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High Level of Cross-Resistance between Kanamycin, Amikacin, and Capreomycin among *Mycobacterium tuberculosis* Isolates from Georgia and a Close Relation with Mutations in the *rrs* Gene▽

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The aminoglycosides kanamycin and amikacin and the macrocyclic peptide capreomycin are key drugs for the treatment of multidrug-resistant tuberculosis (MDR-TB). The increasing rates of resistance to these drugs and the possible cross-resistance between them are concerns for MDR-TB therapy. Mutations in the 16S rRNA gene (*rrs*) have been associated with resistance to each of the drugs, and mutations of the *tlyA* gene, which encodes a putative rRNA methyltransferase, are thought to confer capreomycin resistance in *Mycobacterium tuberculosis* bacteria. Studies of possible cross-resistance have shown variable results. In this study, the MICs of these drugs for 145 clinical isolates from Georgia and the sequences of the *rrs* and *tlyA* genes of the isolates were determined. Of 78 kanamycin-resistant strains, 9 (11.5%) were susceptible to amikacin and 16 (20.5%) were susceptible to capreomycin. Four strains were resistant to capreomycin but were susceptible to the other drugs, whereas all amikacin-resistant isolates were resistant to kanamycin. Sequencing revealed six types of mutations in the *rrs* gene (A514C, C517T, A1401G, C1402T, C1443G, T1521C) but no mutations in the *tlyA* gene. The A514C, C517T, C1443G, and T1521C mutations showed no association with resistance to any of the drugs. The A1401G and C1402T mutations were observed in 65 kanamycin-resistant isolates and the 4 capreomycin-resistant isolates, respectively, whereas none of the susceptible isolates showed either of those mutations. The four mutants with the C1402T mutations showed high levels of resistance to capreomycin but no resistance to kanamycin and amikacin. Detection of the A1401G mutation appeared to be 100% specific for the detection of resistance to kanamycin and amikacin, while the sensitivities reached 85.9% and 94.2%, respectively.

Although the first-line anti-tuberculosis (anti-TB) drugs rifampin (RMP; rifampicin), isoniazid (INH), ethambutol (EMB), pyrazinamide (PZA), and streptomycin (SM) were discovered several decades ago, they are still used today in standard short-course regimens for the treatment of TB. These regimens are, however, ineffective for the treatment of multidrug-resistant (MDR) TB (defined as resistance to at least the two most powerful anti-TB drugs, RMP and INH), leading to the use of less effective and more toxic second-line drugs (SLDs). Injectable drugs such as kanamycin (KAN), amikacin (AMK), and capreomycin (CAP) are the key SLDs for the treatment of MDR-TB (17). The emergence of extensively drug-resistant TB, defined as MDR-TB with additional resistance to any fluoroquinolone and at least one of the injectable drugs (10), once again underlines the importance of fast and reliable testing for susceptibility to these antibiotics.

Mutations in the 3′ part of the 16S rRNA gene (*rrs*), particularly at positions 1401, 1402, and 1484 (1, 7, 11, 12), have been associated with resistance to each of the drugs. It has also been suggested that mutations in the *tlyA* gene are responsible for resistance to CAP (8). Additionally, reports of cross-resistance among various aminoglycosides and CAP have been variable (1, 4, 6, 16). Most of the previous investigations were done with laboratory-generated mutants and with only a limited number of clinical isolates. In this work, we investigated the correlation between mutations in the *rrs* and *tlyA* genes and the in vitro resistance to the three injectable drugs of clinical *Mycobacterium tuberculosis* isolates.

MATERIALS AND METHODS

Sample. The isolates used for this study were chosen on the basis of routine SLD susceptibility testing at the Georgian National Reference Laboratory, Tbilisi, Georgia. Isolates from 80 cultures with known or suspected resistance to KAN and/or CAP and 70 sensitive isolates were selected from the Georgian National Reference Laboratory culture collection, which contains isolates from all regions of the country. All isolates were subjected to MIC testing, sequencing of the 1,400-bp region of the *rrs* gene (see below for the definition) and the complete *tlyA* gene, and mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing. In addition, a selection of 57 isolates, which included both aminoglycoside-resistant and -sensitive strains, was sequenced to detect mutations in the 500-bp region of the *rrs* gene (see below for the definition).

