Diagnostic Microbiology and Infectious Disease xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Genotypic and phenotypic characteristics of aminoglycoside-resistant *Mycobacterium tuberculosis* isolates in Latvia

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ARTICLE INFO

Article history:
Received 17 April 2014
Received in revised form 3 December 2014
Accepted 10 December 2014
Available online xxxx

Keywords:
Mycobacterium tuberculosis
Aminoglycosides
Latvia

ABSTRACT

Mutations causing resistance to aminoglycosides, such as kanamycin (KAN), amikacin (AMK), and streptomycin, are not completely understood. In this study, polymorphisms of aminoglycoside resistance influencing genes such as *rrs*, *eis*, *rpsL*, and *gidB* in 41 drug-resistant and 17 pan-sensitive *Mycobacterium tuberculosis* clinical isolates in Latvia were analyzed. Mutation A1400G in *rrs* gene was detected in 92% isolates with high resistance level to KAN and diverse MIC level to AMK. Mutations in promoter region of *eis* were detected in 80% isolates with low-level MIC of KAN. The association of K43R mutation in *rpsL* gene, a mutation in the *rrs* gene at position 513, and various polymorphisms in *gidB* gene with distinct genetic lineages of *M. tuberculosis* was observed. The results of this study suggest that association of different controversial mutations of *M. tuberculosis* genes to the drug resistance phenotype should be done in respect to genetic lineages.

1. Introduction

As a barely curable disease and an important barrier to the worldwide control of tuberculosis (TB), multidrug-resistant (MDR) and extensively drug-resistant (XDR) forms of TB are critical problems for today's health care (WHO, 2006). Aminoglycosides, such as streptomycin (STR), kanamycin (KAN), and amikacin (AMK), are useful as alternative drugs to combat MDR-TB. However, the resistance to these drugs is getting more common; thus, rapid drug susceptibility tests are required for designing regimens of the treatment (WHO, 2010). *Mycobacterium tuberculosis*, the causative agent of the disease, is a slow-growing pathogen. Thus, it takes 4–6 weeks to detect possible drug resistance by the standard absolute concentration method using the cultivation of bacteria on Lowenstein–Jensen (LJ) solid medium (Kaufmann and van Helden, 2008). Rapid detection of resistances can be achieved by molecular diagnostic techniques aimed to detect gene mutations related to resistance. These methods do not require cultivation of bacteria and can be adapted to direct clinical material (such as sputum or bronchial secretions) (Kaufmann and van Helden, 2008). However, such strategies require complete knowledge about the resistance-related genes for the different anti-TB drugs and their sites of mutation.

Mutations causing resistance to aminoglycosides are not completely understood. The most common mechanism of resistance to KAN and AMK is a point mutation from A to G in the 1400th nucleotide (also referred to as A1401G in the literature) or, in very rare cases, from G to T in the 1483rd nucleotide in the 16S ribosomal RNA gene (*rrs*) that leads to a high-level resistance to both drugs. This polymorphism is associated with 67.4–85.9% of TB resistance cases to KAN and 76.5–94.2% to AMK (Alangaden et al., 1998; Jugheli et al., 2009; Suzuki et al., 1998).

An alternative mechanism of the resistance to aminoglycosides is overexpression of the aminoglycoside acetyltransferase Eis due to a polymorphism in the promoter region of *eis* gene or by increased expression of the transcriptional activator WhiB7 (Reeves et al., 2013; Zaunbrecher et al., 2009). This mechanism causes lower but still important levels of resistance to KAN and detectable but clinically unimportant resistance to AMK. Several types of *eis* mutations were found to be responsible for 80–96.2% of low-level KAN resistance cases (Engström et al., 2011; Zaunbrecher et al., 2009).

The molecular structure of STR is slightly different from 2-deoxystreptamine aminoglycosides (KAN and AMK), and resistance to this drug usually arises via different mechanisms. The first described and the most prevalent mechanism is a substitution of K43 in the ribosomal protein S12 encoded by the *rpsL* gene, which causes high-level resistance (Honore and Cole, 1994; Nair et al., 1993; Sreevatsan et al., 1996). K88 substitution in the same protein causing the same degree of resistance has also been observed, but this mutation is much rarer and is independent of the known genotypic lineages (Lipin et al., 2007; Sun et al., 2010). Additionally, changes in the 530 loop and nucleotide region 915 of the *rrs* gene were associated with STR resistance (Honore and Cole, 1994; Meier et al., 1996; Nair et al., 1993; Sreevatsan et al., 1996; Tracevska et al., 2004). However, the physiological role of many mutations, frequently found in these regions, is controversial. For example,

