *rrl* and *rrs* genes.

The bulk of the isolates with mutations associated with drug resistance originated from the Lake Victoria, Coastal, and Nairobi regions, with 5% (6/122) NTM showing mutations in the *rrl*, *rrs*, or *rpoB* genes (p=0.012)) (Table 2). The age group was statistically significant (p=0.012), with isolates from participants aged 21 to 35 years old having the highest (n=10, 35.7%) number of NTM with target gene alterations for *rrl*, *rrs*, and *rpoB* genes. Males accounted for 76.5% (21/28) of the NTM isolates with mutations in the drug target genes and indicated a strong statistical significance (p=0.000). The majority of NTM with these mutations were detected in new (n=8; 28.5%) and TB relapse patients (n=8; 28.5%) patients, which had a significant statistical significance (p=0.001) (Table 2).

Table 2. Sociodemographic characteristics of patients with drug-resistant NTM.

Variables	NTM (n, %)	Genes with mutations associated with drug resistance to NTM			P value
		rrl (n, %)	*rrs* (n, %)	*rpoB* (n, %)	
Regions					
Mt. Kenya	23(18.9)	2(16.7)	-	-	
North Rift Valley	13(10.7)	1(8.3)	-	-	
South Rift valley	9(7.3)	2(16.7)	-	2(20)	
Lake Victoria	8(6.6)	-	2 (33.3)	4(40)	0.012
Lower Eastern	8(6.6)	1(8.3)	-	2(20)	
Coast	33(27)	2(16.7)	2 (33.3)	2(20)	
Northeastern	4(3.3)	-	-	-	
Nairobi	24(19.6)	4(33.3)	2 (33.3)	-	
Subtotal	122(100)	12 (100)	6 (100)	10(100)	
Age group					
<21	5(4)	-	-	-	
21-35	49(40.1)	4(33.3)	1(16.7)	5(50)	
36-50	40(32.8)	5(41.7)	3(50)	1(10)	0.012
51-65	21(17.3)	2(16.7)	2 (33.3)	3(30)	
>65	7(5.8)	1(8.3)	0	1(10	
Subtotal	122(100)	12(100)	6(100)	10(100)	
Gender					
Female	32 (27)	4(33.3)	2 (33.3)	1(10)	
Male	90(73)	8(66.7)	4(66.7)	9(90)	0.000
Subtotal	122(100)	12(100)	6(100)	10(100)	
Type of patient					
New	33(27)	3(25)	2 (33.3)	3(30)	
Retreatment	21(17)	3(25)	1(16.7)	2(20)	
Relapse	30(25)	2(16.7)	2(33.3)	4(40)	0.001
MDR follow-up	38(31)	4(33.3)	1(16.7)	1(10)	
Subtotal	122(100)	12(100)	6(100)	10(100)	

Mutations in *rrl* gene for the NTM were highly significant with a p value of <0.001. Of the NTM with mutations in their drug target genes, 10.4% (12/122) had mutations in the *rrl* gene (Table 2) with 58.3% (7/12) comprising RGM (Table 3) and 41.7% (5/12) being SGM (Table 3). Point mutation at position 2058 (A2058G, A2058C, A2058T) of the *rrl* gene was seen for 83.3% (10/12) of NTM including *M.intracellulare*, *M. abscessus subsp abscessus, M. nebraskense, M. massiliense, M. kumamototense, M. heraklionense,* and *M. bourgelatii.* A *rrl* A2059G mutation was seen for M. abscessus subsp abscessus representing16.6% (2/12) of the NTM (Table 3).

Of the 54 RGM included for rrs characterization, 11.1% (6/54) exhibited mutations at position 1408(A1408G), The NTM species presenting with these mutations include *M. abscessus subsp abscessus, M. chelonae,* and *M. alsense* (Table 4). The NTM species had a low likelihood (p=0.06) of developing mutations in *rrs* gene for aminoglycoside resistance.

A low association (p=0.89) for *rpoB* mutation in SGM was observed. Mutations within codons between 503-533 of the *rpoB* were seen for 14.7% (10/68) of SGM. These SGM included *M. avium subsp avium*, (S531W S531L, S531Y) *M. intracellulare* (F506L), *M. yongonense* (E509H) and *M. gastri* which had multiple mutations at positions D516V, H526D and, S531F (Table 5).

Table 3. NTM showing mutations associated with macrolide resistance.

