Screening for Streptomycin Resistance-Conferring Mutations in *Mycobacterium tuberculosis* Clinical Isolates from Poland

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**Abstract**

Currently, mutations in three genes, namely *rrs*, *rpsL*, and *gidB*, encoding 16S rRNA, ribosomal protein S12, and 16S rRNA-specific methyltransferase, respectively, are considered to be involved in conferring resistance to streptomycin (STR) in *Mycobacterium tuberculosis*. The aim of this study was to investigate the spectrum and frequency of these mutations in *M. tuberculosis* clinical isolates, both resistant and susceptible to STR. Sixty-four *M. tuberculosis* isolates recovered from as many TB patients from Poland in 2004 were included in the study. Within the sample were 50 multidrug-resistant (32 STR-resistant and 18 STR-susceptible) and 14 pan-susceptible isolates. Preliminary testing for STR resistance was performed with the 1% proportion method. The MICs of STR were determined by the Etest method. Mutation profiling was carried out by amplifying and sequencing the entire *rrs*, *rpsL*, and *gidB* genes. Non-synonymous mutations in either *rrs* or *rpsL* gene were detected in 23 (71.9%) of the STR-resistant and none of the STR-susceptible isolates. Mutations in the *gidB* gene were distributed among 12 (37.5%) STR-resistant and 13 (40.6%) STR-susceptible isolates. Four (12.5%) STR-resistant isolates were wild-type at all three loci examined. None of the *rrs*, *rpsL*, or *gidB* mutations could be linked to low, intermediate or high level of STR resistance. In accordance with previous findings, the *gidB* 47T→G (L16R) mutation was associated with the Latin American-Mediterranean genotype family, whereas 276A→C (E92D) and 615A→G (A205A) mutations of the *gidB* gene were associated with the Beijing lineage. The study underlines the usefulness of *rrs* and *rpsL* mutations as molecular markers for STR resistance yet not indicative of its level. The *gidB* polymorphisms can serve as phylogenetic markers.

**Introduction**

Streptomycin (STR), an aminocyclitol glycoside antibiotic, was the first drug used successfully for the treatment of tuberculosis (TB) in the 1940s. Since then, STR has become the drug of first choice for all forms of TB, and, after the introduction of isoniazid (INH), rifampicin (RMP), pyrazinamide (PZA), ethambutol (EMB), and other anti-TB drugs, an important component of combination therapies for the disease. However, the high rates of STR resistance, primarily due to the historical use of the drug in a monotherapy application, the risk of toxicity, including serious neurotoxic reactions, the parenteral route of administration, and, above all, the lower efficacy relative to the succeeding drugs, lead to a gradual decline in the use of STR. Under the current WHO guidelines, the standard treatment regimen for drug-susceptible TB excludes STR and comprises a combination of INH, RMP, PZA, and EMB during the intensive phase of treatment, followed by INH together with RMP in the continuation phase [1]. Nevertheless, STR continues to be an integral part of chemotherapy for TB. Not only is the drug a component of the retreatment regimens, but also, in many parts of the world, it is among the first-line agents to be administered in drug-resistant TB (DR-TB) cases, yet susceptible to aminoglycosides [1].

The mechanism of action of STR in mycobacteria, largely inferred from the research on *Escherichia coli*, involves irreversible binding to the ribosomal protein S12 and 16S rRNA, which are the constituents of the 30S subunit of the bacterial ribosome. Through this interaction, STR interferes with translational proofreading and thereby inhibits protein synthesis [2].

Resistance of tubercle bacilli to STR appears to be mediated by mutations in the *tpsL* and *rs* genes, encoding the ribosomal protein S12 and the 16S rRNA, respectively. Amino acid substitutions in the S12 protein affect STR binding by perturbing the higher-order structure of 16S rRNA. Alterations in the 16S rRNA itself result in a severely decreased affinity for STR [2].

Furthermore, mutations in the *tpsL* gene have been associated with a high-level of STR resistance, whereas mutations in the *rs* gene have been shown to confer an intermediate-level of resistance [3–7]. Quite recently, mutations in the *gidB* gene, coding for a 7-
methylguanosine methyltransferase (m7G) specific for the 16S rRNA, have been implicated in STR resistance in *M. tuberculosis*. In addition, the *gidB* polymorphisms have been correlated with a low-level of STR resistance [8–10].

