

Sequence analysis of the drug-resistant *rpoB* gene in the *Mycobacterium tuberculosis* L-form among patients with pneumoconiosis complicated by tuberculosis

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Abstract. The aim of the present study was to investigate the mutational characteristics of the drug-resistant *Mycobacterium tuberculosis* L-form of the *rpoB* gene isolated from patients with pneumoconiosis complicated by tuberculosis, in order to reduce the occurrence of the drug resistance of patients and gain a more complete information on the resistance of the *Mycobacterium tuberculosis* L-form. A total of 42 clinically isolated strains of *Mycobacterium tuberculosis* L-form were collected, including 31 drug-resistant strains. The genomic DNA was extracted, then the target genes were amplified by polymerase chain reaction and the hot mutational regions of the *rpoB* gene were analyzed by direct sequencing. The results revealed that no *rpoB* gene mutation was present in 11 rifampicin (RFP)-sensitive strains, while conformational changes were identified in 31 RFP-resistant strains. The mutation rate was 93.55% (29/31) in the resistant strains, and was frequently concentrated in codons 531 (51.61%; 16/31) and 526 (32.26%; 10/31), mainly occurring by case substitutions, including 27 unit point mutations and two two-point mutations. The novel mutation identified in codon 516 had not been previously reported. The substitution of highly-conserved amino acids encoded by the *rpoB* gene resulted in the molecular mechanism responsible for RFP resistance in the *Mycobacterium tuberculosis* L-form. This also demonstrated that the *rpoB* gene is diversiform.

Introduction

Tuberculosis is a chronic infectious disease caused by the bacterium *Mycobacterium tuberculosis*, and it remains a significant public health risk worldwide. Cosmopolitan tuberculosis pestilence has quickly returned due to the misuse of antituberculosis drugs and infection with HIV that has been apparent since the 1980's (1,2). Since becoming the first elected antituberculosis drug, the clinical therapeutic efficacy of RFP has severely degraded due to the appearance of drug-resistant strains and the L-form mutations of *Mycobacterium tuberculosis*. At present, drug resistance is an issue preventing the elimination of tuberculosis (3). According to the statistics, mutations in the *rpoB* gene in *Mycobacterium tuberculosis* account for 90% of the RFP-resistant strains, but there are no studies regarding the resistance of the L-form (4,5). In the present study, a DNA sequence analysis technique was applied for investigating the mutational characteristics of *rpoB* in the *Mycobacterium tuberculosis* L-form, in order to provide a more complete understanding of its resistance.

Materials and methods

Experimental subjects. Subjects included male patients with pneumoconiosis complicated by tuberculosis (n=114) who were aged between 41 and 70 years old, with a mean age of 53.35±9.28 years, and who were treated in the Affiliated Hospital of Anhui University of Science and Technology (Hefei, China) between July 2010 and July 2012. All the subjects were instructed to expectorate phlegm originating from the bottom of the trachea into sterile wide-mouthed bottles subsequent to gargling several times. The H37Rv quality control strain was provided by the Center of Biological Products of the Department of Health (Beijing, China). Patient consent was obtained from all the subjects. Approval was obtained from the Ethics Committee of the School of Medicine, Anhui University of Science and Technology (Huainan, China).

Experimental methods

DNA extraction. The sputum specimens of the patients were inactivated by autoclave. Genomic DNA was extracted using a

