

Short Communication

Molecular Characterization of Rifampicin- and Isoniazid-Resistant *Mycobacterium tuberculosis* Strains Isolated in Kazakhstan

Ulan Kozhamkulov*, Ainur Akhmetova, Saule Rakhimova, Elena Belova¹,
Ariake Alenova¹, Venera Bismilda¹, Lyailya Chingissova¹, Shahimurat Ismailov¹,
Erlan Ramanculov, and Kuvat Momynaliev

National Center for Biotechnology, Astana; and

¹National Center for Tuberculosis Problems, Almaty, Republic of Kazakhstan

(Received December 17, 2010. Accepted March 23, 2011)

SUMMARY: Kazakhstan is one of the 14 countries with a high rate of morbidity due to multidrug-resistant tuberculosis (MDR TB) in WHO European region. The aim of our study was to characterize mutations associated with drug resistance to rifampicin and isoniazid in *Mycobacterium tuberculosis* isolates from Kazakhstan. *M. tuberculosis* strains were isolated from TB patients in different regions of Kazakhstan. A drug susceptibility test was performed on Lowenstein-Jensen medium using the absolute concentration method. Sequencing analysis was performed of the *rpoB* rifampicin resistance-determining region and the *katG* gene, the *oxyR-ahpC* intergenic region, and the *inhA* promoter region in 259 MDR *M. tuberculosis* isolates, in 51 isoniazid-resistant isolates, and in 13 rifampicin-resistant isolates. The mutational analysis revealed that the most frequent mutations associated with rifampicin and isoniazid resistance in *M. tuberculosis* are the substitutions at codons 531 (82.7%) and 315 (98.4%) in the *rpoB* and *katG* genes, respectively. In addition, we have found mutations with lower frequency at codons 526 (8.4%), 533 (1.5%), and 516 (1.1%) in the *rpoB* gene. In 6.2% of the isolates, no mutations were found in the *rpoB* gene. The findings of this study provide useful data for a better understanding of the mutation spectrum of isoniazid and rifampicin resistance among strains isolated from patients in Kazakhstan. Our results are also useful for the development of diagnostic tests of MDR *M. tuberculosis*.

In Kazakhstan, the incidence of tuberculosis (TB) in 2008 was 125.5 cases per 100,000 people, and the mortality rate was 16.9 per 100,000 people (1). The incidence of multidrug-resistant (MDR) TB increased by a factor of 1.7 between 2003 and 2008 (from 5.0 to 8.5 cases per 100,000 people). In Kazakhstan, primary multidrug-resistance was reported in 21.3% of new cases and acquired multidrug-resistance was reported in 39.0% of recurrent cases in 2008 (1). The number of MDR isolates among all cases in 2009 was 2,858 (2). Thus, high rates of morbidity and mortality exist, and the incidence of MDR TB is constantly increasing, complicating efforts to battle this epidemic.

In a previous work (3) conducted in 2001, analysis of the *rpoB* gene of MDR strains from Kazakhstan revealed 10 different mutations in 5 codons, with the most frequent mutations being found in the *rpoB* codons 531 (65.2%), 526 (23.9%), and 516 (7.6%). All MDR isolates carried a mutation in codon 315 of the *katG* gene (S315T). The purpose of our study was to estimate the spectrum and prevalence of mutations in genes implicated in resistance to basic anti-TB drugs in *Mycobacterium tuberculosis* strains isolated in Kazakhstan over an 8-year period. It should be noted that we used the same protocol for the primary isolation of *M. tuberculosis*

spp. and drug susceptibility testing as previously described (3).