Isolate ID	NTM species	Growth rate	Position of mutation in *rrl*	X^2, p value
C21, C64	*M. intracellulare*	SGM	A2058G	
C31	*M. nebraskense*	SGM	A2058C	
C109	*M. kumamototense*	SGM	A2058T	
C132	*M. heraklionense*	SGM	A2058T	
C36, 63	*M. abscessus subsp abscessus*	RGM	A2058C	87, <0.001
C47	*M. abscessus subsp abscessus*	RGM	A2058G	
C88	*M. massiliense*	RGM	A2058T	
C133	*M. bourgelatii*	RGM	A2058C	
C46, C71	*M. abscessus subsp abscessus*	RGM	A2059G	

Table 4. Rapid-growing NTM showing mutations associated with aminoglycoside resistance.

Isolate ID	NTM species	Position of mutation in *rrs*	X^2, p value
C12, C53	*M. chelonae*	A1408G	
C58	*M. alsense*	A1408G	25, 0.06
C46, C47, C70	*M. abscessus subsp abscessus*	A1408G	

Table 5. Slow- growing NTM showing mutations associated with rifampicin resistance.

Isolate ID	NTM species	Codons with mutation in *rpoB*	X^2, p value
C66	*M. avium subsp avium*	S531W	
C78, C94	*M. avium subsp avium*	S531L	
C128	*M. avium subsp avium*	S531Y	26, 0.89
C10, C57, C64, C166	*M. intracellulare*	F506L	
C74	*M. yongonense*	E509H	
C151	*M. gastri*	D516V, H526D, S531F	

Discussion

Non-tuberculous mycobacteria (NTM) are an important cause of pulmonary disease worldwide, and are being isolated increasingly (Rivero-Lezcano & Carolina González-Cortés, 2019). They are often mistakenly treated as *M. tuberculosis* in countries devoid of laboratory competence for mycobacterial species differentiation (Pokam *et al.*, 2022). Recently, there has been a considerable rise in infections caused by NTM (Saxena *et al.*, 2021). These mycobacteria, which comprise a large and diverse range of species, have developed resistance to most conventional antibiotics, rendering their treatments unsatisfactory (Brown-Elliott *et al.*, 2012).

This study established that 23% of NTM harbored mutations associated with resistance to at least one of the antibiotics in the macrolide-based therapy. Regional distribution of NTM with mutations in the target genes had a significant correlation (p=0.012) with the bulk of the isolates originating from the Lake Victoria (n=6, 5%), Coastal (n=6, 5%), and Nairobi (n=6, 5%) regions. The observed regional diversity of NTM harboring drug resistance mutations across Kenya could be attributed to NTM evolution to evade natural antibiotics secreted as secondary metabolites by nearby environmental bacteria in various geographical landscapes (Moore *et al.*, 2019). The 21-35 years age group had the highest number of NTM isolates presenting with drug resistance associated mutations while males comprised the majority (n=21, 76.5%) of cases with NTM showing these mutations. These mutations were associated with a history of previous TB infection as seen in the high number of TB relapse cases recorded in this study (n=8; 28.5%, p=0.001). This is a common observation in sub-Saharan Africa given the high incidence *of M. tuberculosis* disease and the frequent misdiagnosis of NTM infection with TB seen in this region (Aliyu *et al.*, 2013; Hoza *et al.*, 2016; Pokam *et al.*, 2022; Mwangi *et al.*, 2022a).

Despite NTM demonstrating high levels of resistance to a broad range of antibiotics, macrolides including clarithromycin, erythromycin, and azithromycin remain the most effective antibiotic with >80% of isolates showing susceptibility (Ananta *et al.*, 2018). However, some NTM including *MAC* and *M. abscessus* have been associated with increased resistance to macrolides leading to treatment failure (Saxena *et al.*, 2021). The mechanisms of macrolide resistance have been studied at the molecular level and has consistently demonstrated that 80-100% of phenotypic macrolide resistant clinical isolates contained point mutations at positions A2058 and A2059 in the 23S rRNA gene for NTM (Huh *et al.*, 2019). According to 23S rRNA crystallography investigations, A2058 and A2059 serve as important contact sites for macrolide binding since they are conveniently situated on the surface of the peptide exit tunnel of ribosomes. The architecture of the macrolide binding site is changed by a point mutation of A2058/A2059, which creates a smooth, sunken interaction surface that weakens the interaction of macrolides with their binding site hence leading to drug resistance (Poehlsgaard *et al.*, 2005). Our study demonstrated that majority of macrolide-resistant isolates (88.3%) including *M. intracellulare* (A2058G), *M. nebraskense* (A2058C), *M. massiliense* (A2058T), *M. kumamototense* (A2058T), *M. heraklionense* (A2058T), and *M. bourgelatii* (A2058C) had a point mutation at position 2058 in the rrl, while two (16.7%) *M. abscessus subsp abscessus* isolates had mutation at position A2059G. The increased potential for development of these mutations in *MAC* species including *M. intracellulare,* and *M. abscessus* could be attributed to inherent factors such as a high propensity for genetic mutation in the drug target region, and high drug tolerance levels (Park *et al.*, 2020), environmental factors facilitating the emergence of mutations in the *rrl* and gene subsequent ease of transmission to humans (Beverley Cherie Millar, 2019). Other mutations that could confer resistance to macrolides include T2419 in *M. intracellulare* (Huh *et al.*, 2019). However, this mutation was not demonstrated in the Kenyan isolates of this study.