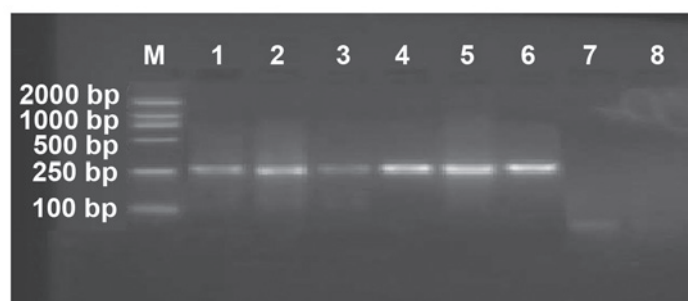
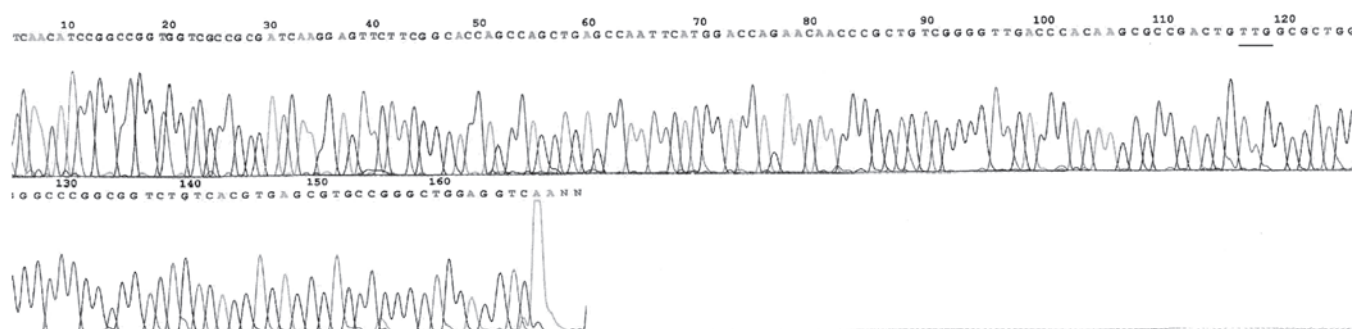
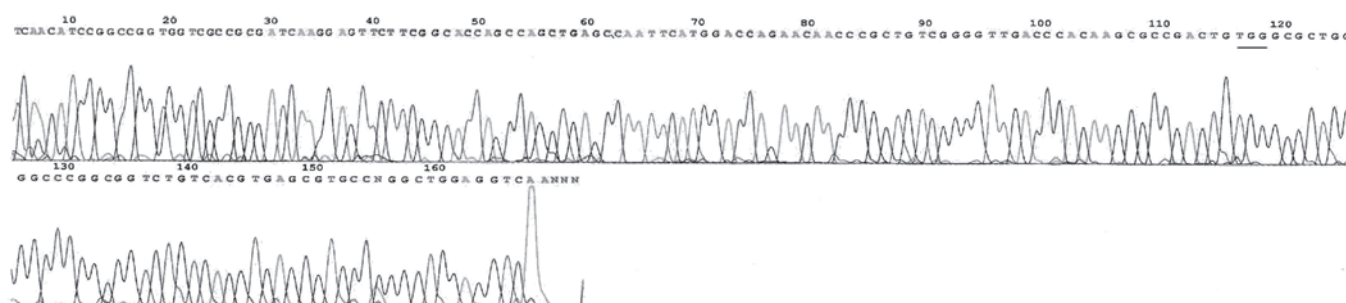
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Table I. Mutation characteristics of the *rpoB* gene in 31 clinical drug-resistant strains of *Mycobacterium tuberculosis* L-forms.

Strain	Location of amino acids, codon	Change of codon	Change of amino acids	Percentage, %
10	531	TCG→TTG	Ser→Leu	32.26
6	531	TCG→TGG	Ser→Trp	19.35
5	526	CAC→GAC	Gln→Asp	16.13
2	526	CAC→TAC	Gln→Tyr	6.45
1	526	CAC→CTC	Gln→Leu	3.23
1	526	CAC→CGC	Gln→Arg	3.23
1	526	CAC→GTC	Gln→Val	3.23
1	516	GAC→GGC	Asp→Gly	3.23
1	511, 526	CTG→CCG, CAC→CTC	Leu→Pro, Gln→Leu	3.23
1	526, 531	CAC→CTC, TCG→TGG	Gln→Leu, Ser→Trp	3.23
2	-	-	-	6.45

Figure 1. Detection of the amplification products of *rpoB* by polymerase chain reaction in clinical sputum specimens. Lane M, DL2000 marker (2000, 1000, 500, 250 and 100 bp); lane 1, the quality control strain (H37Rv); lane 2-6, clinical rifampicin-resistant strains with *rpoB* mutation; lane 7, clinical rifampicin-sensitive strain to *rpoB*; and lane 8, negative control.Figure 2. Sequence analysis of *rpoB* DNA in the clinically isolated *Mycobacterium tuberculosis* L-form; codon 531, TCG→TTG.Figure 3. Sequence analysis of *rpoB* DNA in the clinically isolated *Mycobacterium tuberculosis* L-form; codon 531, TCG→TGG.