The rapid determination of drug resistance patterns of *M. tuberculosis* clinical isolates is an essential prerequisite for the prompt implementation of effective chemotherapy and early interruption of DR-TB transmission. Currently, screening for mutations in genetic *loci* associated with drug resistance is the most promising approach for rapid and reliable diagnosis of DR-TB. In case of STR, the association between specific mutations in the two *aforesaid* genes (*rpsL* and *rrs*) and drug-resistant phenotype, and, consequently, the utility of these mutations as highly-specific predictive markers of STR resistance has been well recognized. Since the type and frequency of STR resistance mutations vary depending on the population and geographical area studied, it is imperative to establish the global prevalence of each particular mutation, before it can be used as an accurate predictor for resistance to this drug.

The purpose of this study was to investigate the spectrum and frequency of mutations in the *rpsL*, *rrs*, and *gidB* genes in STR-resistant and STR-susceptible *M. tuberculosis* clinical isolates from Poland.

**Materials and Methods**

**Strains**

Sixty-four clinical isolates of *M. tuberculosis*, deposited in the culture collection of the National TB and Lung Diseases Research Institute in Warsaw, were used for the study. Included in that number were 50 multidrug-resistant (MDR) (32 STR-resistant and 18 STR-susceptible) and 14 pan-susceptible isolates. The isolates were obtained from pulmonary TB patients diagnosed in different parts of Poland and collected during the third national survey of DR-TB (i.e. from 1 January to 31 December 2004). The study sample included all MDR-TB cases collected in the survey. Primary isolation, cultivation, and species identification were done by using standard mycobacteriological methods, essentially described elsewhere [11].

**Drug Susceptibility Testing**

The first-line drug susceptibility testing (DST) was performed with the conventional proportion method on Lowenstein-Jensen (L–J) medium, strictly according to the WHO guidelines [12]. The following critical drug concentrations were used: 0.2 μg/ml (INH), 40 μg/ml (RMP), 4 μg/ml (STR), and 2 μg/ml (EMB). Resistance was defined by the growth of ≥1% of a bacillary inoculum on drug-containing medium compared to the drug free control.

The minimal inhibitory concentrations (MICs) of STR were determined by the Etest method ([bioMérieux, Marcy-l’Etoile, France), following the instructions of the Etest technical guide, ([http://www.biomérieux.com](http://www.biomérieux.com)). The breakpoint MICs for the Etest method were the same as the point where the inhibition ellipse intersected the scale on the Etest strip. The breakpoint MICs for the Etest method were the same as those used for the proportional method. To validate the performance of the DST, including the Etest assay, the reference strain *M. tuberculosis* H37Rv, susceptible to all the drugs tested, was used as the quality control strain. All isolates were tested in duplicate to assure the accuracy of the results.

The use of the Etest method to determine the MICs of STR was supported by previous studies showing an overall high agreement with the traditional techniques, such as the proportion method in L–J or Middlebrook agar media [13,14].

**DNA Extraction**

Genomic DNA was extracted from *M. tuberculosis* cultures on L–J slants by the lysozyme/protease K-cetyl-trimethyl ammonium bromide (CTAB) method [15]. The purified DNA was dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and quantified with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). The DNA samples were stored at −20°C until used.

**PCR Amplification and Sequence Analysis**

The complete open reading frames of the *rpsL*, *rrs*, and *gidB* genes were subjected to mutational analysis by direct sequencing. The oligonucleotide primers (Table 1) used for PCR amplification of the three genes were newly designed in this study, based on the sequences of the respective genes of *M. tuberculosis* reference strain H37Rv, available at the TubercuList database ([http://genolistpasteur.fr/TubercuList/](http://genolistpasteur.fr/TubercuList/)). The 30-μl PCR mixtures contained 25 μl of the HotStarTaq Master Mix (Qiagen), 0.1 μM of each primer, and 1 μl (ca. 20 ng) of template DNA. All PCRs were run on a T100 Thermal Cycler (Bio-Rad) under the following amplification conditions: initial denaturation at 95°C for 3 min, followed by 34 cycles of denaturation at 95°C for 30 s, annealing at 57°C (rpsL), 55°C (gidB) or 54°C (rrs) for 30 s, and extension at 72°C for 20 s (rpsL), 50 s (gidB) or 1 min and 10 s (rrs), with a final extension at 72°C for 10 min. The products of amplification were analyzed by electrophoresis in 1.5% agarose gels, purified with the Clean-Up kit (A&A Biotechnology) and sequenced by using the BigDye ver. 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems) in the ABI 3130xl Genetic Analyzer (Applied Biosystems). In order to exclude any sequence ambiguities, all sequencing reactions were carried out in both forward and reverse directions using the same primer pairs as for the PCR amplifications and, only for the *rrs* gene, an additional pair of internal sequencing primers (Table 1). The sequence data were assembled and analyzed with the ChromasPro (ver. 1.7.1) software (Technelysium), and the resulting consensus sequences were aligned with the corresponding, wild-type sequences of the reference *M. tuberculosis* H37Rv strain (TubercuList; [http://genolistpasteur.fr/TubercuList/](http://genolistpasteur.fr/TubercuList/)) using the BLASTN software ([http://blast.ncbi.nlm.nih.gov/](http://blast.ncbi.nlm.nih.gov/)).