We analyzed 323 drug-resistant strains of *M. tuberculosis*, including 259 MDR isolates, 51 isoniazid-resistant (INH^r) isolates, and 13 rifampicin-resistant (RIF^r) isolates. Clinical isolates of *M. tuberculosis* were collected from nine regional TB dispensaries during 2009 and deposited in the Reference Laboratory of the National Center for Tuberculosis Problems for surveillance studies of anti-TB drug resistance in Kazakhstan. After primary isolation, mycobacteria were subcultured on solid Lowenstein-Jensen (LJ) medium in regional laboratories, as described previously (4). Species differentiation as *M. tuberculosis* and drug susceptibility testing were performed at the Reference Laboratory. Rifampicin and isoniazid susceptibility tests were carried out on LJ medium containing 40 mg/L rifampicin, 0.2 mg/L isoniazid, or 1 mg/L isoniazid using the absolute concentration method according to the World Health Organization (WHO) recommendations. The results of these microbiological tests were recorded 28 days after inoculation. Isolates were considered to be resistant if more than 20 colonies grew on the media containing antibiotics (5).

DNA extraction procedure, polymerase chain reaction (PCR), and sequence analysis of *rpoB*, *katG*, and promoter regions of the *fabG-inhA* and *oxyR-ahpC* loci was performed as described previously (6–10). Sequencing of the PCR products was carried out with an ABI 3730 Genetic Analyzer automated DNA sequencer (Applied Biosystems, Foster City, Calif., USA) using

*Correspondence author: Mailing address: National Center for Biotechnology, Valikhanov str., 13/1, Astana, 010000, Republic of Kazakhstan. Tel: +7(7172)21 40 20, 21 41 26, Fax: +7(7172)21 46 33, E-mail: kzulan@gmail.com

the BigDye terminator kit (Applied Biosystems) according to the manufacturer's instructions. The sequences were compared with their respective wild-type sequences using the SeqScape software (Applied Biosystems).

Among the 259 MDR isolates, 191 (73.7%) were also resistant to ethambutol and streptomycin (HRES), 66 (25.5%) were resistant to streptomycin (HRS), and 2 (0.7%) were resistant to ethambutol (HRE). The INH^r clinical isolates ($n = 51$) were divided into 2 major categories: 31 isolates (60.8%) were resistant to isoniazid and streptomycin (HS), and 14 isolates (27.4%) were resistant to isoniazid, ethambutol, and streptomycin (HES). Only 5 isolates (9.8%) were resistant to isoniazid, and 1 isolate (1.9%) was resistant to both isoniazid and ethambutol (HE). Among the 13 RIF^r clinical isolates, 7 (53.8%) were resistant to rifampicin and streptomycin (RS), 3 (23.0%) were resistant to rifampicin and ethambutol (RE), 2 (15.4%) were resistant to rifampicin, ethambutol, and streptomycin (RES), and 1 (7.7%) was resistant to rifampicin.

We attempted to identify specific rifampicin resistan-

ce-causing mutations in the *rpoB* gene of 272 *M. tuberculosis* clinical isolates, including 259 MDR strains. Nucleotide polymorphisms leading to RIF^r are mainly found between *rpoB* codons 507 and 533 in the rifampicin resistance-determining region (RRDR). Among the 272 RIF^r clinical isolates, mutations were detected in 255 isolates (93.7%), and 13 different variants were found in 4 codons (Ser531, Asp516, His526, and Leu533) (Table 1). The majority of nucleotide substitutions ($n = 225$, 82.7%) occurred at codon 531 of the *rpoB* gene, where 4 different types of single nucleotide substitutions were identified, predominantly replacing serine with leucine (80.9%). In the remaining isolates, mutations were observed in less than 10% of cases.

The INH^r *M. tuberculosis* isolates ($n = 310$) were studied to identify resistance-causing mutations in *katG* and the promoter regions of the *fabG-inhA* and *oxyR-ahpC* loci (Table 1). Mutations conferring INH^r were detected in 309 (99.7%) out of 310 INH^r clinical isolates. Analysis of the *katG* gene revealed 305 isolates (98.4%) with mutations at codon 315 leading to the

Table 1. Mutations in genes involved in rifampicin and isoniazid resistance of *M. tuberculosis* strains isolated in Kazakhstan