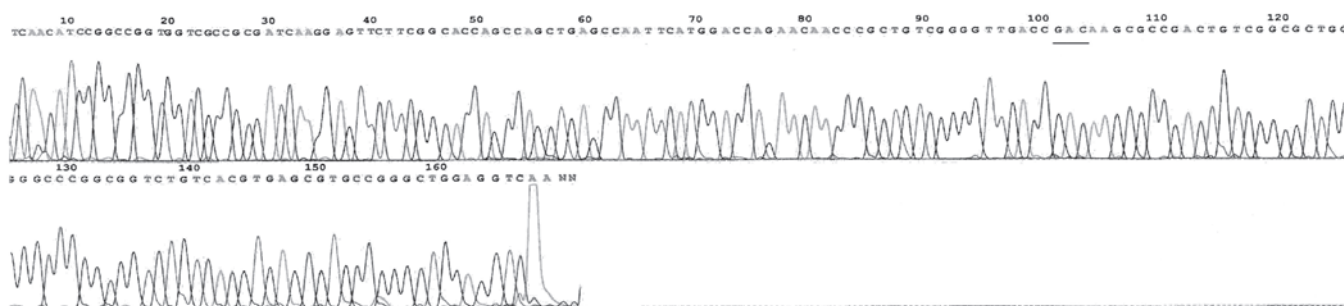


Figure 4. Sequence analysis of rpoB DNA in the clinically isolated *Mycobacterium tuberculosis* L-form; codon, 526 CAC→GAC.

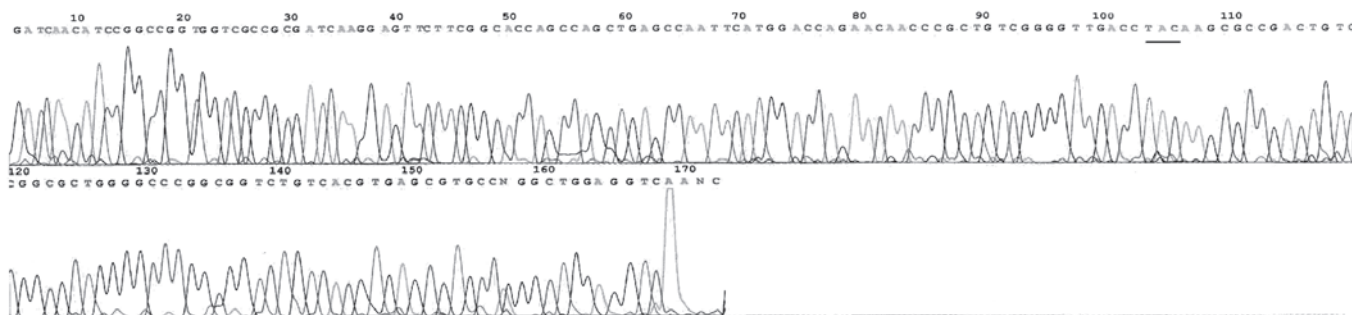


Figure 5. Sequence analysis of rpoB DNA in the clinically isolated *Mycobacterium tuberculosis* L-form; codon, 526 CAC→TAC.