**Nucleotide Sequence Accession Numbers**

The nucleotide sequences of the *rpsL*, *rrs*, and *gidB* genes containing novel mutations were deposited in the GenBank database ([National Center for Biotechnology Information; [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)]) under the following accession numbers: KF740612, KF740621, and KF740622 for the *rpsL* gene mutants, KF796660 to KF796662, and KF796665 for the *rrs* gene mutants, and KF740589, KF740590, KF740598 to KF740601, KF740603 to KF740605, KF740607, KF740609, KF740611, KF796668, and KF796669 for the *gidB* gene mutants.
Genotyping

All isolates were subjected to spoligotyping, essentially as described elsewhere [16]. Spoligotype signatures and phylogenetic (sub-)clades were assigned according to the SITVIT WEB database housed by the Institut Pasteur de Guadeloupe (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/). Clade assignment of the spoligotypes not found in SpolDB4 was done with SPOTCLUST, an algorithm based on the SpolDB3 database, described previously [17] and available online (http://cgi2.cs.rpi.edu/~bennek/SPOTCLUST.html).

Statistical Analysis

The associations between mutations and MICs of STR and differences in the proportion method for determining susceptibility of tubercle bacilli to STR between mutations and spoligotyping families were assessed with aFw and Rev stand for forward and reverse (primers), respectively. All starters used for amplification were also used for sequencing. An asterisk (*) indicates additional intragenic starters used for sequencing.

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Table 1. Primers used for PCR amplification and sequencing.

| Gene | Primer* | Nucleotide sequence (5′→3′) | Product size [bp] |
|------|---------|-----------------------------|-----------------|
| rpsL | Fw: rpsLF | GGCATGGCGCAGAAGAACGAG | 501 |
|      | Rev: rpsLR | ACTGCGTACCACTGCGATCC | |
| rrs  | Fw: rrsF | TGGCCATGCTCTTGATGC | 1707 |
|      | Rev: rrsR | CGGCCACTACAGACAAGAAC | |
|      | rrs-seqF* | TTAGCCGCGTAATGCC | |
|      | rrs-seqR* | CAGTAATCTACGGTACGGAG | |
| gidB | Fw: gidBF | GGAGTGCGTAATGTCC | 785 |
|      | Rev: gidBR | GTCGGTGTTGCTATTC | |

*Fw and Rev stand for forward and reverse (primers), respectively. All starters used for amplification were also used for sequencing. An asterisk indicates additional intragenic starters used for sequencing.

Results

Among the 32 M. tuberculosis isolates examined, defined as MDR and STR-resistant by the proportion method, 27 (84.4%) were also identified as STR-resistant by the Etest methodology. All analyses were performed using the SPSS ver. 22.0 (SPSS Inc., USA) statistical software package.

Four isolates had a C→T transition at nucleotide (nt) position 517. Two isolates had an A→T transversion at nt position 907. One isolate had a transversion A→C at nt position 514, and another one – a transition A→G at nt position 906. Two isolates with 517C→T or 907A→T mutation had their STR MICs below the resistance breakpoint value (0.25 and 0.75 μg/ml, respectively). For the remaining 6 mrr mutants, the MICs were ≥96 μg/ml.

Single mutations in the rpsL gene were detected in 16 (50%) and 2 (6.3%) of the MDR isolates that were resistant (MIC range: 0.75–1024 μg/ml) and susceptible (MICs of 0.125 and 0.38 μg/ml) to STR, respectively. Of the former, all but two isolates had an A→G mutation at nt position 128, resulting in a K43R amino acid substitution. One isolate had a similar amino acid change (K88R), due to an A→G mutation at nt position 263. One isolate had a silent mutation (117C→T; T39T). This mutation was also present in two STR-susceptible isolates. Two isolates with a K43R or K88R mutation had their STR MICs below the resistance breakpoint value (3 and 0.75 μg/ml, respectively).