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http://dx.doi.org/10.1016/j.diagmicrobio.2014.12.004
0732-8893/

C491T is typical of the LAM3 subfamily and is not related to drug resistance (Tudo et al., 2010; Victor et al., 2001). Mutations in the GidB protein are suspected to be the third mechanism of STR resistance in *M. tuberculosis*. First described by Okamoto et al. (2007), these mutations were detected in 33% of STR-resistant isolates. However, later studies have revealed that *gidB* gene is a highly polymorphic region of the *M. tuberculosis* genome, and many mutations in this gene are present in STR-sensitive isolates as well (Spies et al., 2011; Via et al., 2010; Wong et al., 2011).

The average incidence of TB in Latvia, a country in the Baltic region of the northeastern Europe, is 40 cases per 100,000 population. A total of 776 TB cases (a rate of 38.3 cases per 100,000 persons) were reported in 2013 (data from The Centre for Disease Prevention and Control of Latvia). However, Latvia is among the high MDR-TB–burden countries (66 MDR-TB cases were reported in 2013). KAN, AMK, and STR are used in tailor regimen for MDR-TB according to susceptibility pattern in Latvia; thus, biomedical research is needed for determination of *M. tuberculosis* drug resistance mechanisms. The goal of this study was to determine mutations prevalent in KAN-, AMK-, and STR-resistant *M. tuberculosis* clinical isolates in Latvia.

2. Materials and methods

2.1. M. tuberculosis clinical strains and drug susceptibility testing

The source of the mycobacterial isolates was either sputum or bronchial secretions from TB patients admitted to Center of Tuberculosis and Lung Diseases, Riga East University Hospital, during a period from November 2004 to May 2010. In this time frame, 2993 aminoglycoside-resistant cases (including monoresistant, polyresistant TB and MDR-TB) were recorded. Patient diagnosis was confirmed both microscopically and by culturing on LJ solid medium. Cultures were grown for 4–6 weeks. The drug susceptibility pattern of the cultures was conducted via classical drug susceptibility testing (DST) method using LJ medium with a breakpoint concentration of the drug (Kaufmann and van Helden, 2008). Breakpoints for KAN, AMK, and STR were 30, 40, and 4 μg/mL, respectively. As a positive control, the growth of each strain was also tested on nonantibiotic medium. The validity of the antibiotic-containing LJ media was confirmed by its potency to inhibit the growth of the standard strain H37Rv. For this study, available aminoglycoside-resistant and pan-sensitive isolates were randomly selected. The selection criteria were: a) different time of isolation, b) first isolate from each patient at time of diagnosis, and c) isolates with an available DST result. Only successfully recultivated isolates were further evaluated. A total of 41 aminoglycoside-resistant and 17 pan-sensitive *M. tuberculosis* clinical isolates were used in this study.

2.2. Measurement of MIC values for KAN, AMK, and STR

KAN, STR, and AMK MIC values of *M. tuberculosis* strains were measured by the resazurin microtiter assay (REMA) plate method, as previously described (Martin et al., 2011). The growth of each mycobacterial isolate was tested on 96-well plate with lid (Becton Dickinson, Franklin Lakes, New Jersey, USA) filled by 7H9 Middlebrook medium with following drug concentrations:

KAN: 0 (control), 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5, 10, 15, 20, 40, and 100 μg/mL;
AMK: 0 (control), 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5, 10, 15, 20, 40, and 100 μg/mL; and
STR: 0 (control), 0.3, 1.5, 2.5, 5.0, 10, 20, 40, and 100 μg/mL.

The MICs were determined by visual reading of the REMA plate with results obtained after 8 days of incubation. All *M. tuberculosis* strains were tested twice at every drug concentration, and a final MIC value was calculated as an average of 2 results. MIC value of 5 μg/mL was delineated as the minimal, clinically important level for KAN and AMK resistance, as defined by Martin et al. (2011). On the basis of the DST data, 2.5 μg/mL was used as minimum clinically important value for STR resistance. The H37Rv strain was used as a susceptible standard and a quality control for the experiment.

