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Survey and Molecular Characterization of Drug-Resistant *M. tuberculosis* Clinical Isolates from Zunyi, Guizhou Province of China

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1. Introduction

Tuberculosis (TB) is one of the leading infectious disease killers in the world, especially in developing countries. The increasing frequency of human-to-human transmission of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* poses challenges for effective therapeutic options and infection control. MDR-TB is defined as a form of TB that is resistant to at least isoniazid and rifampin, which are used to treat all TB patients. XDR-TB is a form of TB that is resistant to at least rifampicin and isoniazid in addition to any fluoroquinolone, and at least one of the three injectable second-line anti-TB drugs (amikacin, capreomycin and kanamycin). Based on the WHO Report 2010, China was ranked number one among high burden countries in terms of the estimated number of MDR-TB cases in 2008, and number two in terms of total numbers of TB cases in the world with the incidence rate per capita of 99 per 100,000 populations (WHO, 2011). Guizhou province is one of the highest-incidence-rate areas in China, and its prevalence of drug-resistant TB is higher than most of other provinces of China (Chen et al., 2011). The Affiliated Hospital of Zunyi Medical College is one of the specialized centers in Guizhou certified by the provincial government for the treatment of MDR-TB patients. In 2010, more than 15,000 TB patients were treated at the hospital. In order to determine the molecular characteristics of drug-resistant TB and assist in making informed TB treatment decisions for TB patients in the Zunyi area of Guizhou province, hundreds of *M. tuberculosis* clinical isolates were collected at the Affiliated Hospital of Zunyi Medical College and used for the systematic surveillance and other studies. We have made some progresses in the detection and molecular characterization of drug-resistant *M. tuberculosis* clinical isolates using different research methods such as *M. tuberculosis* culture, spoligotyping, gene sequencing, proteomics and drug susceptibility testing against first-line and second-line anti-tuberculosis drugs.

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In this chapter, we will briefly review the recent advances in the area of MDR-TB research; provide detailed descriptions about our research methods and the summary of our current research progresses. This chapter is written for researchers, scientists and physicians in academic institutions, clinical laboratories, pharmaceutical companies and research hospitals. This chapter will also be suitable for readers such as undergraduate, graduate and medical students who wish to learn more about the drug-resistant *M. tuberculosis*, especially MDR/XDR-TB, and some of the current research methods used for the determination of the genetic diversity and anti-tuberculosis drug susceptibility profile of *M. tuberculosis* clinical isolates, and the detection of drug-resistant *M. tuberculosis*.

2. Survey of drug-resistant *M. tuberculosis* clinical isolates

Mycobacteria are aerobes and grow most successfully in tissues with high oxygen content. The suitable growth temperature for mycobacteria is 37°C. Mycobacteria are "acid-fast bacilli" (AFB) because of their lipid-rich cell walls, which are relatively impermeable to various basic dyes unless the dyes are combined with phenol. Once stained, mycobacteria are resistant to decolorization with acidified organic solvents. *M. tuberculosis* is an intracellular pathogen usually infecting mononuclear phagocytes, and slow-growing with a generation time of 18 to 22 hours. The diagnosis of tuberculosis usually requires the detection of acid-fast bacilli in sputum via the acid-fast staining method (Ziehl-Neelsen method), which uses carbolfuchsin as the stain, acid-alcohol as the destaining solution and methylene blue as the counterstain. The culture confirmation of *M. tuberculosis* is the gold standard for diagnosis of tuberculosis and the drug susceptibility testing (DST) provides the basis for surveillance of drug-resistant TB and for physicians to adjust chemotherapy.

Even though TB laboratory services formed an essential part of the DOTS (Directly Observed Treatment Short course) strategy for National Tuberculosis Programs, it was often the most neglected component of these programs because of the absence of standardized techniques which complicated the activities of new laboratory services. Based on those considerations, WHO prepared guidelines in 1998 for laboratory services for the framework of National Tuberculosis Programs (WHO, 1998). The guidelines included three detailed manuals, two of which focused on the technical aspects of TB microscopy and culture, and a third one dealt with laboratory management including lab safety and proficiency testing. These manuals were specifically developed for use in low- and middle-income countries with high TB prevalence and incidence rates (WHO, 1998). Most of the methods used in our laboratory at the Affiliated Hospital of Zunyi Medical College and described in this chapter are based on these three manuals.

2.1 Collection of sputum specimens from patients

M. tuberculosis may be isolated from various clinical specimens, including respiratory specimens such as sputum, body fluids and body tissues (Goodwin, 2007). TB clinical strains from sputum of patients with active pulmonary tuberculosis were collected at the Affiliated Hospital of Zunyi Medical College. Sputum specimens were collected into a sterile single-collection Universal container (28ml) with a tightly fitted lid. A good sputum specimen is considered to be recently-discharged materials from the bronchial tree of the patient, with a minimum amount of oral or nasal materials (WHO, 1998). In an ideal situation, a sputum

specimen produced by a deep cough of the patient should have a volume of 5 to 10 ml, although less volumes are acceptable if the quality is good. All sputum specimens should be transported to the laboratory and processed as soon as possible after collection. If delay is unavoidable, the sputum specimens should be refrigerated to inhibit the growth of unwanted microorganisms.

2.1.1 Solutions for acid-fast staining

Staining solution: 0.8% carbolfuchsin solution

Destaining solution: 3% hydrochloric acid- alcohol solution

Counterstaining solution: 0.3‰ methylene blue solution

2.1.2 Acid-fast staining procedure

The procedures for acid-fast staining were based on the modified method described by Hu et al. (Hu et al., 2008). To prepare a smear for AFB, appropriate specimens were spread uniformly on a microscope slide, which was then fixed at 80°C for 15 minutes. Smears were stained with a carbolfuchsin stain and were examined using a 100× oil immersion objective on a light microscope. In smears stained with carbolfuchsin, AFB typically appear as purple to red slightly curved, short or long rods (2-8μM). They may also appear beaded or banded. Following is the acid-fast staining procedure:

1. Turn on the thermostat-controlled water-bath and set up the temperature to 56°C.
2. Air dry smear on the slide, flame fix, and transfer it to the water-bath.
3. Add carbolfuchsin dye to the heat-fixed smear on slide for 15min, and take out the slide.
4. Allow the slide to cool to room temperature, then, decolorize slide by adding the fresh destaining solution, and wait until red color disappears.
5. Add counterstaining solution for 1 min, wash, and dry.
6. Examine the slide by a 100× oil immersion objective on a light microscope.
7. Interpret acid-fast staining results

Smears should be carefully examined with a minimum of 300 fields, and three horizontal sweeps of a smear should be performed (Goodwin, 2007). The positive smear requires the cut-off of at least 5000 bacilli/ml. The overall sensitivity of the acid-fast staining method varies from 20% to 80% (Goodwin, 2007).