Mutations in the mrr and rpsL genes were mutually exclusive, that is isolates with mrr mutations had no rpsL mutations and vice versa.

Sixteen different mutation types in the gidB gene were identified, distributed among 23 (72.5%) MDR isolates (12 (37.5%) out of 32 STR-resistant and 11 (61.1%) out of 18 STR-susceptible) and 2 (11.1%) pan-susceptible isolates. Ten different types of gidB mutations were observed only in STR-susceptible isolates (9 MDR isolates and one pan-susceptible isolate; MIC range: <0.064–2 μg/ml). These mutations included 5 frameshift mutations due to a single nt deletion (112delG in 2 isolates, 154delT in 2 isolates, 40delC in one isolate, and 98delG in one isolate) or insertion (258insA in one isolate) and 5 substitution mutations: 119A→G (E40G), 155G→A (C52Y), 483C→G (A161A), 523C→G (R175G), and 559A→C (R187Y), which all occurred in single isolates, except for 155G→A and 523C→G mutations which co-existed in 2 isolates. The 559A→C polymorphism was found in a pan-susceptible isolate.

Four gidB mutation types were found exclusively in STR-resistant isolates (MICs of 512 and >1024 μg/ml in one, and 3 isolates, respectively). Two different substitutions, i.e. 276A→C (E92D) and 615A→G (A205A) co-occurred in 2 isolates, whereas substitution 62G→A (R121Q) and a frameshift mutation (60insC) were found in single isolates. Two gidB mutation types were detected in 13 isolates, both STR-susceptible and STR-resistant. A substitution 47T→G (L16R) was present in 10 MDR isolates (8 STR-resistant and 2 STR-susceptible) and one pan-susceptible
isolate, whereas a frameshift mutation caused by a two-nt deletion (del351-2GC) – in 2 MDR isolates, one with and the other without resistance to STR.

Of the 25 isolates with an altered gidB gene sequence, 17 (68%) had a single mutation, 6 (24%) had two, and 2 (8%) had three mutations simultaneously. Ten (15.6%) of the STR-resistant isolates contained mutations in two loci, i.e. in gidB and rrs (2 isolates) or gidB and rpsL (8 isolates). Four (12.5%) STR-resistant isolates whose MICs were of 2, 512, and 1024 mg/ml had wild-type sequences at all three loci analyzed. In one STR-resistant (MIC of 512 mg/ml) isolate a silent mutation (117C>R; T39T) in the rpsL gene was the only mutation detected.

The MICs for STR did not differ between the gidB mutation-containing and gidB mutation-free isolates (P>0.05). Likewise, neither the number of mutations in the gidB gene nor the number of mutated loci were correlated with the MIC values for STR. However, the gidB mutation-containing isolates were considerably more susceptible to STR than the rrs or rpsL mutation-containing isolates. For these three groups of isolates, the MICs were of 1.5, 256, and 256 mg/ml, respectively (Table S1).

The results of mutation profiling at the three loci analyzed (rrs, rpsL, and gidB) and the spoligotyping results of all the 64 M. tuberculosis isolates studied were shown in figure 2.

Figure 1. Schematic representation of the distribution of mutations in the rrs, rpsL, and gidB gene, with regard to MIC values of STR. del, deletion; ins, insertion; fs, frameshift mutation. Numbers in white or grey-shaded boxes, below mutation designations, represent isolates with a particular mutation type and MIC of STR. doi:10.1371/journal.pone.0100078.g001

Discussion

In M. tuberculosis resistance to STR is associated with alterations within the rrs, rpsL, and gidB genes whose products are involved in the 30S ribosomal subunit composition. Although a wide spectrum of distinct mutations in those genes has been characterized, there is still relatively little information on their frequency and distribution among STR-resistant and STR-susceptible isolates from geographically different localities, as well as on the relationship of the mutations with the level of STR resistance.

