Diagnostic Microbiology and Infectious Disease 81 (2015) 177–182

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

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

Mycobacteriology

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 15 December 2014

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