2.3. DNA extraction and DNA sequencing analysis

Extraction of DNA was performed, as previously described (Van Soolingen et al., 1999). DNA samples were further subjected to PCR amplification and DNA sequencing in specific hot-spot target regions of the following genes: the *rrs* regions 1400 and 530, the *eis* gene promoter region, and the entire sequences of the *rpsL* and *gidB* genes. The PCR was carried out with 2x PCR MasterMix (Fermentas, Vilnius, Lithuania) according to the following conditions: an initial denaturation step at 98 °C for 2 minutes, 35 cycles of denaturation at 96 °C for 30 seconds, primer annealing for 30 seconds (*rrs* gene, region 1400: 58 °C; *rrs* gene, region 530: 57 °C; *eis* gene promoter: 51 °C; *rps*L gene: 63 °C; *gid*B gene: 54 °C) and elongation at 72 °C for 30 seconds and a final extension step at 72 °C for 5 minutes. Primer sequences (Metabion, Steinkirchen, Germany) are listed in Table 1. PCR products were sequenced in both directions by a Big Dye DNA sequencing kit (Applied Biosystems, Foster City, California, USA) and analyzed by a standard technique using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). One exception was region 1400 of the *16S rrs* gene when mutations were detected by digestion of PCR amplicons with several restriction enzymes followed by electrophoresis in agarose gel, as previously described (Suzuki et al., 1998). Obtained sequences were compared by BLAST analysis to the genome of the *M. tuberculosis* H37Rv reference strain, which was described by Cole et al. (1998) and is available at the TubercuList World Wide Web Server (genolist.pasteur.fr/TubercuList).

2.4. Determination of the M. tuberculosis lineages

Spoligotyping was used to identify *M. tuberculosis* isolate genotypes. Spoligotype patterns were determined by a commercially available kit (Isogen Life Science, De Meern, The Netherlands) following the manufacturer's instructions. Spoligotype names (SIT numbers, subfamilies, and families) were identified via comparison to SITVIT (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/) and SpolDB4 (http://www.biomedcentral.com/content/supplementary/1471-2180-6-23-s1.pdf) databases.

2.5. Statistical analysis

Statistical analysis was performed to compare the prevalence of various mutations in different *M. tuberculosis* lineages. The 2-sided Fisher's exact test was employed to determine a *P* value (GraphPad Software, La Jolla, California, USA). A *P* value equal to or less than 0.05 was considered significant.

3. Results

3.1. Phenotypic drug resistance

The full spectra drug susceptibility pattern was not available for all *M. tuberculosis* isolates; particularly, DST for AMK was introduced in routine practice in year 2006 in Latvia.

Table 1
Primers used in this study.

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Reference
rrs, region 1400	ttaaaagccggtctcagttc	tacgccccaccattggggc	Suzuki et al. (1998)
rrs, region 530	gatgacggccttcgggttgt	tctagtctgcccgtatcgcc	Tracevska et al. (2004)
eis, promoter region	gcgtaacgtcacggcgaaat	gccttcagaactcgaacg	Zaunbrecher et al. (2009)
*rps*L	ccaaccatccagcagctggt	atccagcgaaccgcggatga	Tracevska et al. (2004)
*gid*B	gagcggagaatgtttcac	ggttcgatagttgaagcc	This study

Please cite this article as: Bauskenieks M, et al, Genotypic and phenotypic characteristics of aminoglycoside-resistant *Mycobacterium tuberculosis* isolates in Latvia, Diagn Microbiol Infect Dis (2014), http://dx.doi.org/10.1016/j.diagmicrobio.2014.12.004

Table 2
Mutation analysis in aminoglycoside-resistant *M. tuberculosis* strains.