2.2 Drug susceptibility testing against different drugs

WHO has endorsed commercial liquid culture systems and molecular line-probe assays as gold standards for rapid detection of MDR-TB; however, because of technical complexity, cost and the requirement of sophisticated lab infrastructure, their uses have been limited in many resource-constrained settings (WHO, 2010). Several noncommercial culture and DST methods have been developed for those resource-constrained settings, and assessed by WHO. Based on the testing results, WHO recently recommended MODS (microscopic observation of drug susceptibility) and NRA (nitrate reductase assay) under certain conditions for direct testing of sputum speciments (WHO, 2010).

M. tuberculosis drug susceptibility testing methods using solid media include proportional, resistance ratio and absolute concentration, which are inexpensive and highly standardized for testing susceptibility to many drugs (WHO, 2009). The proportion method is the most commonly used worldwide. Sputum specimens were digested, decontaminated and used to inoculate egg-based media such as L-J slants for DST. The instructions for the preparation of the egg-based media such as L-J, and detailed protocols for performing standardized bacteriological services for detecting infectious cases of pulmonary tuberculosis, monitoring treatment progress and documenting cure at the end of treatment by means of microscopic examination were well described by WHO in Part III (Culture) of the Laboratory Services in Tuberculosis Control (WHO, 1998) and should be followed strictly.

2.2.1 Preparation of culture media

The recommended TB diagnostic laboratory procedures (WHO, 1998; Chinese Antituberculosis Association, 2006) were followed for the preparation of media and suspension, inoculation and mycobacterium culture in a biologic safety cabinet. Löwenstein-Jensen (L-J) solid media were prepared in the laboratory of the Affiliated Hospital of Zunyi Medical College. All the samples were cultured on L-J solid media and grown colonies were identified to the species level using TCH (2-thiophene carboxylic acid) and PNB (paranitrobenzoicacid) selective media or by standard biochemical procedures.

2.2.1.1 Procedure for preparation of L-J culture medium (for 1632 ml)

1. Dissolve salts from the following ingredients in order in about 300ml of distilled water by heating (WHO, 1998):

Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	2.4g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.24g
Magnesium citrate	0.6g
Sodium glutamate	7.2g
Glycerol (reagent grade)	12ml
Malachite green (2% solution)	20ml
Distilled water	600ml

2. Add glycerol, malachite green solution and make up to 600ml with distilled water.
3. Sterilize the solution by autoclaving at 121°C for 30 minutes.
4. Cool the solution to room temperature.
5. Rinse fresh hen's eggs (not more than 7 days old) thoroughly in running water and soak them in 70% ethanol for 15 minutes.
6. Crack the eggs with the edge of the beaker and pour into a sterile beaker.
7. Stir them with an old-fashioned sterile egg beater until completely blended.
8. Mix the sterilized solution containing all the ingredients (600ml) with homogenized eggs (1000ml) in a large sterile beaker (2 L capacity) and let it stand at room temperature for 1 hour.

2.2.1.2 Preparation of drug-containing solid media

Drug stock solutions at recommended concentrations (Table 1) were made in distilled water. To make media containing the end concentration of different drugs (Table 1), 0.4ml of stock solution for each drug was diluted separately with 400ml of L-J mixture mentioned above.

Media containing different drugs were distributed to sterile universal screw-cap centrifuge tubes (7ml medium per tube); tubes were labeled and caps were tightened. The labeled tubes were placed in an oven at a slanted position (30° angle) and baked at 85°C for 50 min. Baked tubes were cooled down to room temperature and stored at 4°C refrigerator until use. Since the medium was prepared with sterile precautions, this heating process was to solidify the medium instead of sterilizing it. The quality of the egg-based media deteriorates when the baking temperature is too high or the baking time is too long. The L-J medium should be dated and stored in the refrigerator for up to 4 weeks if the caps are tightened to prevent drying of the medium (WHO, 1998).

Drug	Dissolvent	Diluent	Concentration (μg/ml)	
			Stock solution	Drug Medium
Capreomycin	dH_2O	dH_2O	40000	40
Ciprofloxacin	dH_2O	dH_2O	2000	2
Ethambutol	dH_2O	dH_2O	2000	2
Isoniazid	dH_2O	dH_2O	200	0.2
Levofloxacin	dH_2O	dH_2O	2000	2
Rifampicin	DMF	dH_2O	40000	40
Streptomycin	dH_2O	dH_2O	4000	4

Table 1. Recommended concentrations of anti-TB drugs used in the drug susceptibility testing (WHO, 2009). dH_2O, distilled water; DMF, dimethylformamide.

2.2.1.3 Digestion and decontamination

M. tuberculosis grows slowly and takes four to eight weeks or longer to give visible colonies. Cultures are usually made in bottles because of the long incubation time required. The bottles are tightly capped to prevent drying of the cultures. The majority of clinical specimens submitted to the clinical laboratory are contaminated to varying degrees by more rapidly growing normal flora. These would rapidly overgrow the entire surface of the medium before the *M. tuberculosis* start to grow (Goodwin, 2007). Therefore, specimens must be subjected to digestion and decontamination that liquefies the organic debris and eliminates the unwanted normal flora. All currently available digesting and decontaminating methods are to some extent toxic to *M. tuberculosis*. Therefore, to ensure the survival of the maximum number of *M. tuberculosis* in the specimen, the digestion and decontamination procedure must be precisely followed (WHO, 1998).

