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RESEARCH ARTICLE

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

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Abstract

Background: Nontuberculous 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 the 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 95% confidence interval tested the association of NTM to mutation patterns for each gene.

Results: Twenty-eight (23%) of the NTM were resistant to at least one of the antibiotics used in the macrolide-based treatment. Twelve (10.4%) of NTM were macrolide resistant, with 7(58.3%) of RGM and 5(41.7%) of SGM having mutations in the rrl gene. For ten (83.3%) NTM, mutations were found at position 2058, while for two (16.6%) NTM, mutations were found at position 2059. Six (11.1%) of the 54

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version 2 (revision) 24 Oct 2022	✗ view	✓ view
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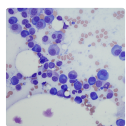1. **Leena Al-Hassan** [ID], Brighton and Sussex Medical School, Brighton, UK

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RGM exhibited mutations in the aminoglycoside target gene rrs at location 1408. Ten (14.7%) of the 68 SGM were resistant to rifampicin, with 40 percent having mutations at codon 531 in the rpoB gene. Conclusion: We demonstrated a significant level of 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

This article is included in the Cell & Molecular Biology gateway.

> **_REVISED_** **Amendments from Version 1**
>
> -Details about the following have been included in the methodology section: number of slow-growing and rapid-growing NTM in the 122 NTM isolates, and positive and negative control details in PCR analysis.
> -Under results, the variables in the first column of table 2 have been corrected to add up to 122 and the percentages calculated correctly.
> -In discussion, mutations identified for each gene and their role in drug resistance have been described.
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> **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, NTM varies in their growth characteristics, with rapid growing NTM (RGM) forming visible colonies within seven days of incubation and slow-growing NTM (SGM) taking up to forty 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 determining the best treatment plan (Chalmers *et al.*, 2019; Mwangi *et al.*, 2022).

NTM treatment consists of a macrolide-based antibiotic regimen, such as clarithromycin or azithromycin, combined with other selected antibiotics that work synergistically to disrupt NTM metabolic processes and growth (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).

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 mostly 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 including those caused by *M. tuberculosis* (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 occurring 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.*, 2022). The NTM isolates included 54 RGM and 68 SGM. The samples were obtained from the National Tuberculosis Reference Laboratory (NTRL) in Kenya between January to November 2020. All the 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 (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.

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 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.

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)

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 NTM with drug sensitivity to macrolides, aminoglycosides, and rifampicin was used, as well as a negative control containing drug sensitive NTM 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 fifteenminutes 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 twenty-eight (23%) of NTM are resistant 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 drug-resistant isolates originated from the Lake Victoria, Coastal, and Nairobi regions, with six (5%) NTM showing mutations in the *rrl*, *rrs*, or *rpoB* genes (p=0.012). 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 twenty one (76.5%) of the 28 drug-resistant NTM identified, indicating a strong statistical significance (p=0.000). The majority of drug-resistant NTM isolates 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).

Mutations in *rrl* gene for the NTM were highly significant with a p value of <0.001. Twelve (10.4%) of NTM were resistant to macrolides (Table 2) with seven (58.3%) of RGM (Table 3) and five (41.7%) of SGM (Table 3) showing mutations within *rrl* gene. Point mutation at position 2058 was seen in *M.intracellulare*, *M. abscessus subsp abscessus, M. nebraskense, M. massiliense, M. kumamototense, M. heraklionense,* and *M. bourgelatii*. Only for *M. abscessus subsp abscessus* was a mutation at 2059 observed (Table 3).

Resistance to aminoglycosides was demonstrated by mutation at position A1408G of the *rrs* gene for six (11.1%) of the 54 RGM. The NTM with aminoglycoside resistance 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 rifampicin resistance in SGM was observed. Mutations within codons between 503-533 of the *rpoB* were seen for ten (14.7%) SGM. These SGM included *M. avium subsp avium, M. intracellulare, M. yongonense* and *M. gastri* with mutations at codons 506, 509, 516, 526, and 531 respectively (Table 5).

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

| Variables | NTM (n, %) | Genes with mutations conferring 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)	

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

Nontuberculous 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 are resistant to at least one of the antibiotics in the macrolide-based therapy. Regional distribution of drug resistant NTM had a significant correlation (p=0.012) with the bulk of drug-resistant isolates originating from the Lake Victoria (n=6, 5%), Coastal (n=6, 5%), and Nairobi (n=6, 5%) regions. The observed regional diversity in drug resistant NTM 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 while males comprised the majority (n=21, 76.5%) of the drug-resistant NTM identified. NTM drug resistance was 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.*, 2022).

Despite NTM demonstrating high levels of resistance to a broad range of antibiotics, macrolides including clarithromy-cin, 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 has been studied at the molecular level and has consistently demonstrated that 80-100% of phenotypic macrolide resistant clinical isolates contain point mutations at positions A2058 and A2059 in the 23S rRNA gene among (Huh *et al.*, 2019). Our study demonstrated a similar pattern where *M. abscessus subsp abscessus* (A2058G/C, A2059G) and *M. intracellulare* (A2058G) formed the majority of isolates (58.3%) with macrolide resistance. Other NTM presenting with high levels of resistance to macrolides were *M. nebraskense* (A2058C), *M. massiliense* (A2058T), *M. kumamototense* (A2058T), *M. heraklionense* (A2058T), and *M. bourgelatii* (A2058C). The increased potential for development of drug resistance 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 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 aminoglycoside resistance with A1408G mutation. Genotypic characteristics in *rrs* that indicate 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).

Similar to *M. tuberculosis*, resistance to rifampicin in NTM is mediated by 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 ten (11.6%) rifampicin-resistant 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 drug resistance 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 a significant level of 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 macrolide resistance, and most macrolide-resistant NTM harbored mutation at position 2058. Majority of RGM had mutation at position 1408 of the *rrs* gene, while rifampicin resistant SGM had mutations within the RRDR of the *rpoB* gene.

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 sensitivity of NTM to antibiotics.

Despite this limitation, the study documents drug resistance 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 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 Nontuberculous Mycobacteria Isolates from Kenya. DOI: https://doi.org/10.6084/m9.figshare.20331378.v2.

This project contains the following underlying data:

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

Author contributions

Acknowledgments

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Version 2

Reviewer Report 22 November 2022

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

✗ **Leena Al-Hassan** iD

Department of Global Health and Infection, Brighton and Sussex Medical School, Brighton, UK

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.

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.

Reviewer Report 03 November 2022

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

Utpal Sengupta

Stanley Browne Laboratory, The Leprosy Mission Community Hospital, New Delhi, Delhi, India

The manuscript can be accepted now.

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.

Version 1

Reviewer Report 10 October 2022

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

Utpal Sengupta

Stanley Browne Laboratory, The Leprosy Mission Community Hospital, New Delhi, Delhi, India

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**, Meru University of Science and Technology, Meru, Kenya
>
> I appreciate your review. The issue mentioned above has been addressed.
>
> *Competing Interests:* No competing interests were disclosed.

F1000Research

Reviewer Report 22 September 2022

?

Leena Al-Hassan iD

Department of Global Health and Infection, Brighton and Sussex Medical School, Brighton, UK

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, Meru University of Science and Technology, Meru, Kenya

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.

Competing Interests: No competing interests

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