Nr	Isolate	Phenotypic drug susceptibility and MIC (μg/mL)						Mutations in aminoglycoside resistance–associated loci				Spoligotype	
		KAN		AMK		STR		*rrs*	*eis* promotor	*rpsL*	*gidB*	SIT Nr.	Subfamily
1	#E01	R	>100	R	>100	R	>100	A1400G	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
2	#E02	R	>100	R	>100	R	>100	A1400G	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
3	#E03	R	>100	R	>100	R	>100	A1400G	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
4	#E04	R	>100	R	>100	R	>100	A1400G	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
5	#E05	R	>100	ND	>100	R	>100	A1400G	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
6	#F01	R	>100	ND	>100	R	>100	WT	G10A	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
7	#E06	R	>100	R	>100	R	100	A1400G, A513C	WT	WT	CTT/CGT L16R	42	LAM9
8	#E07	R	>100	R	>100	R	40	A1400G, A513C	WT	WT	CTT/CGT L16R	42	LAM9
9	#E08	R	>100	R	>100	R	40	A1400G, A513C	WT	WT	CTT/CGT L16R	42	LAM9
10	#E09	R	>100	R	7.5	R	>100	A1400G	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
11	#E10	R	>100	R	7.5	R	>100	A1400G	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
12	#E11	R	>100	ND	7.5	ND	20	A1400G, A513C	WT	WT	CTT/CGT L16R	42	LAM9
13	#G01	R	40	R	7.5	R	>100	G1483T	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
14	#H01	R	7.5	ND	2	R	>100	WT	G10A	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG, A205A	1	Beijing
15	#H02	R	7.5	ND	2	R	>100	WT	G10A	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
16	#H03	R	7.5	ND	1.5	R	>100	WT	G10A	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
17	#H04	R	7.5	ND	1	R	>100	WT	G10A	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
18	#H10	R	5	S	1.5	R	>100	WT	G10A	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
19	#H11	R	5	ND	1	R	>100	WT	G10A	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
20	#H12	R	5	ND	1	R	>100	WT	G37T	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
21	#H13	R	5	S	1	R	>100	WT	G37T	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
22	#H14	R	5	S	0.5	R	>100	WT	G10A	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
23	#H15	R	5	S	0.5	R	>100	WT	G10A	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
24	#H08	R	5	S	2	R	100	A513C	C12T	WT	CTT/CGT L16R	42	LAM9
25	#H16	R	5	ND	1	R	100	A513C	C8T	WT	CTT/CGT L16R	1176	LAM9
26	#I01	R	5	S	2.5	R	100	A513C	WT	WT	CTT/CGT L16R	Unique[a]	
27	#I04	R	5	S	2	R	100	A513C	WT	WT	CTT/CGT L16R	42	LAM9
28	#I02	R	5	ND	2.5	R	20	WT	WT	WT	GGG/GAG G34E	53	T1
29	#I03	R	5	S	1.5	R	10	WT	WT	WT	CGC/TGC R96C	373	T1
30	#H06	R	7.5	S	1	R	5	WT	G37T	WT	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
31	#H09	R	5	ND	1.5	R	5	WT	G10A	WT	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
32	#H07	R	7.5	S	0.5	S	1.5	WT	C14T	WT	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
33	#H05	R	7.5	S	1	S	≤0.3	WT	C14T	WT	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
34	#N04	S	1.5	S	1.5	R	>100	WT	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
35	#N05	S	1	ND	0.5	R	>100	WT	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
36	#N06	S	1	S	1	ND	>100	WT	WT	AAG/AGG K43R	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
37	#N07	S	2.5	ND	1	R	>100	WT	WT	AAG/AGG K88R	CTT/CGT L16R	262	H4
38	#N01	S	2.5	S	1	R	100	A513C	WT	WT	CTT/CGT L16R	42	LAM9
39	#N12	S	2.5	ND	1.5	R	40	WT	WT	WT	GCG/GTG A138V	283	H1
40	#N13	S	2.5	S	1	R	20	WT	WT	WT	GGA/GAA G71E	283	H1
41	#N15	S	2.5	S	1	R	2.5	WT	WT	WT	CGC/TGC R96C	205	T1

R = resistant; S = sensitive; ND = not determined.
[a] Octal code: 776000000160771.

A general cross-resistance between AMK and KAN is usually considered; however, later studies of possible cross-resistance have shown variable results (Jugheli et al., 2009; Krüüner et al., 2003). Thus, in this study, 41 randomly selected *M. tuberculosis* isolates with confirmed resistance to any of aminoglycosides by the DST method were tested for MIC values of STR, KAN, and AMK (Table 2). In the cases of KAN and AMK, all *M. tuberculosis* isolates could be clearly divided into phenotypic groups: highly resistant, R^H (MIC >100 μg/mL); medium-resistant, R^M (MIC = 40 μg/mL); low-resistant, R^L (MIC = 5.0–7.5 μg/mL); and sensitive strains, S (MIC < 5 μg/mL). For STR, the results of REMA showed much higher variation of MICs between levels of susceptibility and high resistance. Further analysis of MICs demonstrated that among aminoglycoside-resistant *M. tuberculosis* isolates, 13 (31.7%) strains had clinically important MIC level of all the 3 aminoglycosides; 18 (43.9%) strains were resistant to KAN and STR but sensitive to AMK. Eight (19.5%) *M. tuberculosis* isolates were resistant only to STR, and 2 (4.9%) were resistant only to KAN.

