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MECHANISMS OF RESISTANCE

Evidence for Expanding the Role of Streptomycin in the Management of Drug-Resistant *Mycobacterium tuberculosis*

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ABSTRACT In 2019, the WHO tuberculosis (TB) treatment guidelines were updated to recommend only limited use of streptomycin, in favor of newer agents or amikacin as the preferred aminoglycoside for drug-resistant *Mycobacterium tuberculosis*. However, the emergence of resistance to newer drugs, such as bedaquiline, has prompted a reanalysis of antitubercular drugs in search of untapped potential. Using 211 clinical isolates of *M. tuberculosis* from South Africa, we performed phenotypic drug susceptibility testing (DST) to aminoglycosides by both critical concentration and MIC determination in parallel with whole-genome sequencing to identify known genotypic resistance elements. Isolates with low-level streptomycin resistance mediated by *gidB* were frequently misclassified with respect to streptomycin resistance when using the WHO-recommended critical concentration of 2 μg/ml. We identified 29 *M. tuberculosis* isolates from South Africa with low-level streptomycin resistance concomitant with high-level amikacin resistance, conferred by *gidB* and *rrs* 1400, respectively. Using a large global data set of *M. tuberculosis* genomes, we observed 95 examples of this corresponding resistance genotype (*gidB-rrs* 1400), including identification in 81/257 (31.5%) of extensively drug resistant (XDR) isolates. In a phylogenetic analysis, we observed repeated evolution of low-level streptomycin and high-level amikacin resistance in multiple countries. Our findings suggest that current critical concentration methods and the design of molecular diagnostics need to be revisited to provide more accurate assessments of streptomycin resistance for *gidB*-containing isolates. For patients harboring isolates of *M. tuberculosis* with high-level amikacin resistance conferred by *rrs* 1400, and for whom newer agents are not available, treatment with streptomycin may still prove useful, even in the face of low-level resistance conferred by *gidB*.

KEYWORDS *Mycobacterium tuberculosis*, aminoglycosides, drug resistance mechanisms, multidrug resistance, tuberculosis, whole-genome sequencing

Despite recent advances, tuberculosis (TB) remains the number one infectious killer worldwide (1). The ongoing global epidemic of drug-resistant TB and limited effective treatment regimens for drug-resistant *Mycobacterium tuberculosis* have resulted in significant morbidity and mortality (1). Recognition of the inadequacy of the current antitubercular drug development pipeline, and the emergence of resistance to new drugs—including bedaquiline (2–9), delaminid (3, 4), clofazimine (5, 7), and linezolid (6)—has prompted a reanalysis of the existing arsenal of antitubercular drugs in search of untapped potential. Streptomycin may be one such underutilized drug.

Discovered in 1944, streptomycin, an injectable streptidine aminoglycoside antibiotic, was the first antimicrobial agent with proven activity against *M. tuberculosis*. In

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TABLE 1 Distribution of resistance-associated mutations in a South African data set[a]

Drug	Gene	Polymorphism[b]	Number of isolates
Streptomycin	*rpsL*	K43R	31
		K88R	8
	rrs (non-1400)	513	7
		516	6
		907	1
	gidB	nt 62, del 1 bp	1
		nt 103, del 1 bp	1
		nt 108, del 1 bp	3
		nt 116, del 1 bp	3
		nt 282, del 130 bp	78
		nt 368, del 2 bp	1
		A134E	3
		A138V	2
		A141E	2
Kanamycin/amikacin	*rrs*	1400	50
Kanamycin	*eis*	promoter −14	2

[a]Of the 211 South African isolates, 140 were found to have genotypic streptomycin resistance with mutations in *rpsL*, *rrs* (non-1400), and *gidB*, as detailed in the table. Fifty strains were found to have genotypic amikacin/kanamycin resistance with mutations in *rrs* 1400, and two isolates had kanamycin resistance with an *eis* promoter mutation.
[b]nt, nucleotide; del, deletion; bp, base pairs.

conjunction with isoniazid and para-aminosalicylic acid (PAS), streptomycin formed part of the first multidrug combination chemotherapy for TB, introduced in 1952. Its initial widespread use led to the early emergence of streptomycin resistance, which subsequently limited its clinical utility. Streptomycin remained an integral component of first-line TB therapy until the 1980s, and its empirical use in retreatment TB regimens was recommended until recently (10).