The commonly used aminoglycosides for the treatment of NTM are amikacin, streptomycin, kanamycin, tobramycin, and streptomycin (Krause *et al.*, 2016). In our present study, three *M. abscessus subsp abscessus*, two *M. chelonae,* and one *M. alsense* presented with a A1408G mutation. Genotypic characteristics in *rrs* that are associated with aminoglycoside resistance usually are in concordance with DST broth microdilution and GenoType NTM-DR assays, implying that mutations within *rrs* are the molecular basis of aminoglycoside resistance in NTM (Bouzinbi *et al.*, 2020). For instance, a study that selected *in-vitro* aminoglycoside-resistant *M. abscessus* and *M. chelonae* presented an A→G mutation at position 1408 within the *rrs* upon sequencing. This confirms that a single point mutation at 1408 is adequate to confer high-level aminoglycoside resistance (Nessar *et al.*, 2011). Further, individual mutations at T1406, C1409 and G1491 have also indicated considerable resistance to aminoglycoside in most *M. abscessus* subspecies (Nessar *et al.*, 2011). Similar to other bacteria, NTM present with low-level aminoglycoside resistance through the production of drug-modifying enzymes including acetyltransferases (Sanz-García *et al.*, 2019), phosphorylases, adenylates, and methylases which act at various points on the aminoglycoside scaffold making it less potent (Krause *et al.*, 2016; Munita & Arias, 2016; Sanz-García *et al.*, 2019; Tarashi *et al.*, 2022; Zaragoza Bastida *et al.*, 2017). Why does mutation at 1408 confer more drug resistance compared to other point mutation.

Similar to *M. tuberculosis*, resistance to rifampicin in NTM is associated with mutations in the 81 bp RRDR corresponding to codons 503 to 533 of the *rpoB* gene (Saxena *et al.*, 2021; Zhou *et al.*, 2020). Our study identified 11.6% (10/68) NTM with mutations occurring at codon 531 in *M. avium,* codon 506 in *M. intracellulare*, 509 in *M. yongonense. M. gastri* had amino acid substitutions at positions 516, 526, 531 of the *rpoB* gene. Our findings did not establish mutations outside of the RRDR. However, low-level rifampicin resistance has previously been demonstrated in *M. intracellulare* with mutations occurring outside the RRDR at position N494S (Park *et al.*, 2020). Broth microdilution analysis which is the gold standard for rifampicin drug resistance testing presents a high minimum inhibition concentration (MIC) for isolates with mutations in the RRDR, hence confirming the role of these mutations in conferring high-level resistance to rifampicin (Huh *et al.*, 2019). We further demonstrated that *M. gastri* harbored more than one mutation within the RRDR which is a unique attribute observed for *M. kansasii* complex species to which *M.gastri* belongs (Wu *et al.*, 2018). A similar study obtained rifampicin-resistant *M. kansasii* from clinical isolates and *in vitro* generated mutant, and demonstrated mutations in codons 513, 526, and 531 of *rpoB* which is a common pattern in some SGM and *M. tuberculosis* (Klein *et al.*, 2001).

We found considerable levels of mutations in the drug target genes for macrolides, aminoglycosides and rifampicin in Kenyan NTM. To guide therapy, both species-level identification and drug resistance testing of NTM should be performed before starting treatment for NTM infection.

Conclusion

We demonstrated significant mutations associated with drug resistance for macrolides, aminoglycosides and rifampicin in NTM isolated from symptomatic TB-negative patients in Kenya.

M. abscessus and *MAC* were the dominant NTM with *rrl* mutations, and most mutations occurred at position 2058. Majority of RGM had mutation at position 1408 of the *rrs* gene, while *rpoB* mutations presented within the RRDR.

Limitations of the study

This study investigated the acquired mechanisms of drug resistance for NTM. Other intrinsic factors apart from drug target gene mutation could influence the sensitivity of NTM to antibiotics.