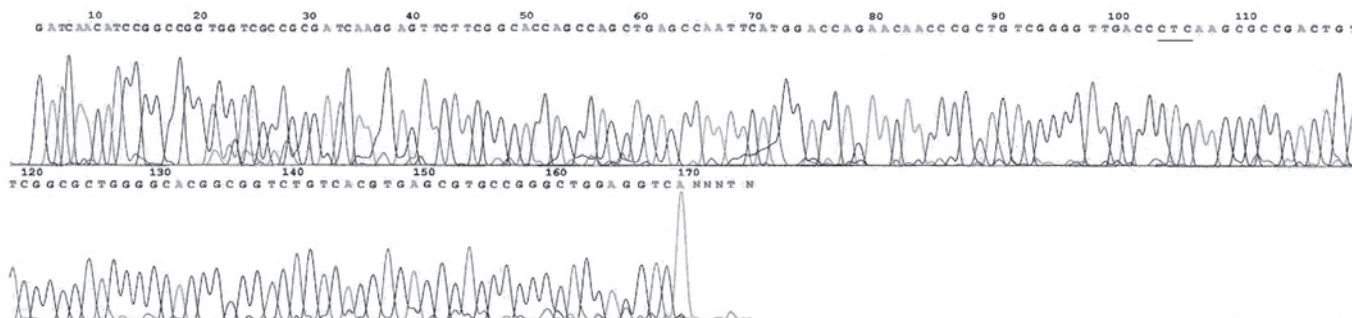


Figure 6. Sequence analysis of rpoB DNA in the clinically isolated *Mycobacterium tuberculosis* L-form; codon, 526 CAC→CTC.

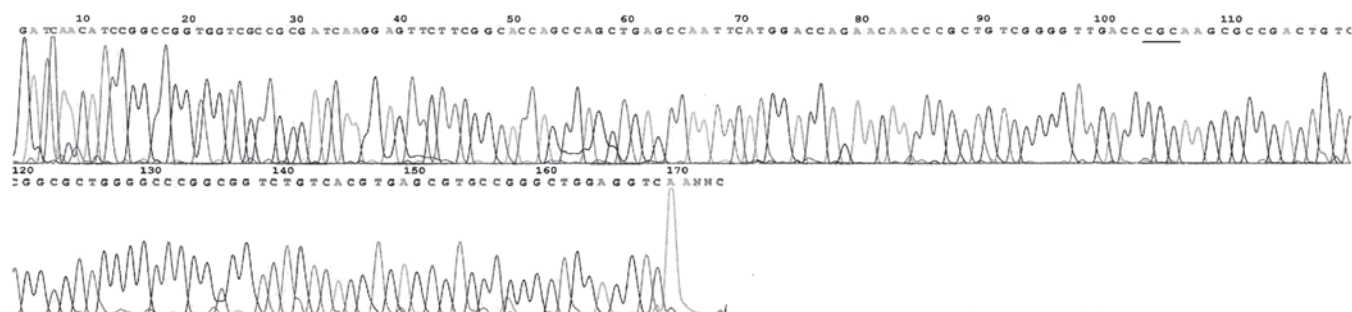


Figure 7. Sequence analysis of rpoB DNA in the clinically isolated *Mycobacterium tuberculosis* L-form; codon, 526 CAC→CGC.

DNA extraction kit (Takara Biotech Co., Ltd., Dalian, China). The inactivated specimens were lysed with 200 μ l DNA lysate (10 mmol/l Tris-HCl, 100 mmol/l NaCl, 25 mmol/l EDTA, 1% SDS, 0.2 mg/ml proteinase K) and incubated at 55°C for

1-3 h, then at 95°C for 5 min. The lysate was extracted twice with phenol:chloroform:isoamyl alcohol (volume ratio 25:24:1). DNA was precipitated by adding ammonium acetate. 2.5 volumes of pure ethanol were added, followed by incubation