3.2. Spoligotyping of M. tuberculosis isolates

All *M. tuberculosis* clinical isolates were genotyped by the spoligotyping method (Tables 2 and 3). The results showed that isolates included in this study belonged to 8 different spoligotype subfamilies. Aminoglycoside-resistant group samples belonged to Beijing (26 isolates), H1 (2 isolates), H4 (1 isolate), LAM9 (8 isolates), and T1 (3 isolates) subfamilies; 1 isolate had unique spoligotype (octal code: 776000000160771). Sensitive control group isolates belonged to Beijing (4 isolates), H1 (1 isolate), H4 (2 isolates), LAM1 (2 isolates), T1 (1 isolates), T5 (1 isolate), and U (1 isolate) subfamilies. Four sensitive isolates had unique spoligotypes different to each other, octal codes: 777760007760731, 777400003760771, 600137737400771, and 757777777720761.

3.3. Identification of mutations in sensitive M. tuberculosis strains

All 17 drug-sensitive *M. tuberculosis* isolates were screened for mutations in 1400th and 530th regions in *rrs* gene, promoter region of *eis* gene, and whole sequences of *rpsL* and *gidB* genes. The results show that genetic regions tested had no mutations in drug-sensitive *M. tuberculosis* isolates; the only exception was the *gidB* gene, when mutations were detected in 11 of 17 (65%) pan-sensitive isolates tested (Table 3).

3.4. Identification of mutations associated with KAN and AMK resistance

In order to characterize KAN and AMK resistance, *rrs* gene and promoter region of *eis* gene were analyzed. In total, A1400G mutation was detected in 92% (11/12) of *M. tuberculosis* isolates with high resistance level (MIC >100 μg/mL) to KAN and resistance to AMK (Table 4). Importantly, a diverse resistance profile to AMK was observed in these isolates (MIC 7.5 and >100 μg/mL, Table 2). By contrast, this mutation was not observed (0/20) in isolates low resistant to KAN (MIC 5–7.5 μg/mL) and sensitive to AMK (MIC <5 μg/mL); this difference was statistically significant ($P < 0.0001$). G1483T, another mutation in 1400th region of *rrs* gene, was found just once; this isolate #G01 belonged to Beijing genotype and had MIC values 40, 7.5, and >100 μg/mL of KAN, AMK, and STR, respectively (Table 2).

The analysis of promoter region of *eis* gene showed 5 types of mutations (C-8T, G-10A, C-12T, C-14T, and G-37T) among 17 aminoglycoside-resistant isolates (Table 2). Most of these isolates (16/17) were low resistant (MIC of 5 and 7.5 μg/mL) to KAN and sensitive to AMK. The exceptional isolate #F01 belonged to Beijing genotype and had high resistance (MIC >100 μg/mL) to both drugs. In total, mutations in the promoter region of *eis* gene were detected in 8% (1/12) of *M. tuberculosis* isolates with high resistance level to KAN and resistance to AMK; by contrast, this mutation was observed in 80%

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Table 3
Mutation analysis in pan-susceptible *M. tuberculosis* strains.

Nr	Isolate	Phenotypic drug susceptibility			Mutations in aminoglycoside resistance–associated loci				Spoligotype	
		KAN	AMK	STR	*rrs*	*eis* promotor	*rpsL*	*gidB*	SIT Nr.	Subfamily
1	#O02	S	S	S	wt	wt	wt	CTT/CGT L16R	UNIQUE[a]	
2	#O03	S	S	S	wt	wt	wt	CTT/CGT L16R	UNIQUE[b]	
3	#O04	S	S	S	wt	wt	wt	CTT/CGT L16R	20	LAM1
4	#O05	S	S	S	wt	wt	wt	CTT/CGT L16R	20	LAM1
5	#O22	S	S	S	wt	wt	wt	CTT/CGT L16R	254/766	T5
6	#O23	S	S	S	wt	wt	wt	CTT/CGT L16R	254	T5
7	#O24	S	S	S	wt	wt	wt	CTT/CGT L16R	1451	U
8	#O28	S	S	S	wt	wt	wt	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
9	#O30	S	S	S	wt	wt	wt	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
10	#O31	S	S	S	wt	wt	wt	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
11	#O32	S	S	S	wt	wt	wt	GAA/GAC E92D, GCA/GCG A205A	1	Beijing
12	#O34	S	S	S	wt	wt	wt	wt	283	H1
13	#O36	S	S	S	wt	wt	wt	wt	262	H4
14	#O37	S	S	S	wt	wt	wt	wt	262	H4
15	#O42	S	S	S	wt	wt	wt	wt	1597	T1
16	#O43	S	S	S	wt	wt	wt	wt	UNIQUE[c]	
17	#O44	S	S	S	wt	wt	wt	wt	UNIQUE[d]	

[a] Octal code: 777760007760731.
[b] Octal code: 777400003760771.
[c] Octal code: 600137737400771.
[d] Octal code: 757777777720761.