While the majority of the molecular determinants of aminoglycoside resistance are known, commercial diagnostic tests that assay for genotypic streptomycin resistance are lacking. Resistance to streptomycin does not contribute to the definition of extensively drug resistant (XDR) TB, which is defined as multidrug-resistant (MDR) isolates with additional resistance to quinolones and other injectable agents (amikacin, kanamycin) (11). Streptomycin is currently classified as a group C second-line agent for use in longer MDR-TB regimens (10), which are recommended in limited circumstances only. While XDR isolates are frequently cross-resistant to second-line injectable agents, there may be untapped potential for continued use of streptomycin for low-level resistance.

In our large collection of *M. tuberculosis* isolates from South Africa, we characterized aminoglycoside-resistance phenotypes in conjunction with whole-genome sequencing to identify patterns of aminoglycoside resistance. Subsequently, we used a global data set of over 5,000 *M. tuberculosis* genomes to assess the occurrence of genotypic low-level streptomycin resistance concomitant with high-level amikacin resistance worldwide.

RESULTS

Using 211 sequenced clinical isolates of *M. tuberculosis* from South Africa (Table S2 in the supplemental material), we performed critical concentration testing for streptomycin and kanamycin, and observed incomplete cross-resistance between these two aminoglycosides (Table S3). Amikacin critical concentration was not performed due to anticipated near complete cross-resistance with kanamycin (12), which was confirmed by our MIC testing (Fig. S1). Using genomic sequences for these 211 isolates, we sought known drug resistance markers for these aminoglycoside drugs. A total of 140 isolates were found to have genotypic markers of streptomycin resistance, with mutations in *rpsL*, *rrs* (non-1400), and *gidB*, whereas 50 isolates had mutations in *rrs* 1400, which confers high-level resistance to both amikacin and kanamycin (Table 1). Two isolates

TABLE 2 Distribution of co-occurring genotypic resistances to streptomycin and amikacin/kanamycin in a South African data set[a]

	Amikacin/kanamycin genotype			
Streptomycin genotype	**WT**	***rrs* 1400**	***eis* promoter**	**Total**
WT	67	2	2	71
rpsL	33	2	0	35
rrs (non-1400)	8	5	0	13
gidB	44	**41**[b]	0	85
Total	152	50	2	204

[a]Of note, 7 isolates were identified to contain more than one streptomycin resistance mutation, as described in Table S4 in the supplemental material.
[b]The boldface type indicates the number of isolates with co-occurrence of *gidB* and *rrs* 1400 mutations that confer low-level streptomycin and high-level amikacin resistance.

contained mutations in the promoter region of *eis*, which confers resistance to kanamycin, but not to streptomycin or amikacin. Co-occurrence of streptomycin and amikacin/kanamycin resistance genotypes was determined (Table 2), including identification of seven isolates with more than one streptomycin-resistance-determining mutation (Table S4).

In comparing MIC data from Sensititre testing with known aminoglycoside resistance genotypes, we evaluated the relationship between genotypic and phenotypic resistance to streptomycin (Fig. 1A), amikacin (Fig. 1B), and kanamycin (Fig. S1). There was a bell-shaped distribution (Fig. 1A) of isolates containing *gidB* mutations with low-level streptomycin resistance (median MIC 4 μg/ml; interquartile range [IQR], 2 to 4 μg/ml) (Table 3). By critical concentration testing per the WHO-recommended guidelines, the majority of isolates with *gidB* mutations (76%, 70/92) were classified as resistant to streptomycin. In contrast, high-level streptomycin resistance was observed in isolates with either *rrs* (non-1400) or *rpsL* mutations, with median MIC 32 μg/ml (IQR, 16 to 32 μg/ml) and 32 μg/ml (IQR, 16 to 32 μg/ml), respectively. Three isolates with no identifiable streptomycin mutations were noted to have high MICs to streptomycin (MIC 16 to 32 μg/ml), suggesting that additional streptomycin resistance mutations remain to be discovered, but this could also be due to errors in phenotyping. Nearly every isolate with high-level amikacin and kanamycin resistance contained an *rrs* 1400 mutation (Fig. 1B, Fig. S2).