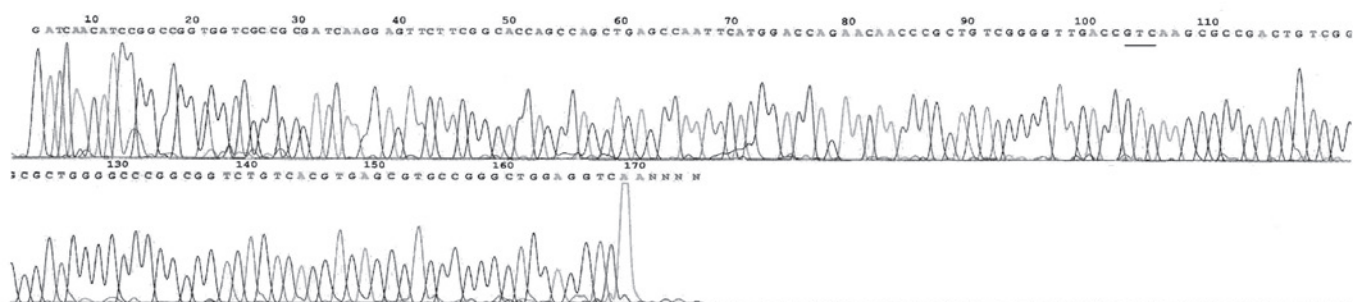


Figure 8. Sequence analysis of *rpoB* DNA in the clinically isolated *Mycobacterium tuberculosis* L-form; codon, 526 CAC→GTC.

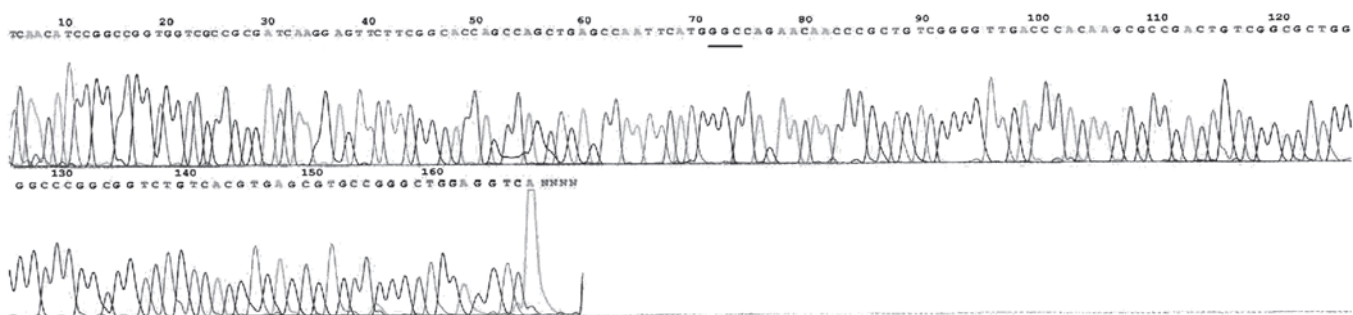


Figure 9. Sequence analysis of *rpoB* DNA in the clinically isolated *Mycobacterium tuberculosis* L-form; codon, 516 GAC→GGC.

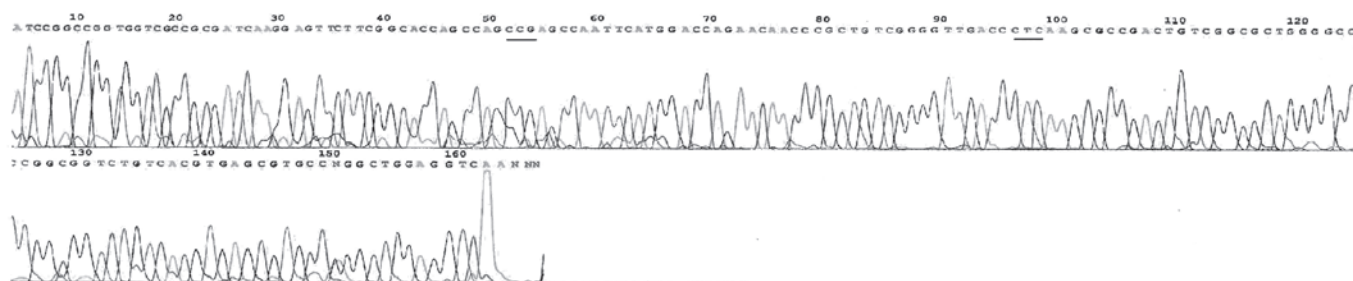


Figure 10. Sequence analysis of *rpoB* DNA in the clinically isolated *Mycobacterium tuberculosis* L-form; codon, 511 CTG→CCG and codon, 526 CAC→CTC.