Despite this limitation, the study documents drug resistance-associated mutation patterns of Kenyan NTM for the first time, and advocates for drug resistance testing before commencement of treatment for NTM infection.

Ethical clearance

This study was approved by the Kenyatta National Hospital-University of Nairobi Ethics Review Committee (Ref: KNH-ERC/A/38) on 30th January 2020. Waiver for individual informed consent was granted as the study utilized remnant clinical samples and the research posed no greater than minimal risk to the study subjects.

Data availability

Figshare. Mutation Patterns of Resistance Genes for Macrolides, Aminoglycosides, and Rifampicin in Non-tuberculous Mycobacteria Isolates from Kenya. DOI: https://doi.org/10.6084/m9.figshare.20331378.v2 (Mwangi et al., 2022b).

This project contains the following underlying data:

-   Mutation patterns for rrl, rrs, and rpoB genes in NTM

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Author contributions

ZMM - Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing

GN - Investigation, Writing – Review & Editing

MWM - Supervision, Writing- Review and Editing

FGO - Conceptualization, Methodology, Supervision, Writing- Review and Editing

WDB - Conceptualization, Methodology, Supervision, Writing- Review and Editing

References

Adékambi T, Colson P, Drancourt M: **rpoB-Based Identification of Nonpigmented and Late-Pigmenting Rapidly Growing Mycobacteria.** *J. Clin. Microbiol.* 2003; **41**(12): 5699–5708.
PubMed Abstract | **Publisher Full Text** | **Free Full Text**

Alffenaar JW, Märtson AG, Heysell SK, *et al.*: **Therapeutic Drug Monitoring in Non-Tuberculosis Mycobacteria Infections.** *Clin. Pharmacokinet.* 2021; **60**(6): 711–725.
PubMed Abstract | **Publisher Full Text**

Aliyu G, El-kamary SS, Abimiku A, *et al.*: **Prevalence of Non-Tuberculous Mycobacterial Infections among Tuberculosis Suspects in Nigeria.** *PLoS One.* 2013; **8**(5): e63170–e63177.
PubMed Abstract | **Publisher Full Text**

Ananta P, Kham-ngam I, Chetchotisakd P, *et al.*: **Analysis of drug-susceptibility patterns and gene sequences associated with clarithromycin and amikacin resistance in serial Mycobacterium**

abscessus isolates from clinical specimens from Northeast Thailand. *PLoS One.* 2018; **13**: 1–15.
Publisher Full Text

Bastian S, Veziris N, Roux AL, *et al.*: **Assessment of clarithromycin susceptibility in strains belonging to the Mycobacterium abscessus group by erm(41) and rrl sequencing.** *Antimicrob. Agents Chemother.* 2011; **55**(2): 775–781.
PubMed Abstract | **Publisher Full Text**

Beverley Cherie Millar JEM: **Antimycobacterial Strategies to Evade Antimicrobial Resistance in the Nontuberculous Mycobacteria.** *Int. J. Mycobacteriol.* 2019; **8**(1): 7–21.
PubMed Abstract | **Publisher Full Text**

Bouzinbi N, Marcy O, Bertolotti T, *et al.*: **Evaluation of the GenoType NTM-DR assay performance for the identification and molecular detection of antibiotic resistance in mycobacterium abscessus complex.** *PLoS*

One. 2020; **15**(9 September 2020): 1–5.
PubMed Abstract | **Publisher Full Text**

Brown-Elliott BA, Iakhiaeva E, Griffith DE, *et al.*: **In vitro activity of amikacin against isolates of mycobacterium avium complex with proposed MIC breakpoints and finding of a 16S rRNA gene mutation in treated isolates.** *J. Clin. Microbiol.* 2013; **51**(10): 3389–3394.
PubMed Abstract | **Publisher Full Text**

Brown-Elliott BA, Nash KA, Wallace RJ: **Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria.** *Clin. Microbiol. Rev.* 2012; **25**(3): 545–582.
PubMed Abstract | **Publisher Full Text**

Chalmers JD, Balavoine C, Castellotti PF, *et al.*: **European Respiratory Society International Congress, Madrid, 2019: nontuberculous mycobacterial pulmonary disease highlights.** *Eur. Respir. J.* 2019; **6**(May 2020): 00317–02020.
PubMed Abstract | **Publisher Full Text**

Falkinham JO: **Environmental Sources of Non tuberculous Mycobacteria.** *Clin. Chest Med.* 2017.
Publisher Full Text

Falkinham JO: **Challenges of NTM drug development.** *Front. Microbiol.* 2018; **9**(JUL): 1–7.
PubMed Abstract | **Publisher Full Text**

