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BRIEF COMMUNICATION

Second-line anti-tuberculosis drug resistance and its genetic determinants in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates

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MDR-TB;
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Abstract *Background:* Mutations in several genetic loci have been implicated in the development of resistance to second-line anti-tuberculosis (TB) drugs (SLDs). The purpose of this study was to investigate the prevalence of resistance to SLDs and its association with specific mutations in multidrug-resistant (MDR) *Mycobacterium tuberculosis* clinical isolates.
Materials and methods: The study included 46 MDR-TB isolates. Mutation profiling was performed by amplifying and sequencing the following six genes: *gyrA*/*gyrB*, *rrs*, *tlyA*, and *ethA*/*ethR*, in which mutations are implicated in resistance of tubercle bacilli to ofloxacin (OFX), amikacin (AMK), capreomycin, and ethionamide (ETH), respectively.
Results: Of the strains analyzed, 14 (30.4%) showed resistance to at least one of the four SLDs tested. Mutations in the *gyrA* gene occurred in 34 (73.9%) strains, with the most common amino acid change being Ser95Thr. The Asp94Asn and Ala90Val substitutions in the *gyrA* were present exclusively in OFX-resistant strains, yet represented only 40% of all OFX-resistant strains. The only mutation in the *gyrB* gene was substitution Ser447Phe, detected in one OFX-resistant isolate. None of the AMK-resistant strains carried a mutation in the *rrs* gene. Mutations in the *ethA*/*ethR* loci were found in one ETH-resistant and 11 ETH-susceptible strains.

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Conclusions: The results of this study challenge the usefulness of sequence analyses of tested genes (except *gyrA*) for the prediction of SLD resistance patterns and highlight the need for searching other genetic loci for detection of mutations conferring resistance to SLDs in *M. tuberculosis*.

Introduction

The two most detrimental forms of drug-resistant tuberculosis (TB) are multidrug-resistant (MDR)-TB, defined as resistance to at least isoniazid (INH) and rifampicin (RMP), and extensively drug-resistant (XDR)-TB, defined as MDR-TB with additional resistance to second-line anti-TB drugs (SLDs), that is any fluoroquinolone (FQ) and one of the three injectable agents: amikacin (AMK), kanamycin (KAN), or capreomycin (CAP).

Mutations in several genetic loci have been implicated in the development of resistance to SLDs. Resistance to FQs has been reported to be associated with mutations in two regions known as "quinolone resistance-determining regions (QRDRs)", in the *gyrA* (codons 74 to 113) and *gyrB* (recently proposed to span from codon 500 to codon 540) genes, which encode the respective subunits of the DNA topoisomerase gyrase.[1,2] Resistance to KAN, AMK, and CAP is thought to be mediated by mutations in the *rrs* gene, coding for 16S rRNA.[3] Furthermore, resistance to CAP has been shown to be caused by alterations in the *tlyA* gene, coding for 2′-O-methyltransferase, an enzyme that methylates riboses in rRNA.[4] Finally, mutations in a two-gene operon (*ethA*/*ethR*) whose products are involved in the activation of ethionamide (ETH) have been implicated in conferring resistance to this drug.[5]

The purpose of this study was to investigate the prevalence of resistance to SLDs in MDR *Mycobacterium tuberculosis* clinical isolates and to search for genetic determinants of SLD resistance.

Materials and methods

The study included 46 MDR *M. tuberculosis* strains, collected at the National Tuberculosis and Lung Diseases Institute in Warsaw during the third national survey of DR-TB. The isolates were recovered from 46 unrelated pulmonary TB patients (40 men and 6 women; age range, 31—79 years; median age, 50.5 years) from across Poland. These patients represented all bacteriologically-confirmed MDR-TB cases reported in Poland in 2004 [TB cases registered (total), $n = 9493$; TB notification rate, $n = 24.9$/100,000 population]. The detailed sociodemographic and clinical characteristics of the patients whose isolates were used in this study had been presented elsewhere.[6] Primary isolation, cultivation, and species identification were performed with standard mycobacteriological methods.[6] Drug susceptibility testing was carried out using the 1% proportion method on the Löwenstein-Jensen medium. The cutoff drug concentrations were as follows: INH, 0.2 μg/mL; RMP, 40 μg/mL; streptomycin (SM), 4 μg/mL; ethambutol, 2 μg/mL; ofloxacin (OFX), 2 μg/mL; AMK, 4 μg/mL; CAP, 40 μg/mL, and ETH, 40 μg/mL. Genomic DNA was extracted using the cetyltrimethylammonium bromide method, as previously described.[7] The presence of mutations possibly associated with resistance to SLDs was determined by amplifying and sequencing six genetic loci, i.e., two hotspot targeted regions: *gyrA* (codons 17 to 213) and *gyrB* (codons 377 to 612) and the following genes: *rrs* (AMK), *tlyA* (CAP), and *ethA*/*ethR* (ETH). All primers used for amplification were newly designed (Table 1). Amplification reactions were performed according to manufacturer's specific recommendations (TopTaq DNA polymerase, QIAGEN, Hilden, Germany). Purified polymerase chain reaction amplicons were sequenced in both directions using the same primers as for polymerase chain reaction amplification and when needed, additional sequencing primers. All alignments were done against the genome sequence of the *M. tuberculosis* reference strain H37Rv (http://www.ncbi.nlm.nih.gov/genbank/; GenBank accession number: AL123456.3) using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/).

Results

A total of 14 (30.4%; 14/46) strains were resistant to at least one of the four SLDs tested. Nine (19.5%) strains were resistant to either OFX (7; 15.2%) or AMK (2; 4.3%), and thus met the definition of pre-XDR-TB, whereas three (6.5%) strains were resistant to OFX and AMK (2; 4.3%) or CAP (1; 2.2%) simultaneously and were categorized as XDR-TB strains. Four (8.7%) strains, including two pre-XDR strains, were resistant to ETH. The overall frequencies of resistance to SLDs were as follows: 21.7% ($n = 10$) for OFX, 8.7% ($n = 4$) for AMK, 8.7% ($n = 4$) for ETH, 2.2% ($n = 1$) for CAP.

Distribution of mutations and percentage of strains carrying mutations in each of the investigated genes among 46 *M. tuberculosis* strains are shown in Figure 1. Nonsynonymous amino acid changes in the *gyrA*/*gyrB* loci, excluding Ser95Thr (*gyrA*), which is known as natural polymorphism[8,9] occurred in seven (15.2%; 7/46) strains, six of which were resistant to OFX. However, only five (out of 10) OFX-resistant strains contained mutations specific for resistance to this FQ. These were Ala90Val, Asp94Asn, and Asp94Gly in *gyrA* locus (four strains) and Ser447Phe in *gyrB* locus (one strain). None of the four AMK-resistant strains carried mutations in the *rrs* gene. The *rrs* mutations, in two highly mutable regions known as the 530 and 912 loops, were found in seven (15.2%; 7/46) strains susceptible to