

## Original Research Article

# Analysis of molecular mechanisms of drug resistance of *Mycobacterium tuberculosis* in patients with pulmonary tuberculosis and its pharmacoeconomics

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### Abstract

**Purpose:** To investigate the molecular mechanisms of drug resistance of *Mycobacterium tuberculosis* in patients with pulmonary tuberculosis and its pharmacoeconomics.

**Methods:** Data pertaining to patients with primary tuberculosis treated in the First Affiliated Hospital of Zhaoqing Medical College, Zhaoqing, China from January 2020 to June 2021 were retrospectively analyzed. Sputum specimens were collected from all eligible patients, and 151 uncontaminated specimens with good bacteriophage activity were screened.

**Results:** A total of 107 *Mycobacterium tuberculosis* strains were isolated from the 151 specimens, 31 of which strains were resistant to varying degrees to rifampicin, isoniazid, streptomycin, and ethambutol with an overall resistance of 28.97%. There were 16 strains with *rpoB* mutation, 22 strains with *katG* mutation, and 8 strains with *inhA* mutation. The difference in the sputum negative rate, lesion absorption rate, and tuberculosis cavity closure rate, and total medical cost between the two groups were not statistically significant ( $p > 0.05$ ). The incidence of adverse reactions in the FDC group was significantly lower than that in the blister pack group ( $p < 0.05$ ).

**Conclusion:** The total resistance of *Mycobacterium tuberculosis* in primary tuberculosis patients remains at a high level, and the development of resistance is associated with mutations in *rpoB*, *katG*, and *inhA* genes. FDC regimen provides more pharmacoeconomic and therapeutic benefits than blister pack regimen.

**Keywords:** Tuberculosis, Molecular mechanism of drug resistance, Fixed-dose combination, Cost-effectiveness analysis

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## INTRODUCTION

China has a heavy clinical burden of tuberculosis, with the third-highest prevalence worldwide [1]. Pulmonary tuberculosis is

managed using first-line anti-tuberculosis drugs, such as rifampicin, isoniazid, streptomycin, and ethambutol. However, clinical practice has revealed an increasing trend in drug resistance in pulmonary tuberculosis, due to factors such as

age and improper anti-tuberculosis treatment [2]. Prior research has shown that drug resistance gene mutations are a significant molecular mechanism of drug resistance in pulmonary tuberculosis [3].

It has been reported that, compared with ordinary tuberculosis, the long course and treatment difficulties for multidrug-resistant tuberculosis impose a serious economic burden on patients and their families, which consequently impairs patient compliance and complicates the treatment [4]. Blister pack and fixed-dose combination (FDC) medications are the current treatment regimens for pulmonary tuberculosis with promising efficacy [5]. Accordingly, this study was designed to compare FDC and blister pack regimen as well as analyze the molecular mechanism of drug resistance of *Mycobacterium tuberculosis* and some pharmacoeconomics outcomes.

## METHODS

### Clinical data

The medical data of patients with primary tuberculosis treated in the First Affiliated Hospital of Zhaoqing Medical College, from January 2020 to June 2021 were retrospectively analyzed. Sputum specimens were collected from all patients, and a total of 151 specimens with no contamination and good bacteriophage activity were screened.

### Inclusion criteria

Patients aged 18 - 60 years, with a confirmed diagnosis of pulmonary tuberculosis [6], with positive acid-fast bacilli smear or a positive mycobacterial culture, with complete clinical data, and who received FDC or blister pack medications (2HRZE/4HR protocol) were included.

### Exclusion criteria

Patients with disseminated tuberculosis, tuberculous pleurisy, or extrapulmonary tuberculosis, with contaminated strains or a low survival of strains after culture, who were pregnant or lactating, with allergies to anti-tuberculosis drugs, or who failed to complete the prescribed course of treatment were excluded.

There were 92 males and 59 females, aged 20 to 58 years, with a mean age of  $51.29 \pm 5.73$  years, and the duration of disease ranged from 1 to 24 months, with a mean duration of  $7.79 \pm 4.96$  months. The study complied with the relevant

regulations of hospital clinical research ethics and it was approved by the ethics committee of the First Affiliated Hospital of Zhaoqing Medical College (approval no. 2019-12-26). The study protocol was conducted in strict accordance with the guidelines of Helsinki Declaration [7].