2.2.2 Drug susceptibility testing

We have collected hundreds of TB culture samples from TB patients, and used them for the DNA extraction and drug-susceptibility testing against seven anti-TB drugs: rifampicin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB), capreomycin (CPM), ciprofloxacin (CIP) and levofloxacin (LVF). The proportion method on L-J media was used to perform the DST against different drugs in most of clinical *M. tuberculosis* isolates. The following drug concentrations in L-J media were used for the DST: RIF, 40μg/ml; INH, 0.2μg/ml; STR, 4μg/ml; EMB, 2μg/ml; CPM, 40μg/ml; CIP, 2μg/ml; and LVF, 2μg/ml.

2.2.3 Quality control

After coagulation, 5% of the slopes were picked up randomly and continued for incubation for 2 days at 37 °C to check for sterility. If no colony was grown on the solid medium after 48 hours of incubation at 37°C, the whole media batch should be good for DST.

2.2.4 Results criteria

The proportion testing results can be recorded at 28 days and again at 42 days as 3+ for confluent growth; 2+ for more than 100 colonies; and 1-99 colonies for the actual number of colonies. The percentage of drug resistance can be expressed as: Number of colonies on drug-containing medium/Number of colonies on L-J medium × 100%. If the percentage >1, the tested bacterium is considered to be drug resistant.

2.2.5 Results and discussion

We analyzed 316 clinical *M. tuberculosis* isolates for DST against four drugs (RIF, INH, STR and EMB). Results showed that 51.3% of isolates were resistant to one or more drugs, 19.0% were MDR, 20.9% were resistant to any single drug, 12.0% were resistant to any two drugs, 10.8% were resistant to any three drugs, and 7.6% were resistant to four drugs (Table 2). The prevalence of single drug-resistance was STR>RIF>EMB>INH in combined cases. There were 209 isolates in the first-treated (new TB cases) group, in which 42.1% were resistant to at least one drug, and 23.9% were resistant to any single drug. In addition, there were 107 isolates in the previously treated group, in which 69.2% were resistant to at least one drug, and 15.0% were resistant to any single drug. Our results indicated that the prevalence of drug-resistance in new tuberculosis cases was very high in the Zunyi area of Guizhou province, with 42.1% of the isolates resistant to at least one drug, and there were obvious differences in the drug susceptibility profiles between new and previously treated TB cases. These results also highlight the importance of surveillance of drug-resistant TB in order to improve the treatment outcomes of TB patients.

Drug Susceptibility*	PT (107 isolates)		FT (209 isolates)		Combined (316 isolates)	
	No. of cases	%	No. of cases	%	No. of cases	%
Resistant to any single drug	16	15.0	50	23.9	66	20.9
Resistant to any 2 drugs	23	21.5	15	7.2	38	12.0
Resistant to any 3 drugs	22	20.6	12	5.7	34	10.8
Resistant to 4 drugs	13	12.1	11	5.3	24	7.6
Total	74	69.2	88	42.1	162	51.3

*Drugs tested: isoniazid, rifampincin, ethambutol and streptomycin; PT, previously treated; FT, First-time treated (new cases).

Table 2. Drug susceptibility profiles of 316 clinical *M. tuberculosis* isolates.

3. Molecular characterization of drug-resistant *M. tuberculosis*

The recommended standard or first-line treatment of tuberculosis includes a combination of four drugs, rifampicin, isoniazid, ethambutol and pyrazinamide, with or without

streptomycin. Resistance to the first-line anti-TB drugs has been linked to mutations in at least 13 genes: *rpoB* for rifampicin resistance, *katG, inhA, kasA, ahpC, ndh, furA*, and *oxyR* for isoniazid resistance, *embCAB* for ethambutol resistance, *pncA* for pyrazinamide resistance, and *rpsL, rrs,* and *gidB* for streptomycin resistance. Resistance to the second-line anti-TB drugs has been linked to mutations in at least 10 genes (Banerjee, et al., 2008). For the detection of mutations in genes linked to rifampicin and isoniazid resistance, we amplified *rpoB* and *KatG* gene fragments from many drug-resistant *M. tuberculosis* clinical isolations and performed DNA sequencing analyses. We also detected mutations in 5 other genes associated with drug-resistance (*inhA, rpsl, rrs, gyrA and gyrB*). Nucleotide sequences of primers used for PCR amplification and DNA sequencing were listed in Table 3.

Gene	Primer Sequence (5′→3′)	Product Size (bp)
rpoB	Forward, TCAGACCACGATGACCGTTCC Reverse, GTCCATGTAGTCCACCTCAGACG Sequencing, TCGGCATGTCGCGGATGGAG	688
katG	Forward, GCTCGGCGATGAGCGTTAC Reverse, CTCGTAGCCGTACAGGATCTCG Sequencing, GCTCGGCGATGAGCGTTAC	409
inhA	Forward, TCGCAGCCACGTTACGCTC Reverse, CCAGCCGCTGTGCGATC Sequencing, TCGCAGCCACGTTACGCTC	175
rpsl	Forward, GGCAGCCCGCAGCGTCGTG Reverse, TGTAGCGCACACCAGGCAGGT Sequencing, GGCAGCCCGCAGCGTCGTG	211
rrs	Forward, TCAGGAGGAACACCGGTGGCG Reverse, AATCCACATGCTCCGCCGCTTG Sequencing, TCAGGAGGAACACCGGTGGCG	253
gyrA	Forward, CAGCTACATCGACTATGCGA Reverse, GGGCTTCGGTGTACCTCAT Sequencing, CAGCTACATCGACTATGCGA	320
gyrB	Forward, CGCAAGTCCGAACTGTATGTCGTAG Reverse, GTTGTGCCAAAAACACATGC Sequencing, CGCAAGTCCGAACTGTATGTCGTAG	346

Table 3. Primers used in this study for PCR amplification and DNA sequencing

3.1 Determination of mutation profiles in drug-related genes

3.1.1 DNA isolation and PCR amplification

The genomic DNA was extracted from *M. tuberculosis* clinical isolates using a Bacterial DNA Kit (Tiangen, China) following the manufacturer's instruction. DNA fragments for 7 drug-related genes were amplified by PCR using synthetic oligonucleotide primers listed in Table 3. The following thermocycler parameters were applied with initial denaturation at 94°C for 5 min; 35-42 cycles of denaturation at 94°C for 30 sec; primer annealing at 58-62°C for 30 sec; extension at 72°C for 30-60 sec; and a final extension at 72°C for 7 min. The obtained DNA

fragments were analyzed by electrophoresis in 2.0% agarose gel and were visualized under UV light on a transilluminator.