Gopalaswamy R, Shanmugam S, Mondal R, *et al.*: **Of tuberculosis and non-tuberculous mycobacterial infections – a comparative analysis of epidemiology, diagnosis and treatment.** *J. Biomed. Sci.* 2020; **27**: 17–74.
PubMed Abstract | **Publisher Full Text**

Goswami B, Narang P, Mishra P, *et al.*: **Drug susceptibility of rapid and slow growing non-tuberculous mycobacteria isolated from symptomatics for pulmonary tuberculosis, Central India.** *Indian J. Med. Microbiol.* 2016; **34**(4): 442–447.
PubMed Abstract | **Publisher Full Text**

Hansen JL, Ippolito JA, Ban N, *et al.*: **The structures of four macrolide antibiotics bound to the large ribosomal subunit.** *Structural Insights into Gene Expression and Protein Synthesis.* 2002; **10**: 117–128.
Publisher Full Text

Hoza AS, Mfinanga SGM, Rodloff AC, *et al.*: **Increased isolation of nontuberculous mycobacteria among TB suspects in Northeastern, Tanzania: public health and diagnostic implications for control programmes.** *BMC. Res. Notes.* 2016; **9**: 109.
PubMed Abstract | **Publisher Full Text**

Huh HJ, Kim SY, Jhun BW, *et al.*: **Recent advances in molecular diagnostics and understanding mechanisms of drug resistance in nontuberculous mycobacterial diseases.** *Infect. Genet. Evol.* 2019; **72**(October 2018): 169–182.
PubMed Abstract | **Publisher Full Text**

Kim SY, Kim DH, Moon SM, *et al.*: **Association between 16S rRNA gene mutations and susceptibility to amikacin in Mycobacterium avium Complex and Mycobacterium abscessus clinical isolates.** *Sci. Rep.* 2021; **11**(1): 6108–6108.
PubMed Abstract | **Publisher Full Text**

Klein JL, Brown TJ, French GL: **Rifampin resistance in Mycobacterium kansasii is associated with rpoB mutations.** *Antimicrob. Agents Chemother.* 2001; **45**(11): 3056–3058.
PubMed Abstract | **Publisher Full Text**

Koh W, (2017). Nontuberculous Mycobacteria — Overview. *Microbiology Spectrum*, 1–7.

Koh W, Kwon OJ, Jeon K, *et al.*: **Clinical Significance of Nontuberculous Mycobacteria Isolated From Respiratory Specimens in Korea.** *Chest.* 2006; **129**(2): 341–348.
PubMed Abstract | **Publisher Full Text**

Krause KM, Serio AW, Kane TR, Connolly LE: **Aminoglycosides: An Overview.** 2016; 1–18.

Li QJ, Jiao WW, Yin QQ, *et al.*: **Compensatory mutations of rifampin resistance are associated with transmission of multidrug-resistant Mycobacterium tuberculosis Beijing genotype strains in China.** *Antimicrob. Agents Chemother.* 2016; **60**(5): 2807–2812.
PubMed Abstract | **Publisher Full Text**

Litvinov V, Makarova M, Galkina K, *et al.*: **Drug susceptibility testing of slowly growing non-tuberculous mycobacteria using slomyco test-system.** *PLoS One.* 2018; **13**(9): e0203108–e0203113.
PubMed Abstract | **Publisher Full Text**

Munita JM, Arias CA: **Mechanisms of antibiotic resistance.** *Virulence Mechanisms of Bacterial Pathogens.* 2016; **4**(2): 481–511.
Publisher Full Text

Mwangi ZM, Mukiri N, Onyambu FG, *et al.*: **Genetic Diversity of Nontuberculous Mycobacteria among Symptomatic Tuberculosis Negative Patients in Kenya.** *International Journal of Mycobacteriology.* 2022a; **11**(1): 60–69.
Publisher Full Text

Mwangi Z, Onyambu F, Mureithi MW, *et al.*: Mutation Patterns of Resistance Genes for Macrolides, Aminoglycosides, and Rifampicin in

Nontuberculous Mycobacteria Isolates from Kenya. figshare. Dataset. 2022b.
Publisher Full Text

Nasiri MJ, Haeili M, Ghazi M, *et al.*: **New insights in to the intrinsic and acquired drug resistance mechanisms in mycobacteria.** *Front. Microbiol.* 2017; **8**(APR)
Publisher Full Text

Nessar R, Reyrat JM, Murray A, *et al.*: **Genetic analysis of new 16s rRNA mutations conferring aminoglycoside resistance in Mycobacterium abscessus.** *J. Antimicrob. Chemother.* 2011; **66**(8): 1719–1724.
PubMed Abstract | **Publisher Full Text**