All rrs gene mutations found in this study localized in two highly mutable regions known as the 530 loop (514A>R; 517C>R) and the 912 loop (906A>R; 907A>R). All these mutations, except one (906A>R), have previously been reported in STR-resistant M. tuberculosis isolates, most of which were MDR [7,18–20]. The 906A>G substitution is novel but other mutations in the same position (906A>R; 906A>T) have already been described [21–24]. According to the literature, neither of these four rrs mutation types were observed in isolates susceptible to STR. Mutations in the rrs gene accounted for a quarter (8/32) of the STR-resistant isolates, reaching the highest mutation frequencies for this gene reported, ranging from 0–28% [6,18,19,22,24–28]. Compared with the rrs gene, mutations in the rpsL gene was associated with twice as many STR-resistant isolates (16/32). Of the three mutation types identified for the rpsL gene, two (K43R and K88R) are the most common genetic alterations present in STR-resistant M. tuberculosis isolates. A single mutation K43R,
representing 87.5% of the \textit{rpsL} mutants and 43.7% of the STR-resistant sample from this work, ranks first in most of the studies characterizing the mutation profiles associated with STR resistance in tubercle bacilli. Yet, the detection rate for this mutation varies considerably between geographical areas, being of 13.2% in Mexico [26], 25% in Brazil [29], 42.9% in North India [30], 52.8% in Korea [18], 70.4% in China [22], and 80.4% in Singapore [31]. Of note is that the highest frequencies of the K43R substitution are in areas where strains of the Beijing genotype family predominate.

Figure 2. Dendrograms (DendroUPGMA, Jaccard model, UPGMA) based on spoligotype patterns (a) and mutation profiles at \textit{rrs}, \textit{rpsL}, and \textit{gidB} genes (b) of the 64 \textit{M. tuberculosis} clinical isolates under the study. A, isolate no.; B, spoligotype pattern (binary); C, spoligotype signature (shared international type, SIT); D, phylogenetic clade; E, mutation profile. Numbers representing isolates resistant or susceptible to STR were framed against grey and white backgrounds, respectively. The presence or absence of an individual nucleotide change was shown as black and white spots, respectively. NF, not found (in the SITVIT WEB database); orphan, spoligotype represented by a single isolate in the SITVIT WEB database. The SPOTCLUST-assigned clades are given in brackets. Asterisks indicate the confirmed assignment to the LAM lineage, based on the presence of LAM-specific mutations, i.e. 309G>A and 1212 C>G substitution, in \textit{Rv0129c} and \textit{Rv3062} genes, respectively [36,37].

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The K43R is also the leading mutation among those STR-resistant isolates that display an MDR phenotype, with the mutation frequency of 24–64.1% [18,19,29,32,33]. The high prevalence of the K43R mutation among STR-resistant M. tuberculosis isolates can be explained by the fact that this particular mutation carries no fitness cost for the bacteria [34]. The K60R substitution usually plays a minor role and occurs much less frequently than K43R. Only a few studies have documented those two mutations equally represented among STR-resistant isolates [4–6,27]. In the great majority of studies in which STR-susceptible isolates were also screened for mutations in the rpsL gene, neither codon 43 nor 88 was changed. However, mutations in these codons, including the commonest K→R amino acid replacements do occur in susceptible isolates, albeit very rarely [27,35]. The only other mutation found in the rpsL gene was a silent mutation in codon 39 (T39T). This mutation, which had only once been described before this study, seems not to be associated with STR resistance, as it was observed in STR-resistant and STR-susceptible isolates.

Double mutants at both rsr and rpsL genes were absent from the sample analyzed. Mutations in both these loci have very rarely been observed to occur concurrently [4,25,26,31]. This may suggest that mutational alterations in either rsr or rpsL may alleviate the need for the modification of the other gene.

An important observation from this study was that rsr and rpsL mutation-containing isolates did not differ from each other in terms of STR resistance level, as evidenced by comparing the MICs for individual isolates, which included MDR-TB isolates. Therefore, STR resistance, if occurring, was always compounded with MDR phenotype.