3.1.2 DNA sequencing analysis

In order to determine mutation profiles of 7 drug-related genes (*rpoB, katG, inhA, rpsl, rrs, gyrA* and *gyrB*), we purified PCR-amplified gene fragments and sent them to Shanghai Invitrogen for DNA sequencing using primers listed in Table 3. The FinchTV program was downloaded from Geospiza's website and used to view the original sequencing data. Sequence alignment analysis was conducted using the BLAST program at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare our sequencing results with those wild-type *M. tuberculosis* genes listed in the GenBank.

3.1.3 Results and discussion

In 32 rifampicin-resistant strains, we identified 13 different types of missense mutations at codons 509, 511, 516, 522, 526, 531, 533, 550 and 572 of *rpoB* gene, and compared the mutation profile with those of rifampicin-resistant *M. Tuberculosis* isolates from different geographical regions of the world. Comparison of the results showed that the *rpoB* gene mutation profile in rifampicin-resistant *M. Tuberculosis* clinical isolates from Guizhou province differed not only from other provinces of China but also from other countries of Asia, Europe and America (Chen et al., 2010). Two new mutations (Val550Leu and Ser509Arg) were identified and deposited in GenBank (GQ250580 and GQ250581). We concluded that mutation profiles of rifampicin-resistant *M. Tuberculosis* isolates were variable depending on the geographical locations, and further studies would be needed to determine the molecular basis for such variations (Chen et al., 2010).

In 30 isoniazid-resistant strains, 18 (60%) of them had mutations in *katG* and/or *inhA* genes and two newly identified mutations in *katG* gene (Val230Met and Pro232Gln) were deposited to GenBank (GQ250582 and GQ250583). Results from this study further confirmed that mutations in *katG* and *inhA* genes are related to the isoniazid resistance in *M. tuberculosis* (Chen Y et al., 2010).

To better compare drug resistance and mutation profiles of clinical *M. Tuberculosis* isolates collected at our hospital, we selected 23 representative isolates and combined the DST results against 4 drugs with mutation profiles in 7 genes related to drug resistance in the same table (Table 4). It is clear that the relationship between drug resistance and mutations in specific genes may be more complex than we expected. For example, no mutations were found in 6 of the 7 drug-related genes for isolate number 2, which was resistant to all 4 drugs tested, suggesting that there must be changes in other regions of the genes or other genes. Further studies will be needed to determine the molecular mechanism underlining the resistance of this kind of isolates to different anti-TB drugs. In another example, two mutations were identified in isolate number 17, one in *rpoB* gene (TCG531TTG) and another in *katG* gene (AGC315ACC). However, isolate number 17 was resistant only to rifampicin but sensitive to 3 other drugs. It will be necessary for us to repeat the DST for this isolate to confirm whether this isolate does have a mutation in *katG* gene but still sensitive to isoniazid. In summary, more studies are need to identify new genes related to MDR-TB.

No.	R	H	S	E	Mutations in specific resistant genes						
					rpoB	*katG*	*inhA*	*rpsL*	*rrs*	*gyrA*	*gyrB*
1	r	r	r	r	TCG531TTG	AGC315ACC CCG232CAG GGC237GAG GAG340AAG	CGT16GGT GGA23CGA	WT	WT	NA	WT
2	r	r	r	r	WT	WT	WT	WT	WT	NA	WT
3	r	r	r	r	CTG533CCG	AGC315ACC	WT	AAG43AGG	WT	NA	WT
4	r	r	r	r	CAC526TAC	WT	WT	WT	WT	NA	WT
5	r	r	r	r	CAC526GAC	WT	WT	WT	WT	GCG90GTG AGC95ACC	NA
6	r	r	r	s	WT	WT	GCC5TCC	WT	WT	AGC95ACC	WT
7	r	r	r	s	GAC516GTC	AGC315ACC	GCC5TCC ACT6AGT	NA	WT	GCG90GTG AGC95ACC	WT
8	r	r	r	r	CTG533CCG	GTG230ATG	GCC5TCC	NA	NA	NA	NA
9	r	r	r	r	WT	WT	GCC5TCC	WT	WT	AGC95ACC	WT
10	r	r	r	s	TCG531TTG	AGC315ACC	GCC5TCC GAA7GCA GGG8GCT	WT	WT	NA	WT
11	r	r	r	r	TCG531TTG	WT	GCC5TCC	WT	WT	NA	WT
12	r	r	r	s	CAC526GAC	WT	WT	AAG43AGG	WT	GCG90GTG AGC95ACC	WT

No.	R	H	S	E	Mutations in specific resistant genes						
					rpoB	*katG*	*inhA*	*rpsL*	*rrs*	*gyrA*	*gyrB*
13	r	r	r	r	CAC526TAC	AGC315ACC	WT	WT	WT	GAC94GGC AGC95ACC	GAG528GCG
14	r	r	s	r	TCG522TTG GTG550TTG	AGC315ACC	WT	WT	WT	ACC80ATC AGC95ACC	WT
15	r	r	r	r	TCG531TTG	AGC315ACC	WT	WT	WT	AGC95ACC	WT
16	r	r	r	r	TCG531TTG	AGC315ACC	WT	AAG43AGG	WT	GAC94GGC AGC95ACC	ACC539CCC
17	r	s	s	s	TCG531TTG	AGC315ACC	WT	NA	NA	NA	NA
18	r	r	r	r	TCG531TTG	AGC315ACC	WT	WT	WT	AGC95ACC	WT
19	r	r	r	s	CTG511CCG GAC516AAC	WT	WT	AAG43AGG	WT	AGC95ACC	WT
20	s	r	r	s	CTG533CCG	AGC315ACC	WT	NA	NA	NA	NA
21	r	r	r	r	TCG522TTG GTG550TTG	AGC315ACC	WT	WT	WT	AGC95ACC	WT
22	r	r	r	r	TCG531TTG	AGC315ACC	WT	AAG43AGG	WT	AGC95ACC	WT
23	r	r	s	r	AGC509AGG CTG511CCG GAC516GTC	Gene loss	WT	NA	WT	NA	WT

Abbreviations: R, rifampicin; H, isoniazid; S, streptomycin; E, ethambutol; s, susceptible; r, resistant; WT, wild type; and NA, data not available.