MIC determination. The MICs of the drugs were determined by using Löwenstein-Jensen medium containing the following concentrations of drugs: 7.5, 15, 30, 60, and 120 μg/ml for KAN; 7.5, 15, 30, 40, 60, and 120 μg/ml for AMK; and 10, 20, 40, 80, and 160 μg/ml for CAP. Pure active substances were obtained from Sigma-Aldrich (Bornem, Belgium) or Acros Organon (Geel, Belgium). All tubes were incubated at 37°C for 28 days. The MIC was defined as the lowest concentration of drug resulting in the complete inhibition of growth or growth that constituted <1% of the inoculum. The resistance cutoff concentration was defined according to World Health Organization recom-

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TABLE 1. Primers used for PCR

Primer name	Target DNA	Sequence
KM-SA	1400 *rrs* region	5′-AAGTACCCCGCCTGGGGAGTACGG-3′
KM-RA	1400 *rrs* region	5′-GGTGGGACAACACCTGGAACAAGTC-3′
KM-SEQ	1400 *rrs* region	5′-CTAGAGATAGGCGTTCCCTTGTGG-3′
P1	500 *rrs* region	5′-TGCTTAACACATGCAAGTCG-3′
P2	500 *rrs* region	5′-TCTCTAGACGCGTCCTGTGC-3′
P7	500 *rrs* region	5′-CATGCAAGTCGAACGGAAAGG-3′
TlyA-SA	*tlyA*	5′-CGACGTCGGTGGTGGTGCGGTA-3′
TlyA-RA	*tlyA*	5′-GTCCGGTCTTCCACCCGGTAATCCT-3′
TlyA-Seq	*tlyA*	5′- CGATCGCACGTCGTCTTTCCGA-3′

mendations (18), as follows: for KAN, 30 μg/ml; for AMK, 40 μg/ml; and for CAP, 40 μg/ml.

Primer design. Three sets of primers have been designed (Table 1).

The first combination of primers, primers KM-SA and KM-RA, was used to amplify an 831-bp sequence containing the 3′ end of the *rrs* gene and part of the adjacent spacer sequence (referred as the "1400 *rrs* region" throughout the text). The second set of primers, primers P1 and P2, yielded a 920-bp amplicon that covered the 5′ end of the *rrs* gene (referred as the "500 *rrs* region" throughout the text). The final set of primers, primers TlyA-SA and TlyA-RA, was used to amplify the complete *tlyA* gene.

Oligonucleotides were designed on the basis of an alignment of the respective sequences from reference strain *M. tuberculosis* H37Rv (GenBank accession no. NC_000962; NCBI bank), some relevant non-*M. tuberculosis* mycobacteria, and nonmycobacterial species by using CLC sequence viewer software (version 4.6.1). Amplify software (version 1.2; University of Wisconsin—Madison) was used to estimate the stabilities and binding capacities of the selected oligonucleotides and to simulate PCRs.

The specificities of the *rrs* primers were evaluated with a set of 24 *M. tuberculosis* complex isolates, 18 non-*M. tuberculosis* mycobacterial isolates, and 10 nonmycobacterial isolates, whereas the sensitivities were determined with a logarithmic dilution of *M. bovis* DNA.

DNA extraction and amplification. DNA extraction was done by suspending a loopful of bacteria in 1× TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA) and boiling the mixture for 5 min. The extracted DNA was analyzed immediately or was stored at <−18°C.

PCRs for both *rrs* regions and the *tlyA* gene were performed in a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.6), 50 mM KCl, 1.65 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 12.5 pmol of each appropriate primer, 1.5 U *Taq* polymerase (Promega, Madison, WI), and 2 μl of DNA extract. PCR was performed with a PTC 100 MJ Research (Waltham, MA) thermocycler.

TABLE 2. Relation between profiles of resistance to KAN, AMK, and CAP in vitro and mutations in the *rrs* gene for 145 *M. tuberculosis* isolates[a]

Susceptibility to:			No. of isolates with the following mutation:			
KAN	AMK	CAP	A1401G	C1402T	NM	Total
R	S	S	0	0	9	9
R	R	S	5	0	2	7
R	R	R	60	0	2	62
S	S	R	0	4	0	4
S	S	S	0	0	63	63

[a] Abbreviations: R, resistant; S, susceptible; NM, no mutation or mutations other than A1401G and C1402T.