### Species identification

Non-tuberculous mycobacteria and *Mycobacterium bovis* were excluded using p-nitro benzoic acid (PNB) selective medium and 2-thiophenecarboxylic acid hydrazide (TCH) selective medium. The colonies of *Mycobacterium tuberculosis* cultured for 2 - 3 weeks were ground in a sterile glass grinder, diluted with 0.9 % saline containing 0.05 % Tween 80, and made into 1 mg/mL bacterial suspension using the McFarland standards to adjust the turbidity of the suspension. After diluting the concentration to 10 - 2 mg/mL using a 22SWG standard inoculation loop, 0.01 mL of the solution was inoculated into PNB and TCH medium and incubated at 37 °C with 5 % CO<sub>2</sub> for 4 weeks. In the case of *Mycobacterium bovis*, no colony growth was observed on PNB and TCH media. Colonies growing only on TCH medium were considered *Mycobacterium tuberculosis*, and those growing on both PNB and TCH media were considered non-tuberculous mycobacteria.

### Drug susceptibility test

In accordance with the National Bacteriological Procedures for Tuberculosis (NBPT), drug-containing media were prepared by dissolving rifampicin (40 µg/mL), isoniazid (0.2 µg/mL), streptomycin (4 µg/mL), and ethambutol (2 µg/mL) in Roche media (Zhuhai Yinke Medical Engineering Co., Ltd.), solidified at 85 °C for 50 min and kept at 4 °C away from light after inspection to ensure no contamination. The bacterial solution was diluted to a concentration of 10 - 2 mg/mL and 10 - 4 mg/mL using a 22SWG standard inoculation loop, and inoculated evenly into the slant of drug-containing media and blank medium respectively by streaking, and incubated at 37 °C and 5 % CO<sub>2</sub> for 4 weeks. Drug resistance (DR) was calculated using Eq 1.

$$DR (\%) = (CD - CM) / CB \times 100 \dots\dots\dots (1)$$

where CD represent the colonies grown in drug, CM is the containing medium and CB are the colonies grown in blank medium

The percentage of drug resistance greater than 1 % was considered resistant to the drug. Mono-resistance cases refer to resistance to a single

first-line anti-tuberculosis drug, poly-resistance cases refer to resistance to two or more first-line drugs but not to both isoniazid and rifampicin, while multidrug resistance cases refer to resistance to first-line anti-tuberculosis drugs including at least isoniazid or rifampicin.

**Drug-resistant gene determination**

The DNA extraction fresh colonies were collected using the inoculation loop, transferred in a centrifuge tube containing 1.5 mL of sterile saline, and inactivated at 100 °C for 30 min. The solution was then centrifuged at 3000 rpm for 5 min, and the supernatant was discarded. A 400 µL volume of TE buffer (Beijing Solabao Biotechnology Co., Ltd., item No. T1120) was added to the solution and the cell pellet was suspended evenly, followed by the addition of lysozyme (Beijing Ita Biotechnology Co., Ltd., Item No. SY3854), and the solution was then incubated overnight at 37 °C. The DNA of *Mycobacterium tuberculosis* was extracted according to the instructions of cetyltrimethylammonium bromide (CTAB) kit (Shanghai JingAn Biological Engineering Co. Ltd., Item No. JK-EA02057), and stored at -20 °C, prior to the determination of DNA concentration using the ultra-micro UV-Vis spectrophotometer (METTLER TOLEDO, Switzerland, model UV5Nano) and 10-fold dilution. The primers for polymerase chain reaction (PCR) amplification were synthesized by Shanghai Bioengineering Co (Table 1).

The PCR reaction system contained 0.5 µL of each upstream and downstream primers, 1.0 µL of DNA template, 2.5 µL of High-Fidelity PCR Master Mix with GC Buffer (Shanghai Hengfei Biotechnology Co., Ltd., No. F-532S), 0.5 µL of dNTP Mix (Shanghai Jizhi Biochemical Technology Co., Ltd., No. PC2200) 0.5 µL, dH2O 19.75 µL, Hot Start II DNA Polymerase (Thermo Fisher, USA, No. F549L) 0.25 µL.

Reaction conditions: 95 °C for 2 min, 1 time, 95 °C for 20 s, 62 °C for 30 s, 68 °C for 40 s, 30 cycles, 68 °C for 10 min. Then 2 µL of amplification products were subjected to agarose gel electrophoresis, and the gel imaging system was used for photography. The PCR products were subjected to DNA sequencing by Shanghai

Bioengineering Co. Ltd and compared with the standard sequences of rpoB, katG, and inhA genes in the NCBI database.