(16/20) of isolates low resistant to KAN and sensitive to AMK. This difference was statistically significant ($P = 0.0001$).

Importantly, the results showed that 4 *M. tuberculosis* isolates with moderate MIC level of KAN had wild-type sequences of 1400th region and promoter region of *rrs* and *eis* genes, respectively. These samples amounted to 12% of all KAN-resistant isolates included in this study and belonged to T1 (#I02 and #I03), LAM9 (#I04), and unique spoligotype (#I01) genotypes (Table 2).

3.5. Identification of mutations associated with streptomycin resistance

Furthermore, mutations in genes that could have an impact on STR resistance in *M. tuberculosis* strains were analyzed. The *rpsL* gene was found to have 2 types of mutations: K43R (22 isolates) and K88R (1 isolate). K43R was detected exclusively in 92% (22/24) of STR-resistant Beijing strains (Table 5). Moreover, all 22 K43R mutation-positive isolates were highly resistant to STR (MIC >100 μg/mL). Those 2 remaining STR-resistant *M. tuberculosis* Beijing isolates with wild-type *rpsL* gene (#H06 and #H09) were low resistant to STR (MIC = 5.0 μg/mL, Table 2). The absence of this mutation in 6 STR-sensitive as well as in 2 low-level STR-resistant isolates of Beijing lineage was statistically significant ($P < 0.0001$ and $P = 0.0036$, respectively). The difference of the K43R mutation prevalence in isolates of Beijing (92%) and LAM9 (0%) lineages was statistically significant ($P < 0.0001$, Table 5). Due to limited sample sizes, statistical analysis was not performed for other spoligotypes. The only isolate with K88R mutation in the *rpsL* gene (#N07) was highly resistant to STR (MIC >100 μg/mL), sensitive to KAN and AMK, and belonged to H4 genotype (Table 2).

As the mutation A513C in the *rrs* gene was detected only in aminoglycoside-resistant isolates but the relationship of this mutation with KAN or AMK susceptibility pattern was not observed, we further analyzed its possible relation to STR resistance. The results show that 100% (8/8) of STR-resistant *M. tuberculosis* isolates of LAM9 family had the mutation A513C in the *rrs* gene (Table 5). By contrast, this mutation was not detected in STR-resistant isolates of Beijing genotype; this difference was statistically significant ($P < 0.0001$). One *M. tuberculosis* isolate that had this mutation (#I01) belonged to a genotype with a unique spoligotype and was highly resistant to STR (MIC = 100 μg/mL), low resistant to KAN (MIC = 5 μg/mL), and sensitive to AMK (Table 2).

Table 4
Detection of mutations in *M. tuberculosis* isolates with variable KAN and AMK susceptibility pattern.

Phenotypic drug susceptibility (MIC value, μg/mL)		No. of isolates, total	No. (%) of *M. tuberculosis* isolates with mutation		
			rrs, A1400G	*rrs*, G1483T	*eis* promoter, any type
KAN	R^H (>100)	12	11 (92)	0	1 (8)
AMK	R (5 to >100)				
KAN	R^M (40)	1	0	1 (100)	0
AMK	R (5 to >100)				
KAN	R^L (5–7.5)	20	0	0	16 (80)
AMK	S (<5)				
KAN	S (<5)	17	0	0	0
AMK	S (<5)				

R^H = highly resistant (MIC >100 μg/mL); R^M = medium-resistant (MIC = 40 μg/mL); R^L = low-resistant (MIC = 5.0 to 7.5 μg/mL); S = drug-sensitive (MIC < 5 μg/mL).