Olivier KN, Griffith DE, Eagle G, *et al.*: **Randomized trial of liposomal amikacin for inhalation in nontuberculous mycobacterial lung disease.** *Am. J. Respir. Crit. Care Med.* 2017; **195**(6): 814–823.
PubMed Abstract | **Publisher Full Text**

Park HE, Kim S, Shim S, *et al.*: **16S and 23S Rrna Gene Mutation Independent Multidrug Resistance of Non-Tuberculous Mycobacteria Isolated From South Korean Soil.** *Microorganisms.* 2020; **8**(8): 1–19.
PubMed Abstract | **Publisher Full Text**

Peixoto S, Maria L, Montenegro L, *et al.*: **Major Article Identification of nontuberculous mycobacteria species by multiplex real-time PCR with high-resolution melting.** *Journal of the Brazilian Society of Tropical Medicine.* 2020; **53**: 1–7.
Publisher Full Text

Pharmd JAS, Escalante P, Wilson JW: **Pharmacotherapy Approaches in Nontuberculous Mycobacteria Infections Jennifer.** *Mayo Clin. Proc.* 2019; **94**(August): 1567–1581.
PubMed Abstract | **Publisher Full Text**

Poehlsgaard J, Pfister P, Böttger E. C, *et al.* (2005). **Molecular mechanisms by which rRNA mutations confer resistance to clindamycin**. *Antimicrobial Agents and Chemotherapy*, **49**(4): 1553–1555.
Publisher Full Text

Pokam BDT, Yeboah-Manu D, Ofori S, *et al.*: **Prevalence of non-tuberculous mycobacteria among previously treated TB patients in the Gulf of Guinea, Africa.** *IJID Regions.* 2022; **3**(January): 287–292.
PubMed Abstract | **Publisher Full Text**

Ramasoota P, Pitaksajjakul P, Phatihattakorn W, *et al.*: **Mutations in the rpoB gene of rifampicin-resistant Mycobacterium tuberculosis strains from Thailand and its evolutionary implication.** *Southeast Asian J. Trop. Med. Public Health.* 2006; **37**(1): 136–147.
PubMed Abstract

Rivero-Lezcano OM, Carolina González-Cortés MM: **The Unexplained Increase of Nontuberculous Mycobacteriosis Octavio.** *Int. J. Mycobacteriol.* 2019; **8**(1): 1–6.
PubMed Abstract | **Publisher Full Text**

Sam AS, Ninan MM, Ranjani R, *et al.*: **Nontuberculous mycobacteria – clinical and laboratory diagnosis: experiences from a TB endemic country.** *Future Science OA.* 2020; **6**.
Publisher Full Text

Sanz-García F, Anoz-Carbonell E, Pérez-Herrán E, *et al.*: **Mycobacterial aminoglycoside acetyltransferases: A little of drug resistance, and a lot of other roles.** *Front. Microbiol.* 2019; **10**(JAN): 1–11.
PubMed Abstract | **Publisher Full Text**

Saxena S, Spaink HP, Forn-Cuní G: **Drug resistance in nontuberculous mycobacteria: Mechanisms and models.** *Biology.* 2021; **10**(2): 1–22.
PubMed Abstract | **Publisher Full Text**

Simons S, Van Ingen J, Hsueh P, *et al.*: **Nontuberculous Mycobacteria in Respiratory Tract Infections, Eastern Asia.** *Emerg. Infect. Dis.* 2011; **17**(3).
Publisher Full Text

Tarashi S, Siadat SD, Fateh A: **Nontuberculous Mycobacterial Resistance to Antibiotics and Disinfectants: Challenges Still Ahead.** *Biomed. Res. Int.* 2022; **2022**: 1–12.
PubMed Abstract | **Publisher Full Text**

WHO: **WHO guidelines for the programmatic management of drug-resistant tuberculosis.** 2014; (pp. 1–430).
Reference Source

Wu ML, Aziz DB, Dartois V, *et al.*: **NTM drug discovery: status, gaps and the way forward.** *Drug Discov. Today.* 2018; **23**(8): 1502–1519.
PubMed Abstract | **Publisher Full Text**

Zaragoza Bastida A, Rivero Pérez N, Valladares Carranza B, *et al.*: **Molecular Identification of Mycobacterium Species of Public Health and Veterinary Importance from Cattle in the South State of México.** *Canadian Journal of Infectious Diseases and Medical Microbiology.* 2017; **2017**: 4–11.
PubMed Abstract | **Publisher Full Text**