When comparing the MIC of each isolate to streptomycin and amikacin, numerous isolates had mismatched phenotypes, indicating that resistance to amikacin did not confer resistance to streptomycin, and vice versa (Fig. 2). In particular, 29 isolates from South Africa exhibited low-level streptomycin resistance (MIC 4 μg/ml or 8 μg/ml) and concomitant high-level amikacin resistance (MIC $\geq$16 μg/ml) (circled area, Fig. 2). These findings suggest that use of streptomycin instead of amikacin would be the preferred aminoglycoside for treatment of these isolates. The vast majority of isolates with this phenotype (93%, 27/29) contained a *gidB* resistance genotype, and 100% (29/29) contained an *rrs* 1400 mutation.

From the genomic data, we constructed a phylogeny to determine the interrelatedness of isolates with (i) low-level streptomycin resistance and (ii) concomitant low-level streptomycin and high-level amikacin resistance in phenotypic testing (Fig. 3). The 57 isolates with low-level streptomycin resistance were distributed throughout the phylogeny. The majority (25/29, 86%) of South African isolates with low-level streptomycin and high-level amikacin resistance belonged to the Tugela Ferry XDR clone, which was responsible for epidemic XDR in the region in the early 2000s (13). However, there were four isolates outside this cluster, indicating that this phenomenon was not unique to this clone.

To determine whether the phenomenon of low-level streptomycin and high-level amikacin resistance occurred outside South Africa, we analyzed our large data set of 5,310 *M. tuberculosis* isolates from 43 countries (14). Within this data set, 257 isolates contained mutations for resistance to all four drugs that define XDR (rifampin, isoniazid,

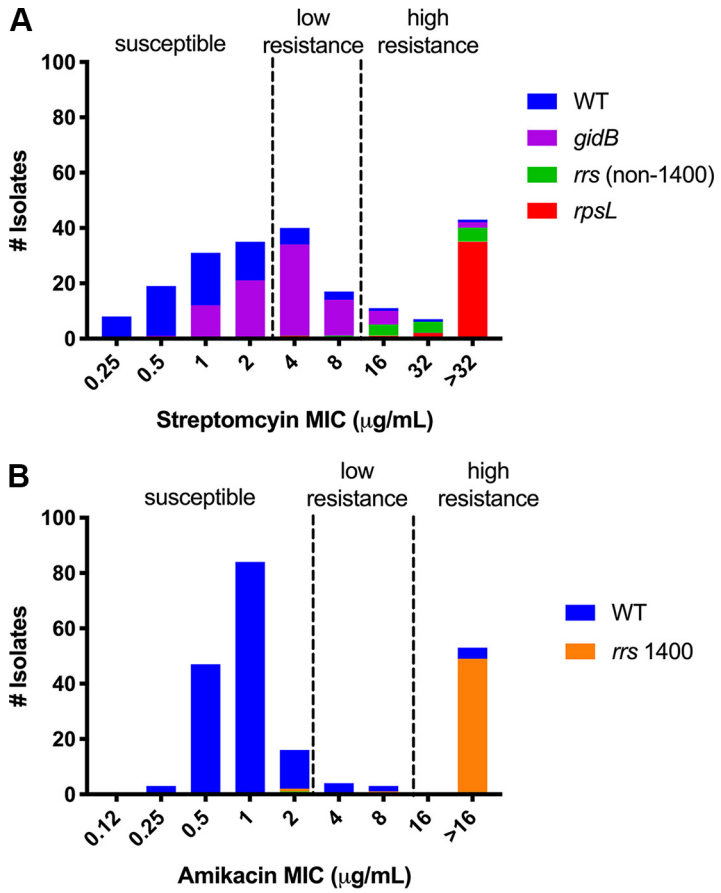

FIG 1 Clinical strains of *M. tuberculosis* were observed to have a range of susceptibility to aminoglycosides, mediated by resistance genotype. A total of 211 isolates of *M. tuberculosis* from South Africa underwent MIC determination and aminoglycoside resistance genotyping to identify mutations that confer resistance to streptomycin or amikacin, respectively. (A) Streptomycin MIC testing revealed a bell-shaped curve distribution of *gidB* strains with low-level streptomycin resistance, whereas strains containing *rrs* (non-1400) or *rpsL* mutations had higher resistance. Of note, three isolates containing resistance elements in both *gidB* and *rpsL* were included among the *rpsL* isolates. (B) Amikacin MIC testing revealed high-level resistance among strains containing the *rrs* 1400 mutation. Kanamycin MIC results mirrored that of amikacin (Fig. S1 in the supplemental material).