Table 4. Drug susceptibility profiles and mutational patterns of 23 representative clinical isolates

3.2 Genotyping

3.2.1 Spoligotyping and its application in surveillance

Spacer oligonucleotide typing (Spoligotyping) is a molecular method used to differentiate *M. Tuberculosis Complex (MTC)* isolates. This method is based on PCR analysis of polymorphisms in the MTC direct repeat (DR) chromosomal region containing multiple 36bp DR loci. Each DR is interspersed by a unique spacer sequence of 35 to 41 bp. After PCR amplification, the fragment containing the whole DR region was hybridized to specific oligonucleotide probes designed according to the different spacer sequences, and genotypes were determined depending on the hybridization patterns. The most widely used 43 sites were developed in 1997 by Kamerbeek et al, including 37 derived from the reference strain H37Rv and 6 spacers derived from *M. bovis* BCG (Kamerbeek et al., 1997). This method is more rapid and easier to perform than the standard genotyping technique based on IS6110 profiling.

Member of M. TB complex	Characterization of Spoligotyping (Missing Spacers)
Mycobacterium tuberculosis	33~36 (Viana-Niero et al., 2001)
Mycobacterium bovis	39~43 (Soini et al., 2000; Filliol et al., 2003)
Mycobacterium africanum	8, 9, 39 (Viana-Niero et al., 2001; Filliol et al., 2003)
Mycobacterium microti	37, 38 (Niemann et al., 2000)

Table 5. Characterization of *M. tuberculosis complex* by Spoligotyping

MTC includes *M. tuberculosis*, *M. bovis*, *M. voles* and *Africa mycobacterium*. *M. tuberculosis* is one of the leading pathogens to human and animal, followed by *M. bovis*. *M. tuberculosis* and *M. bovis* can be distinguished from MTC by using L–J agar slants, PNB and TCH. *M. bovis* can be quickly identified by spoligotyping. Considering the difficulties in determining *M. vole* and *Africa mycobacterium* by traditional methods of bacteriology, Soolingen et al. developed the spoligotyping method to distinguish the *M. vole* from MTC (van Soolinger et al. 1998). Results from Niemann et al. proved that *M. bovis* could be differentiated from *M. Africa* by spoligotyping (Niemann et al., 2000). In addition, Viana-Niero et al. also indicated that the spoligotyping fingerprint of *M. Africa* was between *M. tuberculosis* and *M. bovis* (Viana-Niero et al., 2001). Spoligotyping can be used to distinguish MTC members based on their characteristic spoligotypes (Table 5). Until now, IS6110-RFLP has been the gold standard for genotyping of *M. tuberculosis* (Majeed et al., 2004). However, a comparison study using IS6110-RFLP and spoligotyping indicated that the genotyping capacity of spoligotyping was better than IS6110-RFLP to strains containing low-copy numbers of IS6110 (Bauer et al., 1999).

To study the genotypic diversity, we choose spoligotyping for molecular typing of 100 clinical *M. tuberculosis* isolates collected at the Affiliated Hospital of Zunyi Medical College from 2008 to 2009. Bacterial growth and chromosomal DNA isolation were carried out by the method of Soolingen et al. (van Soolinger et al., 1991). The extracted DNA was used as a template for PCR amplification of the DR region of the genome with the biotinylated forward primer, 5′-GGTTTTGGGTCTGACGAC-3′ and the reverse primer 5′-CCGAGAGGG GACGGAAAC-3′. The following thermocycler parameters were applied with initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s; primer annealing at 56°C for 30 s; extension at

72°C for 30 s; and a final extension at 72°C for 8 min. The PCR amplified products were hybridized to a membrane containing a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. DNAs isolated from *M. tuberculosis* H37Rv and *M. bovis* BCG were used as controls. The hybridized PCR products were incubated with 1:4000-diluted streptavidin-peroxidase conjugate (Boehringer) for 30min at 42°C. Detection of hybridizing DNA was done by using the chemiluminescent ECL (Amersham) detection system followed by exposure to X-ray film (Hyperfilm ECL; Amersham) in accordance with the manufacturer's instructions.

Spoligotypes in binary format were entered in the SITVIT database (Pasteur Institute of Guadeloupe). Twenty-nine distinct spoligotyping patterns were observed. In total, 20 orphan patterns were identified and the remaining 77 were contained within 9 superfamilies: Beijing, T1, T3, T2, MANU2, Beijing-like, U, H3 and H3-T3. Results showed that almost half of the isolates were clustered to the Beijing lineage, and 13 isolates were clustered to the T1 lineage.

To analyze the MTC population structure, evaluate the complicacy of the global TB transmission and provisional evolution of the TB genetic landscape, Institute Pasteur had built a genetic diversity database for the MTC DR locus in 1999. This database was updated to SpolDB4 (Spoligotyping Database 4) in 2006. The updated database contained 1939 shared-types (STs) of 39,295 strains from 122 countries, which were temporarily classified into 62 clades/lineages (Brudey et al., 2006). In this database, the 10 most prevalent clades are ST1 (Beijing family), ST53 (T1), ST190 (Beijing family), ST52 (T2), ST50 (H3), ST54 (MANU2), ST37 (T3), ST742 (H3), ST265 (Beijing family) and ST127 (H4).

The Beijing genotype family was originally identified in China (Brudey et al., 2006) and defined as strains that presence of at least three spacers 35-43 and absence of spacers 1-34 (van Soolinger et al., 1995). Beijing family strains represent at least 13% of strains worldwide and about half of strains in the East Asia. A total of 2,346 *M. tuberculosis* isolates from 13 provinces, excluding Guizhou province, in China were genotyped by spoligotyping, and results showed that 74.08% of the isolates belonged to the Beijing family (Dong et al., 2010). Other studies also examined the relationship of Beijing genotype with drug resistance, however, the association between them was still not clear. The possible reasons for this uncertainty might be: 1) the diversity of treatment programs; 2) compliance to treatment; 3) different quality of anti-TB drugs; and 4) spread of different and not yet distinguished sublineages of the Beijing strains (Parwati et al., 2010).