Zhou L, Xu D, Liu H, *et al.*: **Trends in the Prevalence and Antibiotic Resistance of Non-tuberculous Mycobacteria in Mainland China, 2000–2019: Systematic Review and Meta-Analysis.** *Front. Public Health.* 2020; **8**(July): 1–9.
Publisher Full Text

Open Peer Review

Current Peer Review Status: ❌ ✅ ✅

Version 5

Reviewer Report 04 April 2023

https://doi.org/10.5256/f1000research.146329.r168595

✅ **Clement Likhovole** iD

Department of Microbiology, UZIMA University, Kisumu, Kenya

The authors have revised the manuscript as per the recommended changes. The revised manuscript is adequate in form and content

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: TB molecular biology and immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

> Author Response 05 Apr 2023
> **Zakayo Mwangi**
>
> Thank you for your approval
>
> *Competing Interests:* No competing interests were disclosed.

Version 4

Reviewer Report 24 March 2023

https://doi.org/10.5256/f1000research.144441.r167243

? **Clement Likhovole** iD

[1] Department of Microbiology, UZIMA University, Kisumu, Kenya
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General comments

The manuscript addresses an important area in Tuberculosis research particularly analysis of drug resistant mutations that have been linked to treatment failure.

Abstract:
- The authors to include a hyphen in Non-tuberculous Mycobacterium (NTM).

- Use upper case for the first letters of the following acronyms, SGM and RGM.

- In the results section, percentages should not be in brackets. The proportions to indicated in brackets e.g. 23% (23/100). This should be done all the data presented in this section. When reporting on the mutations, also indicate the amino acid or nucleotide change as well. For example, which mutation is present on codon 2058? Change from what to what?

Introduction:
- Second sentence, the authors to describe in detail the type of culture used to grow NTMs for 40 days. MGIT?

- Second paragraph, authors to indicate the duration of treatment of NTM infections. Also the length of treatment with Rifampicin to be mentioned. Is it the same for patients infected with comorbidities?

- The authors have listed several drugs used for the treatment of NTMs but described only one resistance mechanisms for NTMs, do they all have the same type of resistance mechanism? (Macrolides - Clarithromycon and Azithromycin).

- Paragraph seven, the authors to indicate that Rifampicin is recommended for first-line treatment of MTB alongside other drugs.

Laboratory procedures:
- The authors to estimate the volume of sputum sample collected from each participant.

- For the FM microscopy, how many readers were used before reporting the smear results?

- What volume of DNA was finally collected after extraction? Was quantification of the DNA material done?

Results:
- The authors to mention the table with results indicating that the bulk of the samples were obtained from Lake Victoria, Coastal and Nairobi region.

Discussion:
- The authors to refer to my previous comments on Non-tuberculous mycobacterium.

○ The authors to critically discuss the relevance of the rrl significant mutations reported in the study sites. What are the implications of these finding?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: TB molecular biology and immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 27 Mar 2023
Zakayo Mwangi

Thank you for your valuable feedback.

Competing Interests: No competing interests were disclosed.

Reviewer Report 27 February 2023

https://doi.org/10.5256/f1000research.144441.r163993

Leena Al-Hassan iD

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No further comments. Please see previous comments about the need to correlate findings with MIC.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Infectious diseases and antibiotic resistance

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

- -

Version 3

Reviewer Report 05 December 2022

https://doi.org/10.5256/f1000research.141647.r157361

Leena Al-Hassan iD

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I have read the revised version, but I still see that the manuscript has misleading information that needs to be re-written. The data provided need to associated with phenotypic data on resistance. Identification of mutations in genes is not sufficient to prove resistance, as the researchers have not confirmed any silent mutations.

The positive controls used in the methodology should be isolates that are confirmed resistant and harbour these mutations. This is the definition of a positive control. The negative control is an isolates that is genotypically confirmed having no mutations in the target genes.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Infectious diseases and antibiotic resistance

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

> Author Response 13 Dec 2022
> **Zakayo Mwangi**
>
> Thank you for the feedback.
>
> *Competing Interests:* No competing interests were disclosed.

Version 2

Reviewer Report 22 November 2022

https://doi.org/10.5256/f1000research.139953.r154017

✗ **Leena Al-Hassan** 🆔
   [1] Department of Global Health and Infection, Brighton and Sussex Medical School, Brighton, UK
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Thank you for revising the manuscript and incorporating the comments into the text. However I would highly suggest that you revise the sections where you correlate the genotype with phenotype, since you have not done the AST (MIC) in this study. If you have not done the AST testing, you cannot state that they are resistant. Your study is primarily genotype based, and you have detected mutations in target resistance determinants, and I therefore suggest you change the wording of the results and discussion to reflect that. These mutations may be silent mutations, and if you do not have phenotype data to support it, you cannot state the fact that they are resistant. You can only say that they harbour the mutations which in previous reports have led to resistance.