ofloxacin, and amikacin). As phenotypic data were not available for this data set, we used co-occurrence of a *gidB* resistance mutation and *rrs* 1400 mutation as a genotypic predictor of this combination of low-level streptomycin resistance and high-level amikacin resistance (Fig. 4). We identified 378 unique isolates with *gidB* mutations, including 95 isolates with co-occurrence of *gidB* mutations and *rrs* 1400 mutation (Table S5). All 95 isolates contained resistance-conferring mutations to both isoniazid and rifampin (MDR genotype) in addition to resistance to either ofloxacin or kanamycin (pre-XDR), and 81/95 of these isolates were XDR. Of the 257 XDR isolates in the 5,310-isolate data set, 81 (31.5%) of the XDR isolates contained this *gidB-rrs* 1400

TABLE 3 *gidB* mutations confer low-level streptomycin resistance, whereas *rrs* and *rpsL* mutations confer high-level resistance

Streptomycin genotype	Median MIC to streptomycin in μg/ml (IQR)[a]
WT	1 (0.5–2)
gidB	4 (2–4)
rrs (non-1400)	32 (16–32)
rpsL	32 (32–32)

[a]For each streptomycin resistance genotype, median MIC to streptomycin and interquartile range (IQR) is listed.

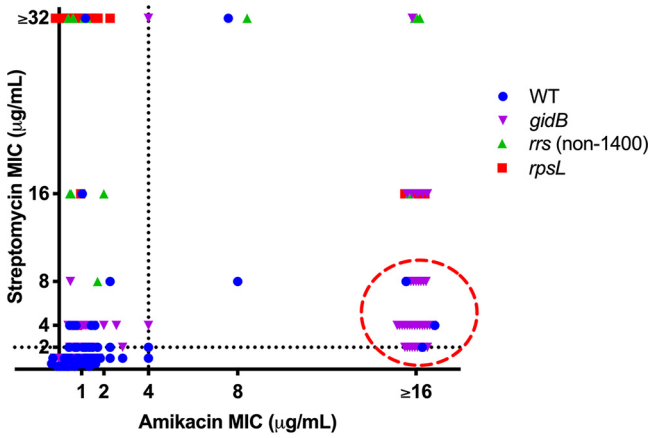

FIG 2 Significant numbers of *M. tuberculosis* isolates exhibit concomitant low-level streptomycin resistance and high-level amikacin resistance. Isolates are represented by streptomycin genotype (see key) and plotted as a function of the relative phenotypic resistance to both amikacin and streptomycin. The red dotted circle indicates the 29 isolates with concomitant low-level streptomycin resistance and high-level amikacin resistance.

combination, indicating frequent occurrence in global XDR-TB. The majority of isolates with the *gidB-rrs* 1400 pattern were LAM4 and likely members of the Tugela Ferry XDR clade. However, there were nine other spoligotypes with isolates containing this pattern, indicating multiple independent evolutionary events. Beyond South Africa, isolates with this resistance pattern were also identified in Belarus, China, Iran, Portugal, Romania, South Korea, and Sweden, indicating that this phenomenon of streptomycin-low and amikacin-high resistance is of global importance for management of drug-resistant TB.

DISCUSSION

In both a South African and a global data set, significant numbers of *M. tuberculosis* isolates contained mutations associated with concomitant low-level streptomycin resistance and high-level amikacin resistance. Current guidelines that recommend only limited use of streptomycin (10) may be unwittingly withholding a potentially lifesaving, inexpensive, and available drug from certain patients with drug-resistant TB. Similarly, current WHO-endorsed laboratory procedures for performing phenotypic DST to streptomycin by critical concentration may obscure the potential utility of streptomycin by not distinguishing between high and low-level resistance.

Given additional newer agents with excellent activity against drug-resistant TB, such as bedaquiline, the updated 2019 WHO guidelines limit use of aminoglycosides (10). Kanamycin is no longer recommended in the treatment of drug-resistant TB patients on longer regimens. Amikacin is now the preferred aminoglycoside, and its use is limited to adults on longer regimens in situations in which DST results confirm susceptibility and for whom high-quality audiometry testing for hearing loss can be performed. Streptomycin use is recommended only when amikacin is not available, and again in situations when DST results confirm susceptibility and in whom safety monitoring can be ensured.