The "T" families were the most prevalent following Beijing family, belonged to modern TB strains, and ill-defined with more than 600 unclassified STs. The "T" families were further divided into 5 subclades (T1-T5) based on single spacer-differences (Brudey et al. 2006). The numbers of T1 and T2 families were ranked the second and third respectively after Beijing family in the updated SpolDB4.

4. Application of proteomics in drug-resistant *M. tuberculosis*

4.1 Proteomics and its application in tuberculosis research

Proteomics is defined as the large-scale study of proteins, their post-translational modifications, and their structures and functions underlying different biological processes.

Proteomics enables the qualitative and quantitative analyses of proteins in complex biological systems, such as cells, tissues, and body fluids under specific conditions or in response to different stimuli (Graham et al., 2011; Lim & Elenitoba-Johnson, 2004; List et al., 2008; Wu & Liu, 2009). Proteomics is an effective means to rapidly identify new proteins as diagnostic or prognostic markers and as therapeutic targets for various diseases including cancers, genetic diseases, and infectious diseases such as tuberculosis (Chung et al., 2007; Kavallaris & Marshall, 2005; Drake et al., 2005; Stulik & Butaye, 2011).

Proteomics has been applied to the research area of *M. tuberculosis* (Bahk et al., 2004; He et al., 2003; Kumar et al., 2010; Lee et al. 1999; Mattow et al. 2001; Mustafa, 2005; Pheiffer et al., 2005; Sharma et al., 2010; Wang et al., 2007; Zhu et al., 2003) and become an important tool to study functional genomics of the bacterium (Mattow et al., 2003; Jiang et al., 2006; Rison et al., 2007; Xie et al., 2009; Jungblut et al., 2001). Proteins from *M. tuberculosis* missing in attenuated *M. bovis* BCG strains were identified by using proteomics (Mattow et al., 2001). Comparative proteome analyses of culture supernatant proteins from virulent *M. tuberculosis* H37Rv and attenuated *M. bovis* BCG (Mattow et al., 2003) or H37Ra (He et al., 2003) were performed to identify new virulent factors. Proteomics was also used for genome-wide analysis of the host intracellular network regulating survival of *M. tuberculosis* (Kumar et al., 2010). It was demonstrated that protein expression by a Beijing strain was different from that of another clinical isolate and *M. tuberculosis* H37Rv (Pheiffer et al., 2005). The major membrane protein of virulent *M. tuberculosis* was characterized using proteomics technology (Lee et al., 1999). Xie et al. used proteomics to compare proteins of MDR-TB isolate with drug-sensitive isolate (Xie et al. 2009). Additionally, proteomics has been successfully used for vaccine design to improve protection against tuberculosis (Mollenkopf et al., 2004).

The development of proteomic profiles for *M. tuberculosis* is needed to identify proteins that are differentially expressed in clinical strains with different drug susceptibility profiles such as MDR-TB and XDR-TB comparing to the standard strain, which can provide insights into the mechanisms by which the mycobacteria resistant to anti-TB drugs and the selection of suitable biomarkers for new diagnostics, new virulent factors and/targets for the development of potential therapeutics and new vaccines.

The most commonly used method for proteomic analysis is the two-dimensional polyacrylamide gel electrophoresis (2-DE), which allows the separation and display of thousands of proteins from a complex mixture by their charges (pI) and relative molecular mass (Mr). Gel-separated proteins can be identified rapidly by mass spectrometry (MS), and such analyses permit the systematic identification of the proteome if genomic information is available. In this preliminary study, we used 2-DE and mass spectrometry to compare protein expression profiles among different clinical *M. tuberculosis* strains such as MDR-TB, XDR-TB and the reference strain H37Rv, and identified some up-regulated, down-regulated genes which might be associated with MDR or XDR.

4.2 Sample preparation for MDR/XDR-TB isolates

The sensitive strain used in this study was the standard *M. tuberculosis* strain H37Rv, which was obtained as a gift from the Chinese Centre for Disease Control and Prevention. Both MDR-TB and XDR-TB strains were clinical isolates collected at the Affiliated

Hospital of Zunyi Medical College. Five to six inoculating loops of *M. tuberculosis* colonies were scraped from improved L-J culture media and transferred to 4 ml of purified water in a screw-top centrifuge tube. The mycobacterium suspension was heated at 80°C for 30 min in a water bath to inactivate *M. tuberculosis*, and centrifuged at 4000*g* for 10min for removing the supernatant. The precipitation was resuspended in the phosphate-buffered saline (pH7.4), and centrifuged at 4000*g* for 10min to remove the supernatant. The inactivated *M. tuberculosis* sample was resuspended in 0.3ml of lysis buffer, and 10μl of proteinase inhibitors were added to the suspension followed by incubation at 4°C for 20 min. The suspension was then sonicated (200W, 1min×30 times), and centrifuged at 4000*g* for 30min at 4°C. Four-fold volume of cold dimethyl ketone were added to the suspension and the mixture was kept at -20°C overnight for precipitating proteins. The protein precipitates were centrifuged at 10000*g* for 5min at 4°C, and resuspended in 7M carbamide and 2M sulfourea solution to dissolve proteins followed by centrifugation at 4000*g* for 30min at 4°C. Concentration of protein samples was measured by the NanoDrop-1000 (Thermo, Germany) and protein samples were stored at -20°C until use.

4.3 Two-dimensional electrophoresis (2-DE)

Protein samples (200-300 μg) were resuspended in 480 μl of rehydration buffer and applied to pH 4-7 IPG strips (Amersham Biosciences) for rehydration. When IPG strips were rehydrated with the protein samples, isoelectric focusing (IEF) was performed in the following voltage mode: 0V 1h, 50V 10h fast voltage, 500V 1.5h linear voltage, 2000V 1.5h linear voltage, 5000V 1.5h linear voltage, 8000V 1.5h linear voltage, 8000V 70000VH fast voltage, and 500V 16h fast voltage.