Sixteen different types of gidB mutations, including nine novel (not reported so far) types, were identified in the sample studied. This is consistent with the highly polymorphic nature of the gidB gene observed by other authors. For instance, Feuerriegel et al. [27] reported 16 gidB mutations among 56 M. tuberculosis isolates, whereas Spies et al. [29] detected 18 mutations in only 19 isolates. In both these studies, as in others, gidB mutations occurred in both STR-susceptible and -resistant isolates [7,9,10,18–20,27,29]. This was also the case in the present study. Twelve gidB mutation types were found in STR-susceptible isolates. The remaining four types were detected only in resistant isolates. However, two of the latter mutations, a deletion mutation (98delG) and a silent mutation (615A→G; A205A), have previously been reported as occurring also in susceptible isolates, and thus cannot be considered as related to STR resistance [7,18–20,27]. Only two newly described mutations, a substitution 62G→A (R21Q) and a frameshift mutation 60insC, might be involved in STR resistance, and in particular the latter, which, apart from being absent in susceptible isolates, did not coincide with any rsr or rpsL mutation. Previous studies have reported the role of gidB mutations in conferring a lower level of STR resistance [8–10]. The results of this study are in line with this observation, where the MICs for all gidB mutation-containing isolates and those without rsr or rpsL mutations were of 1.5 and 0.38 μg/ml, as opposed to 256 μg/ml for gidB mutation-free isolates but with alterations in rsr or rpsL genes. However, when analyzing the MICs for individual gidB mutants (without rsr or rpsL mutations), no consistency between the presence of a mutation type and MIC value was observed. Altogether, it seems that gidB mutations do play a role in STR resistance, as they occur in STR-resistant isolates with no additional mutations in other genes. However, the exact importance of these mutations for STR resistance and its level needs to be further investigated. Although the usefulness of gidB mutations as markers for STR resistance is rather doubtful or at least uncertain, they provide some interesting insights into the phylogeny of tuberculosis bacilli. The most striking finding is that 47T→G (L16R) mutation appears to be specific for the Latin American-Mediterranean (LAM) genotype family whereas 276A→C (E92D) and 615A→G (A205A) polymorphisms seem to be specific for the Beijing genotype family [7,20,27,29]. This relation was supported in the present study. All but two isolates belonging to the LAM genotype harbored the L16R mutation (the remaining two had a wild-type gidB sequence) (Table S2).

Considering the 4 Beijing genotype isolates, two, with an altered gidB allele, displayed a combination of the E92D and A205A mutations (Figure 2).

To better recognize the specificity of the gidB gene mutations detected for the LAM and Beijing families, they were searched against the whole genome sequences of M. tuberculosis strains, deposited in the GenBank database. Screening for the 47T→G (L16R) mutation was performed only on those strains which harbored 309G→A and 1212C→G polymorphisms in Rv0129c and Rv3062 genes, respectively, suggested to be specific for the LAM family [36,37]. Whereas, screening for the 276A→C (E92D) and 615A→G (A205A) mutations was done only for the strains that were positive for the 191A→C mutation in the Rv2629 gene, a specific marker of the Beijing lineage [38]. All six M. tuberculosis strains from the GenBank database, confirmed to represent the LAM family, by the presence of mutations in Rv0129c and Rv3062 genes, did contain the 47T→G mutation in the gidB gene. Likewise, all eight M. tuberculosis strains from the GenBank database, belonging to the Beijing family (mutation at Rv2629), did contain the 276A→C and 615A→G polymorphisms in the gidB gene.

Collectively, although the association of the gidB polymorphisms with specific lineages requires confirmation in broader population samples, the three polymorphisms described could be used as new phylogenetic markers to distinguish between different genogroups, some of which are of high epidemiological relevance. The Beijing and LAM genotypes are the most prominent, having repeatedly been associated with drug resistance, including MDR [39,40]. Interestingly, isolates whose spoligotyping family was unknown had their STR MICs significantly lower than isolates of the LAM, Beijing, and Haarlem (H) genotypes (P<0.04).

Interestingly, 12.5% (4/32) of the STR-resistant isolates had no mutations at any of the three loci investigated. This is much lower than what was observed previously in Poland (51%) [21], in Spain (62.3%) [6], Mexico (52%) [26], or Portugal (33.3%) [33]. An explanation for this difference may lie in the fact that all studies cited above did not perform gidB gene mutation profiling. In those few studies that involved the three-gene panel (rsr, rpsL, and gidB), the proportions of STR-resistant isolates with wild-type alleles at all three loci were similar to that from this study, being 6.9% in Viet Nam [7], 11.8% in Korea [19], 12.5% in Sierra Leone [27], and 22% in Brazil [29]. From these data, it is clear that mechanisms other than mutations in the rsr, rpsL, or gidB genes must exist to mediate STR resistance. One hypothesized mechanism includes changes in the cell envelope leading to decreased permeability, reduced uptake or increased efflux of the drug [4]. Further research are required to confirm this mechanism.
or identify additional mechanisms conferring STR resistance in *M. tuberculosis*.