While treatment-related ototoxicity and nephrotoxicity are well established, streptomycin could still hold therapeutic potential for individuals with drug-resistant TB harboring isolates with low-level streptomycin resistance. If an aminoglycoside is being considered for inclusion in a drug-resistant TB regimen, if the *rrs* 1400 mutation is present, which confers high-level resistance to amikacin, than we recommend selection of streptomycin, even in the face of low-level resistance, such as that conferred by *gidB*. To our knowledge, clinical outcomes for individuals harboring isolates with low-level streptomycin resistance mediated by *gidB* and treated with a streptomycin-containing regimen have not been assessed. An expanded role for streptomycin in drug-resistant

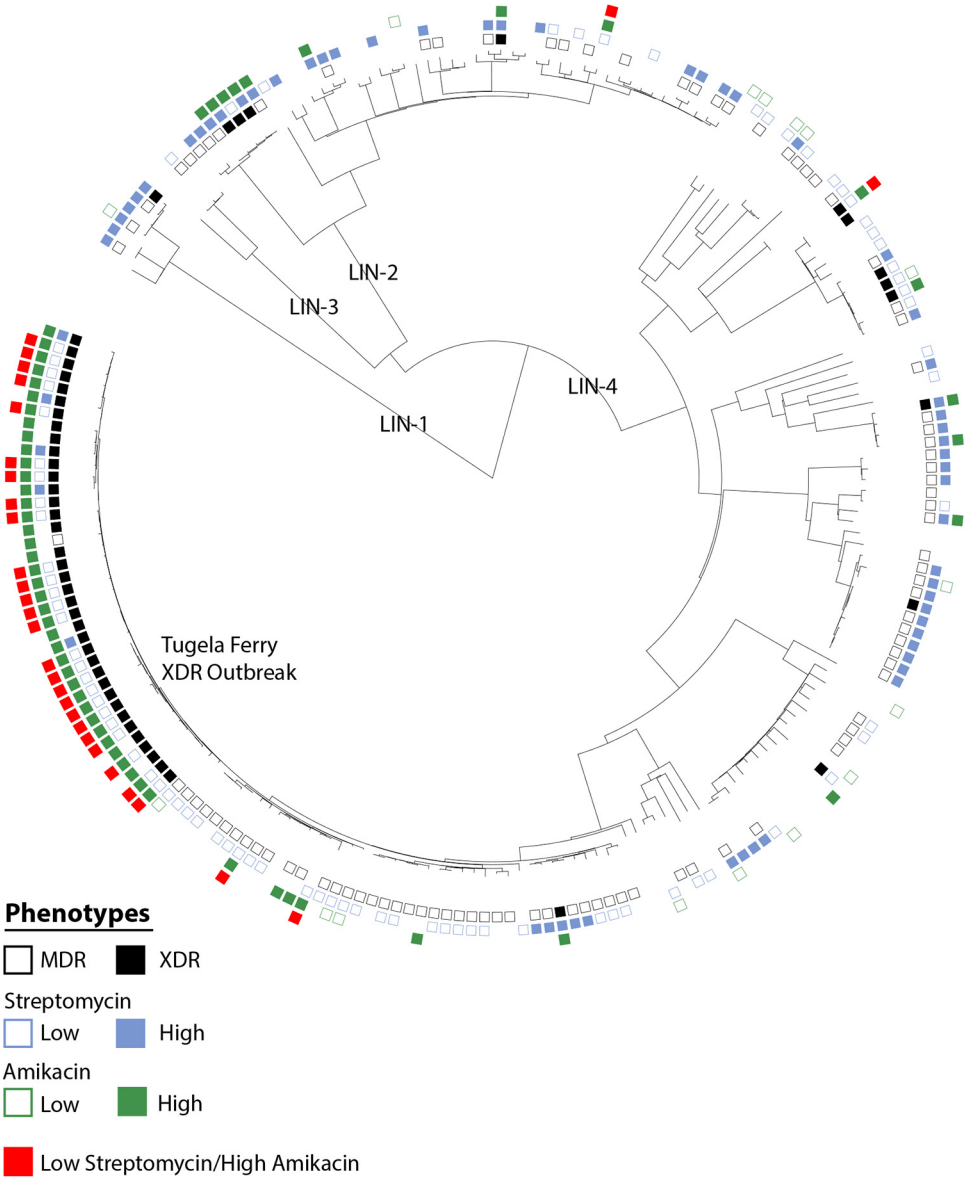

FIG 3 Concomitant low-level streptomycin and high-level amikacin phenotypic resistance in South African *M. tuberculosis* isolates across the phylogeny. Midpoint rooted maximum-likelihood phylogeny of 211 *M. tuberculosis* isolates, containing representatives of four of the seven known *M. tuberculosis* lineages. Phenotypic MDR and XDR are indicated by black and white boxes at the tip of each leaf node. The levels of phenotypic resistance to streptomycin (low, MIC 4 to 8 μg/ml; high, MIC $\geq$16 μg/ml) and amikacin (low, MIC 4 to 8 μg/ml; high, MIC $\geq$16 μg/ml) are indicated by box color, per the key. Strains with concomitant low-level streptomycin resistance and high-level amikacin resistance are indicated in red. While the majority of isolates with low-level streptomycin resistance and high-level amikacin resistance pertained to the Tugela Ferry XDR outbreak clone, four examples were observed outside the outbreak clone, indicating that this was not an isolated evolutionary event.

TB may also increase risk of adverse events related to drug toxicity. Ensuring safety of a streptomycin-based regimen would necessitate implementation of monitoring procedures, including audiometry and measurements of renal function, which constitute an additional burden—especially for resource-limited settings.

In South Africa, due to a clonal outbreak of XDR-TB in Tugela Ferry, a large fraction of circulating XDR-TB isolates contain an 130-bp deletion in *gidB* that confers low-level streptomycin resistance and an *rrs* 1400 mutation that confers high-level cross-resistance to amikacin, kanamycin, and capreomycin (15, 16). In a recent long-term cohort study of XDR-TB treatment outcomes in South Africa, only 1% of patients were treated with streptomycin, whereas 98% received capreomycin (17). As treatment