Equilibration was performed immediately prior to the second-dimension run, in which step IPG strips should be laid in the strip equilibrium solution buffer I (6 mol/L Urea, 2% SDS, 0.375mol/L pH 8.8 Tris-HCl, 20%Glycerol, and 2% DTT), then strip equilibrium solution buffer II (mol/L Urea, 2% SDS, 0.375mol/L pH8.8 Tris-HCl, 20% Glycerol, and 2.5% Iodoacetamide) each for 14 minutes. The equilibrated IPG strip was located into prepared 13% SDS-PAGE with low-melting point agarose smother. Following electrophoresis, proteins were visualized by either silver staining (analytical gels) or Coomassie Brilliant Blue G-250 staining (preparative gels).

The pI and Mr gradient of the 2-DE gels were determined using an iterative calibration method after 2-DE gel images transferred to computer by means of the image scanner. Spot detection and image analysis were performed and compared using the program Progenesis SameSpots (Nonlinear Dynamics, UK) for 2-DE database construction. Images of MDR-TB and XDR-TB were compared to the gel image of the standard strain H37Rv, and protein spots showing differential expression levels of more than two-fold were analyzed.

4.4 MALDI-MS and database

Twenty protein spots of interest (5 spots from H37Rv, 10 spots from XDR-TB, and 5 up-regulated protein spots from MDR-TB) were excised from Coomassie Brilliant Blue G-250 stained two dimensional gels (2-DE) and digested in gel using trypsin for 18h at 37°C. Masses of the peptides extracted from gel slices were applied to the sample plate of a matrix-assisted laser desorption ionization–time-of-flight MS (MALDI-TOF-MS). Peptide

mass fingerprint data were searched using the Mascot search (www.matrixscience.com) of the National Center for Biotechnology Information (NCBI) database.

4.5 Comparison of XDR and MDR patterns

The comparison was repeated at least three times, and only those differences confirmed in all comparisons were accepted as strain specific. This study compared the proteome of XDR-TB clinical isolate to those of MDR-TB clinical isolate and the standard strain H37Rv, and demonstrated that the 2-DE protein expression patterns of XDR-TB and MDR-TB clinical isolates were highly correlated, but there were some visible differences between drug-resistant strains and the reference H37Rv strain. Results also showed that more basic proteins were expressed in drug-resistant *M. tuberculosis* isolates than in the standard H37Rv stain. Figure 1 shows differences among three different mycobacterium strains.

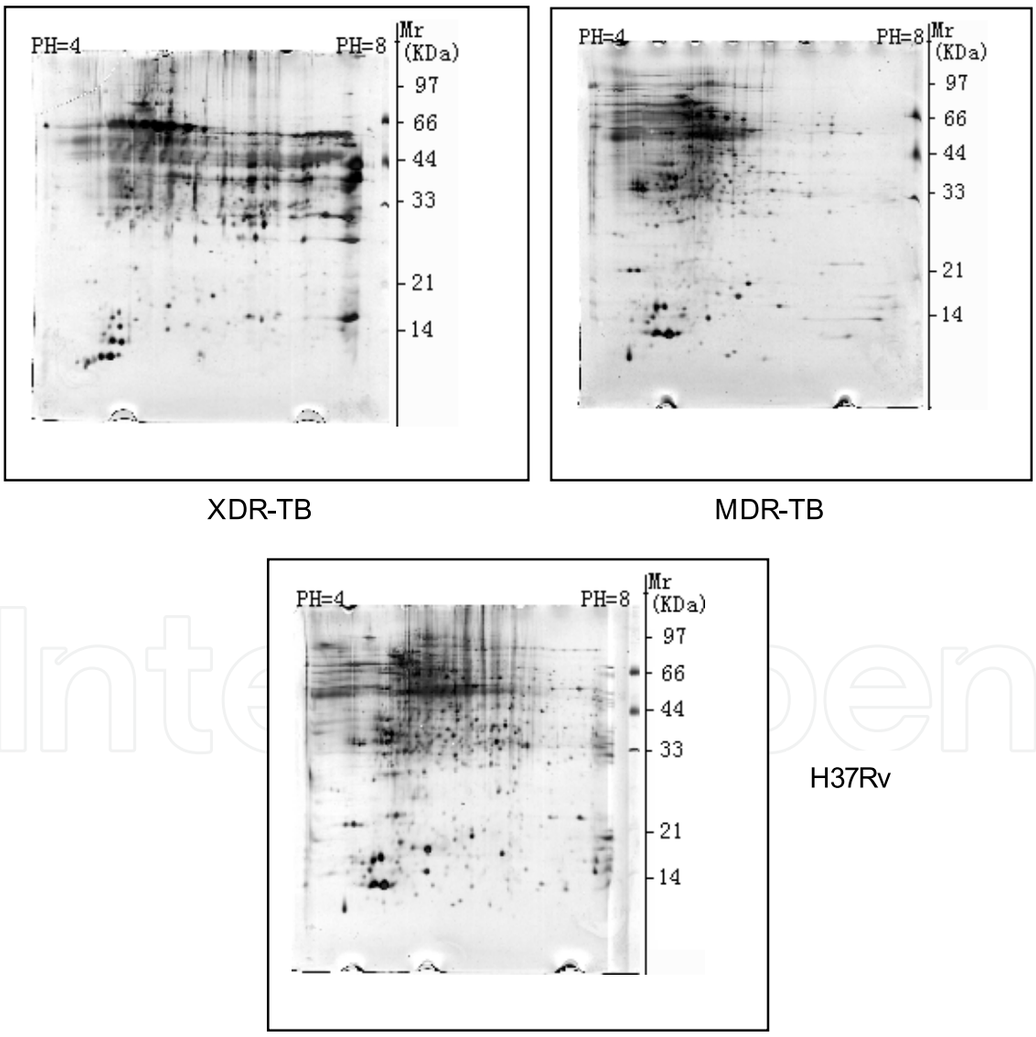

Fig. 1. Protein expression patterns of XDR-TB and MDR-TB clinical isolates compared to the standard strain H37Rv in two-dimensional electrophoresis