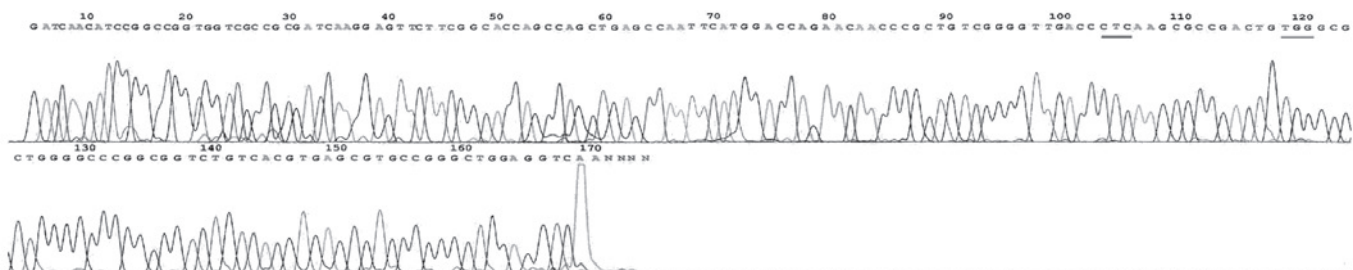


Figure 11. Sequence analysis of *rpoB* DNA in the clinically isolated *Mycobacterium tuberculosis* L-form; codon, 526 CAC→CTC and codon, 531 TCG→TGG.

at -20°C overnight. DNA was pelleted by centrifugation at 12,000 x g for 15 min, washed with 70% ethanol, air-dried and dissolved in the TE buffer [10 mmol/l Tris-HCl (pH 7.4), 1 mmol/l EDTA] (6).

Primer contrivance. The primer was synthesized by Sangon Biotect Co., Ltd., (Shanghai, China) based on the conserved *Mycobacterium tuberculosis* genomic DNA sequence (a

69-bp mutational duplication conserved sequence). The primer sequences of *rpoB* used in this experiment were as follows: Forward: 5'-CGG ATG ACC ACC CAGG AC-3' and reverse: 5'-GGT TTA GAT CGG CAC AT-3'; product size, 258 bp.

PCR analysis and sequence analysis. The total PCR reaction volume was 50 µl and included 5.0 µl 10X buffer (15 mmol/l

MgCl₂), 4.0 μ l dNTPs (200 μ mol/l), 1.0 μ l of each primer (25 pmol/ μ l), 0.5 μ l Taq DNA polymerase (5 U/ μ l) and 4 μ l DNA template. Deionized water was added for a total volume of 50 μ l. The reaction conditions were: 95°C for 5 min of denaturation, then 94°C for 30 sec, 55°C for 90 sec and 72°C for 30 sec, for 33 cycles in total, followed by maintenance at 72°C for 10 min. The amplified product (5 μ l) was determined by electrophoresis with a 2% agarose gel (containing 0.5 mg/ml ethidium bromide). Images were captured and analyzed with Image Master TotalLab software (TotalLab, Ltd., Newcastle, UK). This revealed whether the rpoB mutation was present, indicated by the specific bands at 258 bp. The sequence determination of the DNA sequences of the PCR product was detected by an ABI PRISM 7700 sequencer (Takara Biotech Co., Ltd.).

Statistical analysis. Statistical analyses were performed using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA).

Results

PCR analysis. In the total 42 cases of *Mycobacterium tuberculosis* L-form isolated from the sputum samples, 31 RFP-resistant strains and 11 RFP-sensitive strains were identified (Fig. 1).