3.6. Identification of gidB gene mutations in M. tuberculosis strains

As it was mentioned above, various *gidB* gene mutations were observed both in aminoglycoside-resistant and aminoglycoside-sensitive isolates. Altogether, 7 types of *gidB* gene mutations were found: E92D, L16R, G34E, G71E, R96C, A138V, and a silent polymorphism A205A (GCA/GCG) (Table 5). E92D mutation together with the silent polymorphism A205A was observed only among Beijing isolates; the mutation rate was 100% both for STR-resistant and STR-sensitive samples. By contrast, L16R mutation was absent in Beijing isolates (0/30), but it was strongly associated with subfamily LAM9 (8/8, 100%); this difference was statistically significant ($P < 0.0001$). In addition, L16R mutation was found in *M. tuberculosis* isolates of LAM1, H4, T5, and U spoligotypes; however, due to limited sample sizes, statistical analysis was not performed for these spoligotypes. The remaining 4 types of *gidB* gene mutations were detected in a small number of STR-resistant samples. G34E and R96C were detected in STR-resistant isolates of T1 subfamily, and G71E and A138V mutations were detected in STR-resistant isolates of H1 subfamily (Table 5).

4. Discussion

The present study investigates possible genetic mutations involved in the aminoglycoside resistance mechanisms in *M. tuberculosis* clinical isolates from Latvia. To reach this goal, a collection of aminoglycoside-resistant *M. tuberculosis* strains was screened for well-known resistance associated loci like region 1400 of the *rrs* gene, the promoter region of the *eis* gene, and the entire sequence of the S12 ribosomal protein gene (*rpsL*). In addition, possible role of controversial polymorphism in nucleotide 513 of the *rrs* gene and the entire sequence of the *gidB* gene was studied. Although some additional hypothetical aminoglycoside susceptibility influencing genes, particularly *whiB7*, exist, these mutations were not characterized in the present study. Further investigations are required to address the nature and frequency of these mutations in aminoglycoside-resistant clinical isolates.

Our results indicated that A1400G *rrs* gene mutation was observed in 92% of KAN high-level resistant strains. However, a remarkable diversity of AMK resistance in these isolates was observed, thus possibly indicating to an unknown mutation interacting with A1400G among isolates with higher MIC of AMK. In addition, it could be hypothesized that G1483T mutation in *rrs* gene provides a weaker resistance to KAN than A1400G because much lower MIC in the corresponding strain was observed.

Please cite this article as: Bauskenieks M, et al, Genotypic and phenotypic characteristics of aminoglycoside-resistant *Mycobacterium tuberculosis* isolates in Latvia, Diagn Microbiol Infect Dis (2014), http://dx.doi.org/10.1016/j.diagmicrobio.2014.12.004

Table 5
Detection of mutations in STR-resistant and STR-sensitive *M. tuberculosis* isolates of various genotypic groups.

Genotype	STR resistance	No. of isolates	No. (%) and type of mutation								
			rrs	*rpsL*		*gidB*					
			A513C	K43R	K88R	E92D, A205A[a]	L16R	G34E	R96C	A138V	G71E
Beijing	R	24		22 (92)		24 (100)					
	S[b]	6				6 (100)					
LAM9	R	8	8 (100)				8 (100)				
	S	n.a.									
LAM1	R	n.a.									
	S	2					2 (100)				
T1	R	3						1 (33)	2 (67)		
	S	1									
H1	R	2								1 (50)	1 (50)
	S	1									
H4	R	1			1 (100)		1 (100)				
	S	2									
T5	R	n.a.									-
	S	2					2 (100)				
U	R	n.a.									
	S	1					1 (100)				
Unique[c]	R	1	1 (100)				1 (100)				
	S	4					2 (50)				
Total	R	39	9 (23)	22 (56)	1 (3)	24 (62)	10 (26)	1 (3)	2 (5)	1 (3)	1 (3)
	S[b]	19				6 (32)	7 (37)				

[a] E92D and A205A mutations always appeared together.
[b] Two isolates in STR sensitive group were low resistant to KAN.
[c] All 5 isolates had different spoligotype.

However, additional functional study is needed to address this hypothesis. The distribution of mutations in the promoter region of *eis* gene was similar to previous studies as they were found in 80% of *M. tuberculosis* isolates low-level resistant to KAN and sensitive to AMK (Engström et al., 2011; Zaunbrecher et al., 2009). However, an exceptional case was found where high resistance level both to KAN and AMK (MIC >100 μg/mL for both drugs) coincided with an absence of mutations in 1400th region of *rrs* gene while the promoter region of *eis* gene was found to have a mutation G10A typical to low-level KAN-resistant isolates. Beside that, our results indicated that the molecular mechanism of 12% of KAN-resistant clinical isolates is not dependent on well-known mutations and requires more investigations.