TABLE 3. Relation between resistance to KAN, AMK, and CAP in vitro and mutations in the 1400 *rrs* region for 145 *M. tuberculosis* isolates

Antibiotic	No. of isolates with the indicated susceptibility and the following mutation[a]:									
	A1401G		C1402T		C1443G		T1521C		NM	
	R	S	R	S	R	S	R	S	R	S
KAN	65	0	0	4	0	1	0	1	13	61
AMK	65	0	0	4	0	1	0	1	4	70
CAP	60	5	4	0	0	1	0	1	2	72

[a] Abbreviations: R, resistant; S, susceptible; NM, no mutation.

The following protocol was used to amplify the 1400 *rrs* region: a hot start (90°C), followed by 5 min at 94°C; 40 cycles of 45 s at 94°C, 45 s at 66°C, and 45 s at 72°C; and a final extension of 10 min at 72°C. For the amplification of the 500 *rrs* region and the *tlyA* gene, the same conditions described above were used but the annealing temperatures were 56°C and 64°C, respectively. All PCR products were checked for amplification quality on a 2% agarose gel before they were submitted for sequencing.

Sequencing. Direct single-strand sequencing of the *rrs* and *tlyA* amplicons was done at the Genetic Service Facility of Antwerp University (Antwerp, Belgium) with a capillary sequencer (Applied Biosystems 3730 DNA analyzer) in combination with an ABI Prism BigDye Terminator cycle sequencing kit. Primers KM-SEQ, P7, and TlyA-Seq were used for sense sequencing of the 1400 *rrs* region, the 500 *rrs* region, and the *tlyA* gene, respectively (Table 1). CLC sequence viewer software (version 4.6.1) was used to analyze the final nucleotide sequences in comparison to the *M. tuberculosis* H37Rv wild-type sequence.

MIRU-VNTR typing. Typing of all isolates was performed by using the standard 15 MIRU-VNTR locus format of Genoscreen (Lille, France). Analysis of the patterns was done by using the web application MIRU-VNTRplus (http://www.miru-vntrplus.org).

RESULTS

Of the 150 isolates initially selected, complete MIC and sequencing data were obtained for only 145 isolates, thus defining the final sample size. For five isolates, insufficient growth was observed to allow interpretation of the MIC testing results, and this was also the case after repeated testing.

MIC results. Seventy-eight of the 145 isolates were found to be resistant to KAN. Of these, 9 (11.5%) were susceptible to AMK (MICs, 15 to 30 μg/ml) and 16 (20.5%) were resistant to CAP (MICs, 10 to 40 μg/ml). Four strains were highly resistant to CAP (MICs, >160 μg/ml) but sensitive to the other drugs tested (Table 2). Isolates resistant to both KAN and AMK showed high MICs for both drugs (≥120 μg/ml), whereas three of nine KAN-resistant but AMK-susceptible isolates had lower MICs for KAN (60 μg/ml).

Sequencing results for the 1400 rrs region. The newly developed primers used for the amplification of the 1400 *rrs* region (primers KM-SA and KM-RA) proved to be specific for the *M. tuberculosis* complex, and high-quality amplicons were obtained for all *M. tuberculosis* complex isolates.

Among the 145 isolates sequenced, four types of mutations were observed in the 1400 *rrs* region: A1401G, C1402T, C1443G, and T1521C. The resistance profiles associated with these mutations are shown in Table 3.

The most frequently observed mutation within the region was an A-to-G substitution at position 1401. All isolates with this mutation had high-level resistance to both KAN and AMK (MICs, ≥120 μg/ml) but showed various MICs for

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TABLE 4. Relation between resistance to KAN, AMK, and CAP in vitro and mutations in the 500 *rrs* region for 59 *M. tuberculosis* isolates

Antibiotic[a]	No. of isolates with the indicated susceptibility and the following mutation[a]:					
	A514C		C517T		NM	
	R	S	R	S	R	S
KAN	2	4	3	3	18	29
AMK	1	5	1	5	12	35
CAP	1	5	1	5	14	33

[a] Abbreviations: R, resistant; S, susceptible; NM, no mutation.

CAP (MICs, ≤10 to 160 μg/ml), with five of them being considered CAP sensitive (one isolate had an MIC of ≤10 μg/ml and four isolates had MICs of 40 μg/ml). None of the nine isolates which were resistant only to KAN had a mutation at position 1401 (Table 2). Also, no mutations at position 1401 were seen among the 63 strains not resistant to any of the drugs tested (Table 2). Four isolates carried a C-to-T substitution at position 1402; all of those isolates showed high-level resistance to CAP (MICs, ≥160 μg/ml), while they showed low-level resistance to KAN and AMK (MICs, 15 to 30 μg/ml). Two isolates had the C1443G and the T1521C mutations (Table 3). Both of those isolates had the lowest MICs for all drugs tested.