**Treatments**

The blister pack group was treated with 0.3 g of bulk isoniazid tablets (NMPA approval no. H51020436) daily, 0.45 g of rifampin capsules (NMPA approval no. H51022410) daily, 0.5 g of pyrazinamide tablets (NMPA approval no. H51022733) daily, and 0.75 g of ethambutol hydrochloride tablets (NMPA approval no. H51020720) daily. After 2 months of treatment with the drugs, isoniazid tablets and rifampin capsules were administered for another 2 months. The FDC group was given an anti-tuberculosis fixed-dose combination manufactured by Shenyang Hongqi Pharmaceutical Co. Ethambutol hydrochloride + pyrazinamide + rifampin + isoniazid tablets (NMPA approval no. H20090219) were administered for 2 months, 4 tablets daily, and isoniazid tablets (NMPA approval no. H20103325) were administered for 4 months, 2 tablets daily.

**Evaluation of therapeutic effect**

**Sputum negative rate**

Negative sputum culture for 2 consecutive months and no re-positive cases observed were considered a successful conversion from positive to negative.

**Lesion absorption**

Chest CT showing lesion reduction > 70 % indicates significant absorption, a reduction of 50 to 70 % indicates partial absorption, a reduction of < 50 % indicates no absorption, and a significant enlargement of more than 50 % indicates deterioration. The total effectiveness (T) was calculated with the formula in equation 2.

$$T = S + P \dots\dots\dots (2)$$

Where S is significant absorption, and P is partial absorption.

**Table 1:** PCR primer sequence used

Primer	Upstream (5'-3')	Downstream (5'-3')
rpoB	GTTTAGTTGCGTGCGTGAG	CAATGGTCTCGTCAAGTACA
katG	GGTCTATGTCCTGATTGTTCC	TTCTCCAGATCCCGCTTAC
inhA	TCCGAGGATGCGAGCTATA	ACCTGTTGACCGACTCCAA

### Tuberculosis cavity closure

Closure of the tuberculosis cavity or disappearance of the cavity shown by chest CT is considered a tuberculosis cavity closure. The diameter of the cavity, if equal to 1/2 or less of the original diameter is considered a reduced tuberculosis cavity. Reduction or enlargement of the diameter of the cavity less than 1/2 of the original diameter is considered an unchanged cavity, while an enlargement of the diameter of the cavity by 1.5 times of the original diameter and above is considered increased cavity. The total effectiveness (T<sub>1</sub>) was calculated as in Eq 3.

$$T_1 = C + R \dots\dots\dots (3)$$

where C is the cavity closure, and R is reduced cavity.

### Adverse reactions

The occurrence of gastrointestinal reactions, liver damage, allergic reactions, hyperuricemia, and leukopenia in patients during treatment were recorded.

### Medical cost

The total cost includes direct medical cost, direct non-medical cost, indirect cost, and hidden cost. The direct medical cost includes medication fee, examination fee, bed fee, nursing fee, hospitalization fee, and fee for adverse reaction management. The direct non-medical cost was the escort fee, which was calculated based on the hourly wage standard of part-time hourly workers (RMB 18.4 or USD 2.67), and the mean daily escort fee was about RMB 147.2 or USD 21.33 (8 h of work per day). The indirect cost was the loss of income caused by illness, and the cost of lost wages was calculated using the annual mean wage of employed persons in urban non-private entities in China in 2020 (97,379 Yuan (USD 14,110.44)/year in 2020), with 251 effective working days and an average wage of about 387.96 Yuan (USD 56.22) for working days in 2020. Hidden costs were not considered in this study given the complexity and difficulty of calculation.

### Statistical analysis

The Statistical Packages for the Social sciences (SPSS) 20.0 software was used for statistical analyses. Normally distributed measurement data are expressed as mean ± standard deviation (SD), and independent samples t-test was used for comparison between the two groups. Count data are expressed as

frequencies or composition ratios and analyzed using chi-square test. A difference was considered statistically significant at  $p < 0.05$ .

## RESULTS

### Species identification

After preliminary identification using PNB and TCH selective media, a total of 107 strains of *Mycobacterium tuberculosis*, 39 strains of *Mycobacterium bovis*, and 5 strains of non-tuberculous mycobacteria were isolated from the 151 sputum specimens, of which 16 strains with rpoB mutation (Table 2).