These statements "Twenty-eight (23%) of the NTM were resistant to at least one of the antibiotics used in the macrolide-based treatment " and "Ten (14.7%) of the 68 SGM were resistant to rifampicin, with 40 percent having mutations at codon 531 in the rpoB gene" cannot be proven without AST. You should re-word it to say that 23% harboured mutations associated with resistance to macrolide antibiotics.

Same for the conclusion: you have not demonstrated a significant level of drug resistance for macrolides, aminoglycosides and rifampicin in NTM - you have detected mutations in gene targets that have previously been associated with drug resistance.

I hope my point is clear. I am not doubting your results, and I think it's interesting that you have detected these mutations, however it's not scientifically sound to assume resistance based on genotype only, and you must phrase your results and conclusions according to the methods used and data generated. The way it is written now indicates that you have the AST data for the isolates and you are sure that they are rifampicin or aminoglycoside resistant. I would strongly recommend you perform the AST - even for a subset of isolates - to confirm that the observed genotype correlates with the phenotype.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Infectious diseases and antibiotic resistance

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

> Author Response 28 Nov 2022
> **Zakayo Mwangi**
>
> I appreciate your re-evaluation and the way in which you made the issues that need to be addressed evident.
>
> I revised the paper in accordance with your advice and resubmitted it for review.
>
> *Competing Interests:* No competing interests were disclosed.

Reviewer Report 03 November 2022

https://doi.org/10.5256/f1000research.139953.r154016

✔ **Utpal Sengupta** 🔶
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The manuscript can be accepted now.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Infectious diseases with special reference to tuberculosis and leprosy, Cell Biology, Microbiology, Immunology and Biotechnology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

> Author Response 28 Nov 2022
> **Zakayo Mwangi**
>
> We sincerely appreciate your second reading and approval.
>
> *Competing Interests:* No competing interests were disclosed.

Version 1

Reviewer Report 10 October 2022

https://doi.org/10.5256/f1000research.136168.r150334

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The manuscript has been written on the findings of antimycobacterial resistance of NTM isolated from 122 sputum *M. tuberculosis*-negative patients. The authors have investigated their resistance patterns on the isolated strains using gene sequencing of the drug-resistant genes and noted the mutations in the respective codon positions.

Materials and methods have been well described. Data were properly analyzed and presented adequately in the tables. However, there were a lot of discrepancies in Table 2 in the first column where the total sample number was shown to be 135 instead of 122 and therefore the percentage expressions were wrongly mentioned in the table and in the text. The percentages have to be calculated correctly and Table 2 has to be modified and described accordingly in the text.

The discussion has been adequate, citing adequate references. However, the discussion with respect to the results of Table 2 has to be modified and discussed for a correct understanding of the reader.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Infectious diseases with special reference to tuberculosis and leprosy, Cell Biology, Microbiology, Immunology and Biotechnology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

> Author Response 17 Oct 2022
> **Zakayo Mwangi**
>
> I appreciate your review. The issue mentioned above has been addressed.
>
> *Competing Interests:* No competing interests were disclosed.

Reviewer Report 22 September 2022

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? **Leena Al-Hassan** iD
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This study characterises mutation patterns for resistance genes in NTM from Kenya.

Although the study is interesting, I think the authors need to give more information in the methodology and results section on:

- Identification: 122 isolates were collected, but no details in the results on how many were slow-growing and rapid-growing NTM.

- MIC: No mention of methodology for MIC and results.

- The PCR: Did you include any positive or negative controls in the experiments?

- The discussion on mutations and their role in resistance is quite vague. It's not clear whether the mutations identified can be linked to the observed MIC. Have the researchers checked that these are not silent mutations?

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Infectious diseases and antibiotic resistance

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 27 Sep 2022
Zakayo Mwangi

I appreciate your review.

- Under methods, it has been indicated that of the 122 NTM isolated, 54 were rapid-growing NTM while 68 were slow-growing NTM.

- The phenotypic drug sensitivity by MIC analysis was not done in this study. We performed genotypic characterization of the drug target genes by describing the mutations associated with drug resistance through target sequencing of the drug target genes.

- Positive and negative controls were included during PCR and subsequent sequencing of respective target genes. The controls were based on Genotype NTM-DR results.

- In the discussion, the mutations identified in this study have been compared to those seen in previous studies that had also included MIC in their analysis. The point mutations identified in the drug target genes were seen to be associated with phenotypic drug resistance.

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