Sequence analysis of DNA of *Mycobacterium tuberculosis* L-form. In total, there were 29 mutational strains of the rpoB gene within the 31 RFP-resistant strains. The rate of mutation was observed to be 93.55% (29/31; Table I), mainly concentrated in codon 531 (51.61%, 16/31) and 526 (32.26%, 10/31) and occurring by base substitutions, including 27 unit point mutations and two two-site point mutations. The mutation of codon 516 was a novel observation (Fig. 2-11). No rpoB mutation was identified in the 11 RFP-sensitive strains.

Discussion

Pneumoconiosis is a significant occupational disease in employees of coal mines, and once pneumoconiosis associates with tuberculosis, it develops more rapidly and worsens the tuberculosis of the patient. The prognoses of patients with the disease appear to be poor, and the therapeutic effects on these cases are not good, as may be expected. At present, the therapeutic regimen includes treating the pneumoconiosis and the tuberculosis using antituberculosis drugs, since there is a lack of radically curative drugs for the treatment of pneumoconiosis.

The molecular anti-RFP mechanism of *Mycobacterium tuberculosis* was correlated with the mutation of the rpoB gene, which was coded by the β subunit of RNA polymerase. RFP usually inhibits the transcription of RNA polymerase with the purpose of killing infected cells (7,8). In total, 96% of drug-resistant strains are caused by mutations in the rpoB gene, by an insertion or deletion of basic groups, mainly concentrated around the determining ~80-bp region of RFP. In 35 types of mutations, 43% are missense mutations (9). Mutations in site 513 have been shown to result in greater drug resistance [minimal inhibition concentration (MIC) >32 μ g/ml], while mutations at sites 514, 521 and 533 lead to lower drug resistance (MIC <12.5 μ g/ml) (10-12). In theory, drug-resistant strains of *Mycobacterium tuberculosis*

may occur due to two main reasons. Firstly, resistance may occur due to a mutation in the β subunit of the RNA polymerase of the target molecule by drug action. Secondly, the ingesting capability of the infected cells may be decreased due to a change in the osmosis of the cell wall (13).

The *Mycobacterium tuberculosis* L-form is also known as the *Mycobacterium* cell wall-deficient form; in 1960, Mattmand (14) explained its biological characteristics in detail. It was demonstrated that the changes of the L-form were evident due to the absence of the cell wall either partly or completely, thus affecting the biological characteristics, drug sensitivity and DNA. The L-form is considered a type of mutation. This mutation may be induced by a number of factors, including chemotherapeutics, lysozymes and bacteriophages. Once *Mycobacterium tuberculosis* L form variation occurs, it continues to possess pathogenicity, causing chronic transformation of the disease process, which leads to a worse prognosis. This has resulted in problems in the diagnosis and treatment of tuberculosis (15).

In the present study, the results of the PCR analysis demonstrated that there were 29 mutational strains of the rpoB gene within the 31 RFP-resistant strains; the mutation rate was 93.55% (29/31). In the resistant strains, the mutations were mainly located in codons 531 (51.61%; 16/31) and 526 (32.26%; 10/31), occurring by base substitutions. The former site included 10 strains of Ser→Leu (TCG→TTG) and six strains of Ser→Trp (TCG→TGG), while the latter contained mainly Gln→Asp (CAC→GAC) substitutions. These results revealed that unit point mutations of the rpoB gene of *Mycobacterium tuberculosis* L-form are located in codons 531 and 526 (83.87%; 26/31), as mentioned in previous studies (10,16). The point mutation of codon 516, which has not been previously reported, was a same-sense mutation. A total of 11 RFP-sensitive strains were not mutations of the rpoB gene.

Furthermore, two strains of two-site point mutations were identified, one at codons 526 (Gln→Leu) and 531 (Ser→Trp) and the other at codons 511 (Leu→Pro) and 526 (Gln→Leu). The mutation rate was 6.45% (2/31), appreciably lower than the rate of mutation reported inland and overseas (10,16). This demonstrated that the type of conjoined mutation was a point mutation, and no same-sense mutation was detected. The possible cause may be related to the novel mutational characteristics of the *Mycobacterium tuberculosis* L-form and the effects of sampling errors, and thus further studies on a greater sample size are required in order to verify this conclusion.

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