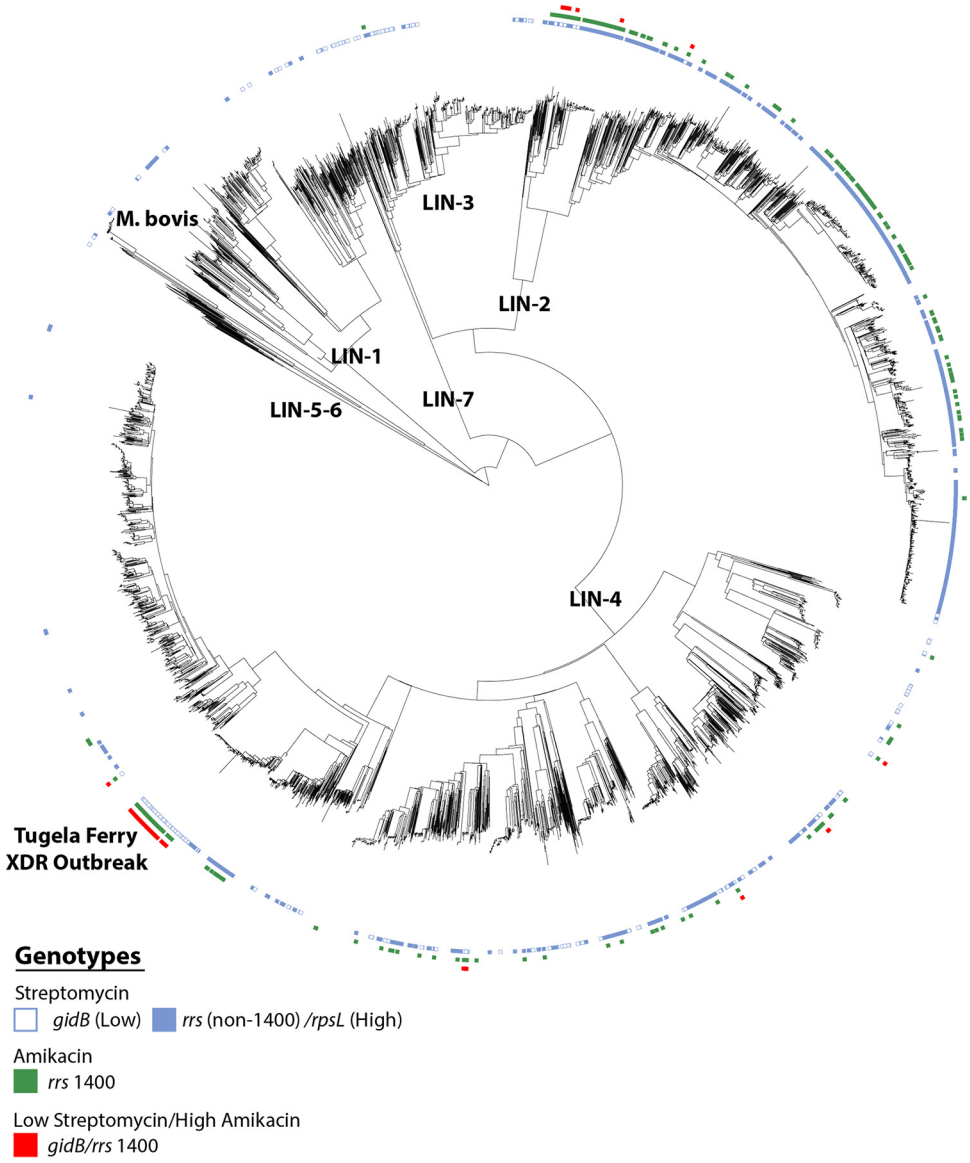

FIG 4 Concomitant low-level streptomycin and high-level amikacin genotypic resistance evolved repeatedly in a global data set of *M. tuberculosis*. Midpoint rooted maximum-likelihood phylogeny of 5,310 *M. tuberculosis* strains from a global data set containing representatives of all seven known *M. tuberculosis* lineages. The presence and levels of genotypic resistance to streptomycin (low, *gidB*; high, *rrs* [non-1400] and *rpsL*) and amikacin (high, *rrs* 1400) are indicated by box color near the leaf nodes. Ninety-five isolates with genotypic mutations predicted to confer both low-level streptomycin resistance and high-level amikacin resistance (*gidB-rrs* 1400) are indicated in red. Concomitant low-level streptomycin resistance and high-level amikacin resistance occurred across the phylogeny, indicating that this phenomenon is of global relevance for TB control.

outcomes for XDR-TB in South Africa were notoriously abysmal (17), including streptomycin may prove useful for patients in whom new drugs are not available because of resistance or contraindications.

Current WHO-endorsed laboratory procedures for performing phenotypic DST to streptomycin by critical concentration fail to provide key information relevant to streptomycin inclusion in a regimen for drug-resistant TB. The MIC distribution for isolates containing *gidB* mutations straddles the WHO-recommended critical concentration of 2 μg/ml (Fig. 1A). This modest increase in MIC among isolates containing *gidB* mutations in comparison to wild-type isolates likely contributes to inconsistencies in testing. Isolates containing *gidB* mutations are frequently misclassified in terms of their susceptibility to streptomycin on critical concentration testing (as occurred in 24% of