Of 74 isolates that showed no mutations in the 1400 *rrs* region, 13 were found to be resistant to KAN, 4 were found to be resistant to AMK, and 2 were found to be resistant to CAP (Table 3). Among the 13 KAN-resistant isolates, high MICs (≥120 μg/ml) were observed for 10 isolates, and 3 isolates showed lower-level resistance (MICs, 60 μg/ml). The four AMK-resistant isolates with no mutations also yielded the highest MIC tested, while the two CAP-resistant isolates with no mutation had MICs of 80 μg/ml.

Sequencing results for the 500 rrs region. To study the relevance of mutations in the 500 *rrs* region for aminoglycoside and polypeptide resistance, we selected 59 isolates, which comprised all isolates that showed in vitro resistance to any of the drugs tested but that had no mutations in the 1400 *rrs* region (n = 35), 10 KAN-resistant isolates with the A1401G mutation, and 14 KAN-susceptible isolates. Of the 59 isolates tested, an A-to-C nucleotide change at position 514 was observed in 6 isolates, and a C-to-T mutation at position 517 was observed in another 6 isolates (Table 4). No mutations were observed in the remaining 47 isolates. Mutations were observed in both resistant and susceptible isolates.

Sequence results for the *tlyA* gene. Sequencing of the *tlyA* gene showed no mutations in any of the 145 isolates.

MIRU-VNTR typing results. MIRU-VNTR typing revealed that the majority (113/145; 77.9%) of isolates belonged to the Beijing lineage, whereas the rest of the isolates were identified as belonging to the LAM, Haarlem, or Ural family (data not shown). Two big clusters showing identical MIRU-VNTR profiles were observed within the Beijing group, with cluster 1 containing 32 isolates and cluster 2 containing 28 isolates. Other smaller clusters ranging from two to six isolates were seen in all genotypes. Aminoglycoside-resistant and -susceptible isolates were observed among the isolates with the various MIRU-VNTR profiles. Nine A1401G mutations were found in cluster 1 (28.1%) and 15 A1401G mutations were found in cluster 2 (53.6%). The remaining mutants with the A1401G mutation were distributed among the various MIRU-VNTR profiles. All four CAP-resistant isolates that yielded a C1402T mutation clustered in a group of six isolates within the Beijing family (cluster 3).

DISCUSSION

The results reported here are the first from a large-scale study of the relation between mutations in the *rrs* and *tlyA* genes and resistance to KAN, AMK, and CAP among isolates from clinical specimens.

Several previous investigations have repeatedly demonstrated general cross-resistance between AMK and KAN, and these drugs were considered interchangeable for drug susceptibility testing (1, 4, 16). As opposed to this, Krüüner et al. (6) presented data showing discordant resistance between KAN and AMK. Our findings support the results of the latter group. An important proportion (11.5%; 9/78) of KAN-resistant isolates still remains susceptible to AMK. The rate of cross-resistance between KAN and CAP was similar to that determined in our previous study of isolates from individuals in the penitentiary system in Georgia, where high levels of resistance to the drug were found, even though there was no history of CAP usage (5). These data question the habit of generalizing resistance to a class of drugs, e.g., cyclic peptides or aminoglycosides, on the basis of resistance to only one drug in the class.

In order to unravel the molecular background of resistance, mutations that most probably do not confer resistance to the drugs tested should be ruled out. In our study, the C1443G and T1521C mutations in the *rrs* gene found in two isolates showed no association with resistance to any of the drugs. To the best of our knowledge, these mutations have not previously been described as conferring resistance.

Similarly, mutations in the 500 *rrs* region (A514C and C517T) have previously been reported by Maus et al. (7) and Krüüner et al. (6), but both groups of researchers suggested that these mutations had no influence on resistance to the drugs mentioned. Our findings support this suggestion, since both mutations showed a clear dissociation with the resistance patterns. Additionally, Victor et al. (15) have proposed that the C-to-T nucleotide change at position 491 of the *rrs* gene (close to the position where we found the thymine-for-cytosine substitution) is a polymorphism not associated with drug resistance. In our study, however, all isolates with these mutations were streptomycin resistant, which is proposed to be linked with nucleotide changes in the 500 *rrs* region (2, 3, 9). These data suggest that the A514C, C517T, C1443G, and T1521C mutations do not play a role in resistance to the drugs studied.