In conclusion, this study adds importantly to the still limited body of evidence on the complexity of the genetic background of STR resistance in TB. The major findings from this study can be summarized in four points. Firstly, all non-synonymous drug-resistant mutations at either *rrs* or *rpsL* gene were found exclusively in STR-resistant isolates, and accounted for 71.9% (23/32) of them, supporting the suitability of *rrs* and *rpsL* mutations as predictive markers for STR resistance. Secondly, the *gidB* gene was highly polymorphic, with most of the polymorphisms having occurred in both STR-resistant and -susceptible isolates. This implies the role of *gidB* mutations as phylogenetic markers rather than markers for STR resistance. Indeed, the *gidB* polymorphisms at codons 16 and 92 or 205 were found to be associated with the LAM and Beijing genotype families, respectively. Thirdly, none of the mutations in any of the three *loci* evaluated could be conclusively linked to low, intermediate or high level of STR resistance. It seems that factors other than genetic alterations, including environmental determinants, may have an impact on the MIC values. Finally, finding of four (12.5%) STR-resistant isolates without any mutation in the three *loci* examined suggests the existence of other mechanisms involved in the development of STR resistance in tubercle bacilli.

References

1. World Health Organization (2010) Treatment of tuberculosis: guidelines – 4th ed. World Health Organization, Geneva, Switzerland. WHO/HTM/TB/2009.420.

2. Muser J (1995) Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Curr Microbiol Rev 8: 496–514.

3. Cooksey RC, Morlock GP, McQueen A, Glickman SE, Crawford JT (1996) Characterization of streptomycin resistance mechanisms among *Mycobacterium tuberculosis* isolates from patients in New York City. Antimicrob Agents Chemother 40: 1186–1190.

4. Muir A, Sander P, Schaper KJ, Schols M, Bottery EC (1996) Correlation of molecular resistance mechanisms and phenotypic resistance levels in streptomycin-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 40: 2452–2454.

5. Abbadi SH, Sameaa GA, Morlock G, Cooksey RC (2009) Molecular identification of mutations associated with anti-tuberculosis drug resistance among strains of *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 13: 673–678.

6. Tudo G, Rey E, Borrell S, Alcaide F, Codina G, et al. (2010) Characterization of mutations in streptomycin-resistant *Mycobacterium tuberculosis* clinical isolates in the area of Barcelona. J Antimicrob Chemother 65: 2341–2346.

7. Nhu NT, Lan NT, Phuong NT, Chau Nv, Farrar J, et al. (2012) Association of *rpsL* and *rrs* loci with ST6 in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 16: 957–962.

8. Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, et al. (2007) Loss of 16S rRNA conformational flexibility in low-level streptomycin-resistant *Mycobacterium tuberculosis*. J Antimicrob Agents Chemother 51: 2218–2225.

9. Shi R, Zhang J, Li C, Kazumi Y, Sugawara I (2007) Detection of streptomycin resistance mutations in clinical isolates of *Mycobacterium tuberculosis*. Clinical Microbiol Rev 20: 1099–1105.

10. Wong SY, Lee JS, Kwak HK, Via LE, Boshoff HIL, et al. (2011) Mutations in *rrs* and *gidB* genes of *Mycobacterium tuberculosis* complex clinical isolates in Poland. J Clin Microbiol 49: 3099–3109.

11. Augustynowicz-Kopec E, Jaworski A, Kostrzewa E, Klatt M (2003) Geographic variation of the predictive values of genomic mutations associated with drug resistance in *Mycobacterium tuberculosis* strains isolated in Poland. Int J Tuberc Lung Dis 7: 1032–1035.

12. Ballif M, Harino P, Ley S, Coscolla M, Niemann S, et al. (2012) Drug resistance-conferring mutations in *Mycobacterium tuberculosis* from Madang, Papua New Guinea. BMC Microbiol 12: 191.

13. Jonhelli H, Hwang SC, Kim HY, Lee YS, et al. (2013) Characterization of mutations in multi- and extensive drug resistance among strains of *Mycobacterium tuberculosis* complex clinical isolates in Republic of Korea. Diagn Microbiol Infect Dis 76: 137–196.

14. Brzostek A, Saigheda A, Slivinski T, Augustynowicz-Kopec E, Jaworski A, et al. (2004) Molecular characterization of streptomycin-resistant *Mycobacterium tuberculosis* strains isolated in Poland. J Clin Microbiol 42: 1032–1035.

15. Shi R, Zhang J, Li C, Kazumi Y, Sugawara I (2007) Detection of streptomycin resistance in *Mycobacterium tuberculosis* clinical isolates from China as determined by denaturing HPLC analysis and DNA sequencing. Microbes Infect 9: 1538–1544.

16. Katukawa C, Tamara A, Miyata Y, Abe C, Makino M, et al. (1997) Characterization of the *rpsL* gene in *M. tuberculosis*-resistant clinical isolates of *Mycobacterium tuberculosis*. J Appl Microbiol 83: 634–640.