isolates in this study). As critical concentration testing is typically performed only at a single concentration, isolates with low-level streptomycin resistance—which may potentially be treated successfully with streptomycin—cannot be distinguished from those with high-level resistance. Similarly, wild-type strains that do not contain genotypes predicted to confer resistance to streptomycin can exhibit low-level streptomycin resistance that is above the critical concentration threshold (as seen in four South African isolates in this study), which may result in withholding a potentially useful drug.

The WHO-recommended critical concentration for streptomycin in *M. tuberculosis* is based on weak scientific evidence (12). The upper limit of wild-type MIC distribution, termed the epidemiological cutoff value (ECOFF), for streptomycin is 2 μg/ml (18). That this is the same value as the critical concentration in DST reflects the lack of clinical and pharmacokinetic/pharmacodynamic data to inform a more practical selection of a critical concentration. Potential strategies to address this issue include: (i) raising the streptomycin critical concentration; (ii) adding a second streptomycin drug concentration to traditional critical concentration testing (e.g., test at both 2 μg/ml and 8 μg/ml to disambiguate between low-level and high-level streptomycin resistance); (iii) performing additional reflex testing when an isolate is identified by traditional critical concentration DST to be resistant to both streptomycin and kanamycin (e.g., more detailed phenotypic analysis or streptomycin resistance genotype determination); or (iv) forgoing critical concentration testing in all forms and instead expanding genotypic aminoglycoside resistance testing.