**Table 2:** Sequencing results of rpoB gene of drug-resistant *Mycobacterium tuberculosis*

Mutation Loci	Codon change	n	%
511	CTG→CCG	3	18.75
531	TCG→TTG	1	6.25
516	GAC→GTC	3	18.75
526	CAC→CGC	9	56.25

### Drug sensitivity

In the present study, 31 strains were resistant to rifampicin, isoniazid, streptomycin, and ethambutol to varying degrees, with an overall resistance rate of 28.97 % (31/107). There were 19 strains (17.76 %) resistant to 1 drug, 6 strains (5.61 %) resistant to 2 drugs, 4 strains (3.74 %) resistant to 3 drugs, and 2 strains (1.87 %) resistant to 4 drugs. The percentage of mono-resistance, poly-resistance, and multi-drug resistance was 17.76 % (19/107), 11.21 % (12/107), and 3.8 % (4/107) of which 22 strains with katG mutation (Table 3).

**Table 3:** Sequencing results of katG gene of drug-resistant *Mycobacterium tuberculosis*

Mutation Loci	Codon change	n	%
315	AGC→ACC	14	63.64
463	CGG→CTG	6	27.27
68	GTC→GAC	2	9.09

### Gene sequencing

A total of 16 strains (51.61 %) had rpoB gene mutations at 511 (CTG→CCG), 531 (TCG→TTG), 516 (GAC→GTC), and 526 (CAC→CGC) loci, respectively (Table 2). 22 strains (70.97 %) had katG gene mutations at 315 (AGC→ACC), 463 (CGG→CTG), and 68 (GTC→GAC) loci with katG gene mutations (Table 3); 8 strains (25.81%) had inhA gene mutations at 145 (GTC→GKC), 268 (TTG→TGG), 175 (GTG→GKG) loci (Table 4).

**Table 4:** Sequencing results of inhA gene of drug-resistant *Mycobacterium tuberculosis*

Mutation	Loci	Codon change	n	%
145		GTC→GKC	5	62.50
268		TTG→TGG	1	7.81
175		GTG→GKG	2	25.00

**Genetic mutations and drug resistance**

The degrees of mutation % of rpoB, katG, and inhA genes were 63.16 % (12/19), 63.16 % (12/19) and 26.32 % (5/19) for 19 mono-resistant strains, 75.00 % (9/12), 58.33 % (7/12), 26.32 % (5/19) for 12 poly-resistant strains, and 33.33 % (4/12) and 75.00 % (3/4), 25.00 % (1/4) for 4 multidrug-resistant strains, respectively (Table 5).

**Efficacy of treatment regimens**

The difference in the sputum negative rate, lesion absorption rate, and tuberculosis cavity closure rate between the two groups did not come up to the statistical standard (P > 0.05) (Table 6).

**Incidence of adverse reactions**

The incidence of adverse reactions in the FDC group (7.89 %) was significantly lower than that

in the blister pack group (18.67 %) (p < 0.05) (Table 7).

**Costs treatment protocol**

The total cost per capita in the blister pack and FDC groups was 9813.14 ± 249.26 Yuan and (9693.28 ± 236.37) yuan, respectively. The FDC group had a lower total cost per capita than the blister pack group, but the difference was not statistically significant (t = 135.27, p = 0.216).

**Cost-effectiveness and sensitivity of the two treatment regimens**

A cost-effectiveness analysis was performed on the two treatment regimens. The cost (C) / effective (E) ratio was 105.15 for the blister pack group and 100.92 for the FDC group, and the ΔC/ΔE for the blister pack group was 125.29 with the lowest cost FDC group as a parameter.

A univariate sensitivity analysis assuming a 15 % decrease in drug price and no change in treatment effect found that the C/E ratios for the blister pack and FDC groups were 89.33 and 85.70, respectively, and the ΔC/ΔE for the blister pack group was 113.87 using the lowest cost FDC group as a parameter (Table 8).

**Table 5:** Relationship between genetic mutations and drug resistance

Resistance	Strains	rpoB gene		katG gene		inhA gene	
		Mutation	No mutation	Mutation	No mutation	Mutation	No mutation
Monoresistance	19	12	7	12	7	5	14
Polyresistance	12	9	3	7	5	4	8
Multidrug-resistance	4	3	1	3	1	1	3

**Table 6:** Comparison of efficacy of treatment regimens (n, %)

Group	n	Sputum negative rate	Lesion absorption rate	Tuberculosis cavity closure rate
Blister pack group	75	70/93.33	71/94.67	75/100.00
FDC group	76	73/96.05	73/96.05	76/100.00
χ <sup>2</sup>		0.556	0.164	0.000
P-value		0.456	0.685	1.000