Previous reports described G-to-T nucleotide changes at position 1484 of the *rrs* gene as being related to resistance to KAN, AMK, and CAP (7, 11, 12). In addition, Maus et al. (7, 8) demonstrated several times that mutations in the *tlyA* gene confer resistance to CAP. Interestingly, none of the isolates tested by us revealed these changes. It is worth noting that most of the previously reported changes in *tlyA* were found among laboratory-generated mutants and only 5 of 18 (7) and

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0 of 16 (8) of the clinical isolates tested. On the other hand, CAP has not been used extensively (if at all) in Georgia (National Tuberculosis Program, personal communication), while both KAN and AMK were widely misused. This suggests that the vast majority of the cases of resistance to CAP emerged as cross-resistance to KAN and/or AMK through a mutation(s) in the *rrs* gene. It is tempting to suggest that if mutations in the *tlyA* gene do play an important role in CAP resistance, they occur as a result of mutant selection due to the direct misuse of CAP. This hypothesis requires further research.

On the contrary, we found a clear relation between the A1401G mutation and resistance to AMK or KAN. Detection of an A1401G substitution appeared to be 100% specific for the detection of KAN and AMK resistance, while sensitivities reached 85.9% and 94.2%, respectively. The latter sensitivities are close to the sensitivity for the detection of mutations in the *rpoB* gene (±95%) in RMP-resistant *M. tuberculosis* isolates (13, 14).

The correlation between a mutation at position 1401 and resistance to AMK and KAN observed in our study was stronger than that reported by other researchers (7, 11). Although this result may be biased by the widespread misuse of KAN and AMK in Georgia, which leads to high-level resistance, it may still represent a reality worldwide, and the A1401G mutation could be the main cause of clinically significant resistance in vivo. Also, even though several large MIRU-VNTR clusters were found within the sample of isolates studied, suggesting recent transmission and the clonal distribution of drug-resistant strains, the A1401G mutation was evenly distributed among clusters and different genotypes. Additionally, reports from other countries identify mutations at the same sites.

Comparison of MIC data and mutations suggests that a nucleotide substitution at position 1401 is closely linked to resistance to both KAN and AMK, but it cannot fully explain the various patterns of CAP MICs. An interesting finding was the relation between high-level CAP resistance and the C1402T mutation. Although all four strains with this mutation were included in one MIRU-VNTR cluster, the results correspond to the findings of Maus et al. (7), and we suggest that this mutation accounts for CAP resistance. Since 16S rRNA is not translated into amino acids but represents a direct portion of the 30S ribosome subunit, we assume that the 1400 region plays an important role in the drug-ribosome interaction for the drugs tested. The fact that various CAP MICs were detected among isolates with a mutation at position 1401 suggests that position 1402 is a greater determinant of the drug-ribosome interaction in the case of CAP and that a nucleotide substitution at the adjacent position, position 1401, only partially confers resistance to CAP. This might explain the high number of isolates with cross-resistance to CAP in settings where the drug is not widely used. Further study is required to address this hypothesis.

Finally, the presence of resistance without associated changes in the *rrs* or *tlyA* gene suggests that other genes or factors may be responsible for resistance in these cases. The absence of mutations in a specific gene is not highly predictive of the absence of resistance to most first-line anti-TB drugs, and often, more than one gene is involved in resistance. To the best of our knowledge, no other genes involved in resistance to the drugs tested have so far been described in general bacteriology. Additionally, the role of an efflux pump cannot be ruled out and should be further studied. These factors may be additive or synergistic when they are combined with mutations in the *rrs* gene, which could also perhaps partially explain the variety of CAP MICs among mutants with the A1401G mutation.

The importance of the fast diagnosis of extensively drug-resistant TB underlines the need for the rapid detection of resistance to aminoglycosides and CAP. As mutations at position 1401 seem to have a relative good value for predicting resistance to AMK (and slightly less value for predicting resistance to KAN), they could be detected by rapid diagnostic systems, such as reverse hybridization assays and multiplex ligation-dependent probe amplification assays. The use of *M. tuberculosis*-specific primers could have an advantage when a detection system is directly applied to clinical specimens.

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