Recent efforts to expand the complement of drug resistance mutation panels included on rapid molecular TB diagnostics have not included streptomycin (19). Whole-genome sequencing (WGS) studies of clinical isolates of *M. tuberculosis* have demonstrated that the majority (92% to 95%) of streptomycin-resistant isolates can be explained by known mutations (20, 21). Thus, omitting streptomycin resistance determinants from rapid drug resistance panels is a missed opportunity to both identify and grade streptomycin resistance relative to amikacin resistance. One potential reason for this exclusion is mutations in *gidB* can occur anywhere in the gene, where they cause frameshift, nonsense, or deletion mutations. Thus, they are difficult to identify with current SNP-based diagnostics and instead require whole-gene-based strategies, such as high-resolution melt analysis (22) or rapid WGS.

It is important to address several limitations of this study. MIC determination was performed with Sensititre, which is not the gold standard for *M. tuberculosis* DST. However, prior investigation comparing Sensititre with traditional methods have shown excellent concordance for aminoglycoside testing (23, 24). In addition, phenotyped isolates derived only from South Africa, and the population structure contained clonal XDR isolates from the Tugela Ferry epidemic. However, the phenomenon of genotypic resistance conferring low-level streptomycin and high-level amikacin resistance was also seen outside this clone. Thus, this observation carries implications for *M. tuberculosis* treatment in other settings.

Our findings suggest that current critical concentration methods for streptomycin resistance determination and the design of molecular diagnostics for resistance may need to be revisited for improved categorization of isolates harboring *gidB* mutations, which confer low-level streptomycin resistance. In the context of limited therapeutic options for drug-resistant *M. tuberculosis*, our results show the potential utility of streptomycin, even for isolates observed to have low-level resistance from *gidB* mutations.

MATERIALS AND METHODS

Clinical isolates. We selected for inclusion a random subset of 211 clinical isolates of susceptible and drug-resistant *M. tuberculosis* from South Africa from our larger sequenced strain set (15).

Drug susceptibility testing by critical concentration. As previously described (15), DST was performed prospectively by critical concentration on Middlebrook 7H11 using the WHO-recommended drug concentrations for streptomycin (2.0 μg/ml) and kanamycin (6.0 μg/ml). Amikacin critical concentration was not performed, as isolates with acquired resistance to amikacin essentially always have resistance to kanamycin (12).

MIC determination. MIC determination for three aminoglycosides (amikacin, kanamycin, and streptomycin) was performed using MycoTB Sensititre plates (TREK Diagnostic Systems), per the manufacturer's instructions. The lowest concentration of drug that did not show visible growth was recorded as the MIC to the respective drug.

Whole-genome sequencing and analysis. Whole-genome sequencing (WGS) and analysis were performed as previously described (15). Genotypic resistance to streptomycin, amikacin, and kanamycin was defined as identification of polymorphisms that are known to be associated with drug resistance, per the refined genotypic resistance definition in Desjardins and Cohen et al. (20) (Table S1). Isolates belonging to the Tugela Ferry XDR clone were identified by phylogenetic clustering with the reference isolates KZN605, collected during the epidemic, as well as the presence of canonical drug-resistance mutations (15). SNP calls from Cohen et al. were used (15). RAxML version 7.3.0 (25) was used to construct a phylogenetic tree from concatenated SNPs, with 1,000 bootstrap replicates.

Data availability. All sequencing data can be found in the Sequence Read Archive NCBI umbrella project identifier PRJNA183624.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.04 MB.

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K.A.C., K.E.S., and A.S.P. conceived of the study and designed the experiments. K.A.C. and V.M. performed the wet-lab experiments. K.A.C., K.E.S., and A.L.M. analyzed the data. K.A.C. and K.E.S. wrote the manuscript. A.M.E. and A.S.P. supervised and coordinated the project. All authors have read the manuscript and confirm that they meet ICMJE criteria for authorship.

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