**Table 7:** Comparison of the incidence of adverse reactions (n, %)

Group	n	Gastrointestinal reactions	Liver damage	Leukopenia	Hyperuricemia	Allergic reactions	Total incidence
Blister pack group	75	4	3	2	4	1	14/18.67
FDC group	76	2	1	1	1	1	6/7.89
χ <sup>2</sup>							4.620
P-value							0.032

**Table 8:** Cost-effectiveness and sensitivity analysis of the two treatment regimens

Group	Cost-effectiveness analysis				Sensitivity analysis			
	C/yuan	E/%	C/E	$\Delta C/\Delta E$	C/yuan	E/%	C/E	$\Delta C/\Delta E$
Blister pack group	9813.14	93.33	105.15	125.29	8337.58	93.33	89.33	113.87
FDC group	9693.28	96.05	100.92	-	8231.36	96.05	85.70	-

Note: C is the total cost, E is the sputum negative rate

## DISCUSSION

The global tuberculosis report released in October 2020 stated that TB remains the leading cause of negative health status of people and one of the top 10 causes of death worldwide [9]. *Mycobacterium tuberculosis* drug resistance or multidrug resistance is a major contributor to mortality in patients with tuberculosis [10]. In this study, an analysis of sputum specimens from patients with primary tuberculosis admitted to the hospital from January 2020 - June 2021 revealed that the overall drug resistance prevalence of pulmonary tuberculosis was 28.97 %, which was higher than that reported by Yuan Wei *et al* [11] and by Li *et al* [12]. The development of drug resistance in *Mycobacterium tuberculosis* is considered to be attributed to mutations in drug resistance genes, and drug resistance-related genes include rpoB, katG, and inhA [13]. Drug resistance gene testing of *Mycobacterium tuberculosis* is of great clinical value to provide guidance for proper clinical regulation of drug use [14]. In the present study, gene sequencing after PCR amplification of drug-resistant *Mycobacterium tuberculosis* DNA from 31 extracted strains revealed that the degree of mutation rates of rpoB, katG, and inhA genes were 51.61, 70.97, and 25.81 %, respectively, which were basically consistent with the results of the previous study [15]. The results suggest an association between the development of drug resistance in *Mycobacterium tuberculosis* patients with primary tuberculosis and mutations in the rpoB, katG, and inhA genes, with higher mutation rates in the rpoB and katG genes in mono- and multidrug-resistant strains and higher mutation % in the rpoB gene in polyresistant strains, in accordance with previous findings [16].

Research has demonstrated that blister pack medication significantly improves compliance and provides high overall clinical efficacy when compared to traditional dosage forms [17]. Fixed-dose combination (FDC) is a synthetic formulation with a fixed-dose based on compounded formulations. An application of FDC to the treatment of patients with pulmonary tuberculosis was found to be moderately effective and to reduce the economic burden of the disease by improving patient compliance [18]. The results of sputum negativity rate, lesion

absorption rate, and cavity closure rate in the present study were consistent with the results of previous studies. The above indexes were slightly higher in the FDC group than in the blister pack group, suggesting that FDC may be a preferred treatment option for the patients. Furthermore, the FDC group showed a significantly lower incidence of adverse reactions than the blister pack group, which was attributed to the reduction of anti-tuberculosis drug doses and the improvement of drug safety by FDC. For tuberculosis patients, especially those with drug resistance, the economic burden is a major factor impacting their treatment efficacy and relapse [19-20]. In the present study, the total cost per capita in the FDC group was lower than that in the blister pack group, but no significant difference was detected, and cost-effectiveness analysis revealed a C/E ratio of 105.15 and 100.92 in the blister pack and FDC groups, respectively, indicating a better performance of FDC regimen versus the blister pack method.

### Limitations of this study

The limitations of this study include the absence of pharmacoeconomic analysis of other treatment regimens for patients with primary tuberculosis and treatment regimens for patients with relapsed tuberculosis, which will be further analyzed in future studies.

## CONCLUSION

The development of resistance is associated with mutations in the rpoB, katG, and inhA genes. The FDC regimen provides more pharmacoeconomic and therapeutic benefits when compared to the blister pack medication regimen. There will be need for further studies for a more detailed pharmacoeconomic analysis.

## DECLARATIONS

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None provided.

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### Ethical approval

This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhaoqing Medical College, China (approval no. 2019-12-26).

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zeqing Bao and Yingyi Bao performed the majority of experiments. Xia Zhang analyzed the data. Xiaocui Qin drew the charts. Weibin Wu designed and coordinated the research while Zeqing Bao and Yingyi Bao wrote the paper. All authors reviewed and approved the manuscript for publication.

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