Spot No.	XDR-TB	MDR-TB	H37Rv	Spot No.	XDR-TB	MDR-TB	H37Rv
2984	156.1	20.42	10.68	1328	36.14	3.39	6.39
1622	145.0	11.43	30.65	2418	31.63	3.22	3.04
2980	99.99	9.90	3.91	1440	31.49	3.43	3.28
1692	85.41	4.34	6.95	2679	26.51	5.71	3.25
1813	78.84	6.39	5.42	1600	26.19	1.36	3.61
1805	67.34	3.31	5.65	2379	13.16	1.821	1.12
2978	49.75	5.09	2.44	2787	11.91	1.79	0.85
1839	46.13	5.60	3.89	1994	9.77	0.90	2.11
1245	38.84	2.79	3.15	2449	5.37	0.72	0.74
2726	36.46	3.16	3.07				

Table 6. Expression levels of 19 proteins specifically overexpressed in the XDR-TB (protein expression level=number in the table$x10^4$)

There were 19 proteins specifically overexpressed in XDR-TB (Table 6), 3 proteins up-regulated (Table 7), 13 proteins down-regulated (Table 8) and 10 proteins disappeared in the XDR-TB clinical isolate compared to the reference H37Rv strain.

Spot No.	XDR- TB	MDR-TB	H37Rv
2561	22.26	29.82	14.69
1228	46.98	4.657	20.38
2378	37.97	9.587	4.238

Table 7. Expression levels of 3 up-regulated proteins in XDR-TB (protein expression level=number in the table$x10^4$)

Out of these forty-five protein spots, twenty were selected for the mass spectrometry analysis, and only three proteins were identified by the MALDI-TOF-MS (Table 9). The differential expression of the ribosomal protein S3 and 19 kda major membrane protein in the XDR-TB strain was validated by the real-time RT-PCR (data not shown). It is not clear how differential expression of these three proteins contributes to the drug resistance of the XDR-TB. Further function analysis of these three proteins will be conducted in our laboratory to determine their relationship with drug resistance of XDR-TB.

Spot No.	XDR-TB	MDR-TB	H37Rv	Spot No.	XDR-TB	MDR-TB	H37Rv
2394	58.18	159.8	122.2	2695	5.16	17.13	13.47
2994	43.26	11.03	92.87	2365	4.76	44.01	48.32
2991	26.93	41.37	65.52	2989	4.56	23.36	13.34
2499	18.92	49.45	49.02	2436	2.67	7.96	13.41
2637	16.45	84.32	53.78	1627	1.87	1.70	11.67
1279	11.77	18.13	36.18	2174	1.40	8.94	16.15
1616	7.60	22.65	12.33				

Table 8. Expression levels of 13 down-regulated proteins in XDR-TB (protein expression quantity=number in the table$x10^4$).

Spot No.	Protein Name	Function
2994	MMP=19 kda major membrane protein	Lipomannan, overlapping peptide sequences
1994	Ribosomal protein S3	Ribonucleoprotein, binding and positioning mRNA for translation
1813	YclD	Unknown

Table 9. Description of three proteins identified by MALDI-TOF-MS

5. Conclusion

In this chapter, we have provided the detailed description about our current progresses in the field of survey and molecular characterization of drug-resistant *M. tuberculosis* clinical isolates using different research methods such as *M. tuberculosis* culture, spoligotyping, gene sequencing, proteomics and drug susceptibility testing against first-line and second-line anti-tuberculosis drugs. We hope this chapter will be useful for researchers, scientists and physicians in academic institutions, clinical laboratories, pharmaceutical companies and research hospitals who have interest in the research related to drug-resistant *M. tuberculosis*, especially MDR/XDR-TB.

6. Acknowledgments

This project was funded in part with federal funds from the CDC, Department of Health and Human Services, under the contract no. 200-2007-M-22792, and by grants of International Cooperation Project from Guizhou Province of China [Qian Science co-G (2009) No. 700120], the Guizhou Science and Technology Fund Project [Qian Science co-J (2007) no. 2223] and Scientific Research Start Foundation from Zunyi Medical College in (F-327). The following people in the Affiliated Hospital of Zunyi Medical College made contributions to the research reported in this chapter: Xin Gan, Yang Chen, Kai-Lun Li, Yuan-Bo Lan, Zhen-Yong Chen, Lin-Mei Zhou, and Yi He. We would like to thank all health care staff in the Affiliated Hospital of Zunyi Medical College who participated in the research. We are also grateful to Professor Kanglin Wan, Zhiguang Liu, and Bing Lv in the Chinese Center for Disease Control and Prevention for their support with the spoligotyping analyses.

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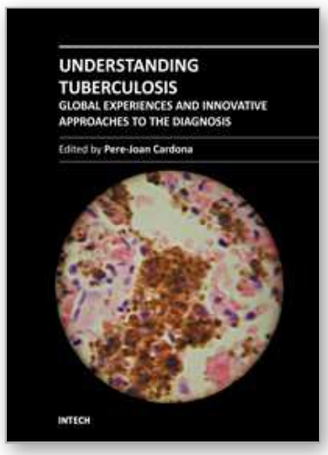

Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis

Edited by Dr. Pere-Joan Cardona

ISBN 978-953-307-938-7
Hard cover, 552 pages
Publisher InTech
Published online 15, February, 2012
Published in print edition February, 2012

Mycobacterium tuberculosis is a disease that is transmitted through aerosol. This is the reason why it is estimated that a third of humankind is already infected by Mycobacterium tuberculosis. The vast majority of the infected do not know about their status. Mycobacterium tuberculosis is a silent pathogen, causing no symptomatology at all during the infection. In addition, infected people cannot cause further infections. Unfortunately, an estimated 10 per cent of the infected population has the probability to develop the disease, making it very difficult to eradicate. Once in this stage, the bacilli can be transmitted to other persons and the development of clinical symptoms is very progressive. Therefore the diagnosis, especially the discrimination between infection and disease, is a real challenge. In this book, we present the experience of worldwide specialists on the diagnosis, along with its lights and shadows.

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