17. Dohner P, Birtzel G, Rüschi-Gerdes S, Feldmann K, Riba M, et al. (1997) Geographic variation of the predictive values of genomic mutations associated with streptomycin resistance in *Mycobacterium tuberculosis*. Mol Cell Probes 11: 123–126.

18. Strekovásan S, Sanvk, Stockhauer KE, Williams DL, Kreiswirth BN, et al. (1996) Characterization of *rrs* and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. Antimicrob Agents Chemother 55: 2513–2522.

19. Carvajal-Cirillo B, Cuellar-Sánchez A, Pasini-Crivelli A, Santana-Álvarez CA, Hernandez-Inclán J, et al. (2011) *Mycobacterium tuberculosis* strains from a high-incidence setting. BMC Microbiol. 12: 90.

20. Spies FS, Ribeiro AW, Ramos DF, Ribeiro MO, Martin A, et al. (2011) Detection of coexistence of multiple drug-resistant isolates in *Mycobacterium tuberculosis* complex clinical isolates. J Clin Microbiol 49: 2625–2630.

Supporting Information

Table S1 Distribution of *rrs*, *rpsL*, and *gidB* mutations among SM-resistant (n = 32) and SM-susceptible (n = 32) *M. tuberculosis* isolates, with regard to MIC values of SM.

Table S2 Spoligotypes, resistance profiles and mutational patterns at *rrs*, *rpsL*, and *gidB* genes of the 64 *M. tuberculosis* isolates studied.

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Author Contributions

Conceived and designed the experiments: TJ. Performed the experiments: HI, HZ, AN. Analyzed the data: TJ. Contributed reagents/materials/analysis tools: EAK ZZ, JB. Wrote the paper: TJ.
31. Sun YJ, Luo JT, Wong SY, Lee AS (2010) Analysis of rpsL and rrs mutations in Beijing and non-Beijing streptomycin-resistant Mycobacterium tuberculosis isolates from Singapore. Clin Microbiol Infect 16: 287–289.

32. Kamaswamy SV, Dou SJ, Rendon A, Yang Z, Cave MD, et al. (2004) Genotypic analysis of multidrug-resistant Mycobacterium tuberculosis isolates from Monterrey, Mexico. J Med Microbiol 53: 107–113.

33. Perdigão J, Macedo R, João I, Fernandes E, Brum L, et al. (2006) Multidrug-resistant tuberculosis in Lisbon, Portugal: a molecular epidemiological perspective. Microb Drug Resis 14: 137–143.

34. Spies FS, von Groll A, Ribeiro AW, Ramos DF, Ribeiro MO, et al. (2013) Biological cost in Mycobacterium tuberculosis with mutations in the rpsL, rrs, rpoB, and katG genes. Tuberculosis 93: 150–154.

35. Van Rie A, Warren R, Mshanga I, Jordaan AM, van der Spuy GD, et al. (2001) Analysis for a limited number of gene codons can predict drug resistance of Mycobacterium tuberculosis in a high-incidence community. J Clin Microbiol 39: 636–641.

36. Homolka S, Projahn M, Feuerriegel S, Ubben T, Diet R, et al. (2012) High resolution discrimination of clinical Mycobacterium tuberculosis complex strains based on single nucleotide polymorphisms. PLoS One 7: e39855.

37. Abadía E, Zhang J, dos Vultos T, Ritacco V, Kroemer K, et al. (2010) Resolving lineage assignment on Mycobacterium tuberculosis clinical isolates classified by spoligotyping with a new high-throughput 3R SNPs based method. Infect Genet Evol 10: 1066–1074.

38. Horolka S, Köser C, Archer J, Rüscher-Gerdes S, Niemann S. (2009) Single-nucleotide polymorphisms in Rv2629 are specific for Mycobacterium tuberculosis genotypes Beijing and Ghana but not associated with rifampin resistance. J Clin Microbiol 47: 223–226.

39. Ignatova A, Dahiley S, Stepamshina V, Shemyakin I (2006) Predominance of multi-drug-resistant LAM and Beijing family strains among Mycobacterium tuberculosis isolates recovered from prison inmates in Tula Region, Russia. J Med Microbiol 55: 1413–1418.

40. Pardini M, Niemann S, Varaine F, Iona E, Meacci F, et al. (2009) Characteristics of drug-resistant tuberculosis in Abkhazia (Georgia), a high-prevalence area in Eastern Europe. Tuberculosis 89: 317–324.