Further, a possible association of detected mutations with STR resistance in *M. tuberculosis* strains in Latvia was analyzed. Mutation K43R in *rpsL* gene was detected exclusively in STR high-level resistant *M. tuberculosis* isolates of Beijing genotype and was completely absent in STR-resistant *M. tuberculosis* isolates belonging to all other lineages included in this study as well as in STR-sensitive and low-level STR-resistant Beijing strains. These data indicate that resistance mechanisms to STR seem to be closely related to genotypic lineages; this finding supports previous observations (Honore and Cole, 1994; Lipin et al., 2007; Nair et al., 1993; Wong et al., 2011). However, Tudo et al. (2010) characterized K43R polymorphism-related resistant isolates with widely diverse phylogenetic spectra. A second *rpsL* gene mutation, K88R, was found only in 1 *M. tuberculosis* isolate belonging to the H4 family; similarly, this isolate was highly resistant to STR.

A mutation in the *rrs* gene at position 513 has frequently been associated with STR resistance as the nucleotide 513 belongs to the region 530 widely known to play an important role in the interactions between STR and bacterial ribosomes (Spies et al., 2011; Sreevatsan et al., 1996; Tracevska et al., 2004). In our study, A513C mutation was detected in 9 STR-resistant *M. tuberculosis* strains; importantly, it was present only in *M. tuberculosis* isolates of LAM9 family, and the prevalence of this mutation in STR-resistant strains of this genotype was 100%. This result is in accordance with previous observation when similar association between the LAM spoligotype family and mutation in the *rrs* gene at position 513 (A → C) was reported (Lipin et al., 2007). These data may indicate another nature of this mutation – genotype-specific marker, although such association was not observed in the study of Spanish *M. tuberculosis* isolates (Tudo et al., 2010). A possible explanation of this discordance could be the fact that having multiple isolates of a circulating strain in a lineage might complicate the statistical analyses of the association of a mutation with a lineage. In this study, *M. tuberculosis* isolates were randomly selected in time frame of 6 years; thus, there is a little possibility for epidemiological linkage for these samples; however, such issue could not be absolutely excluded.

Similarly, E92D and A205A (GCA/GCG) *gidB* gene mutations appear to be sequence polymorphisms instead of a mutation associated with STR resistance because of strong coincidence with Beijing lineage observed in this study as well as the presence of this mutation in STR-susceptible strains. This result is in concordance with previous observations (Spies et al., 2011). Second *gidB* gene mutation L16R previously was characterized as LAM lineage specific (Spies et al., 2011). Indeed, in our study, all *M. tuberculosis* samples of LAM lineages LAM9 (STR-resistant strains) and LAM1 (STR-susceptible strains) harbored this polymorphism. However, the same mutation was observed in *M. tuberculosis* strains belonging to H4, T5, and U lineages as well as in 3 uncharacterized samples in this study. Thus, it could be assumed that this polymorphism could not be used as a LAM lineage–specific marker nor as STR susceptibility marker. In contrast, for STR-resistant *M. tuberculosis* isolates of the T1 genetic family, 2 different *gidB* gene mutation types, i.e., G34E and R96C, were detected despite of the low number of samples analyzed. The same is true for the samples of H1 lineage, when A138V and G71E mutations were found. These results confirmed the highly polymorphic nature of the *gidB* gene and suggest that association of mutations of this gene to the drug resistance phenotype should be done in respect to genetic lineages.

In conclusion, the profile of mutations in *rrs* gene, *eis* gene promoter region, *rpsL*, and *gidB* genes in aminoglycoside-resistant and aminoglycoside-sensitive *M. tuberculosis* isolates of different genetic lineages from Latvia were investigated. This study shows that aminoglycoside resistance mechanisms in *M. tuberculosis* are more diverse than described previously; thus, association of certain genetic mutations with resistance to KAN, AMK, and STR needs to be further investigated.

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Acknowledgments

The authors are grateful for the support of grant of the Latvian Council of Science 09.1296 and VPP "BIOMEDICINE". The authors would like to acknowledge the help of Dr Ivita Šēla given to the study.

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Please cite this article as: Bauskenieks M, et al, Genotypic and phenotypic characteristics of aminoglycoside-resistant *Mycobacterium tuberculosis* isolates in Latvia, Diagn Microbiol Infect Dis (2014), http://dx.doi.org/10.1016/j.diagmicrobio.2014.12.004