Gene	Affected codon	Nucleotide change(s)	Amino acid change	No. (%) of strains with mutation	
RIF-resistant strains					
<i>rpoB</i>	531	TCG→TTG	Ser→Leu	220 (80.9)	
		TCG→TAC	Ser→Tyr	3 (1.1)	
		TCG→CAG	Ser→Gln	1 (0.4)	
		TCG→TGG	Ser→Trp	1 (0.4)	
	Total				225 (82.7)
	526	CAC→CGC	His→Arg	5 (1.8)	
		CAC→CTC	His→Leu	9 (3.3)	
		CAC→GAC	His→Asp	2 (0.7)	
		CAC→TAC	His→Tyr	6 (2.2)	
		CAC→TGC	His→Cys	1 (0.4)	
	Total				23 (8.4)
	533	CTG→CCG	Leu→Pro	Total	4 (1.5)
	516	GAC→TTC	Asp→Phe		1 (0.4)
		GAC→TAC	Asp→Tyr		1 (0.4)
		GAC→GTC	Asp→Val		1 (0.4)
Total				3 (1.1)	
None	None	None (WT)	None (WT)	17 (6.2)	
INH-resistant strains					
<i>katG</i>	315	AGC→ACC	Ser→Thr	280 (90.3)	
<i>fabG-inhA</i>	-15	C→T	—	1 (0.3)	
	-8	T→C	—	1 (0.3)	
<i>oxyR-ahpC</i>	-6	G→A	—	1 (0.3)	
None	None	None	None (WT)	1 (0.3)	
Double mutations INH-resistant strains					
<i>katG</i>	315	AGC→ACC	Ser→Thr	20 (6.5)	
<i>fabG-inhA</i>	-15	C→T			
	315	AGC→ACC	Ser→Thr	1 (0.3)	
	-8	T→C			
	315	AGC→ACC	Ser→Thr	4 (1.3)	
	-8	T→A			
<i>fabG-inhA</i>	-15	C→T	—	1 (0.3)	
<i>oxyR-ahpC</i>	-6	G→A	—		

replacement of serine with threonine. Multiple mutations were identified in 25 strains (8.0%), in which mutations were present in *katG* and at the -8 and -15 positions of the promoter of the *mabA (fabG)-inhA* operon.

Our study revealed the prevalence of specific mutations in drug-resistant *M. tuberculosis* strains circulating in Kazakhstan in 2009. The main mutations in the *rpoB* gene were localized in codon 531 (82.7%) and to a lesser extent in codons 526 (8.4%), 533 (1.5%), and 516 (1.1%). Previously, the prevalence of mutations at 3 codons of *rpoB* (531, 526, and 516) was reported for 92 RIF^r *M. tuberculosis* isolates in 2001 from nine regions of Kazakhstan (3). The mutation frequencies for each codon were 65.2, 23.9, and 7.6%, respectively. Mutations in *rpoB* codon 514 were also identified in 1.1% of cases. Therefore, during the last 8 years, a significant change in the spectrum of mutations in the *rpoB* gene of *M. tuberculosis* clinical isolates has occurred in Kazakhstan.

When comparing the spectrum of mutations in genes that are specific to isoniazid resistance, a predominance of mutations at codon 315 of *katG* was identified. According to previously published data (3), the frequency of mutations in *katG* codon 315 was 97.2% among 142 INH^r isolates circulating in Kazakhstan. Our study revealed similar trends in the mutation frequencies (greater than 90.0% in both studies). In addition, the mutation spectra for *katG* codon 315 were comparable in both studies, with the exception of double mutations in *katG* codon 315 and the promoter region of the *mabA (fabG)-inhA* operon. In contrast to previously published data (3), our studies indicate that 8.4% of isolates contained double mutations, including combinations of mutations in *katG* and the promoter region of the *mabA (fabG)-inhA* operon in 8.0% of cases.

Changes in the type of mutations identified may be a consequence of the prevalent expansion of resistant *M. tuberculosis* strains with mutations at codon 531 of *rpoB*. To confirm this assumption, further genotype studies of resistant *M. tuberculosis* strains are required.

Acknowledgments This study was financially supported by the Ministry of Education and Science of the Republic of Kazakhstan.

Conflict of interest None to declare.

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