JOURNAL OF CLINICAL MICROBIOLOGY, July 2011, p. 2719–2721 Vol. 49, No. 7
0095-1137/11/$12.00 doi:10.1128/JCM.00317-11

Molecular Characterization of Drug-Resistant *Mycobacterium tuberculosis* Isolates Circulating in China by Multilocus PCR and Electrospray Ionization Mass Spectrometry▽

Feifei Wang,[1,3] Christian Massire,[5] Haijing Li,[3] Lendell L. Cummins,[5] Feng Li,[5] Jialin Jin,[1] Xiaoping Fan,[1] Sen Wang,[1] Lingyun Shao,[1] Shu Zhang,[1,3] Shufang Meng,[3] Jing Wu,[1] Chanyi Lu,[1] Lawrence B. Blyn,[5] Rangarajan Sampath,[5] David J. Ecker,[5] Wenhong Zhang,[1,2]* and Yi-Wei Tang[3,4]*

Department of Infectious Diseases, Huashan Hospital,[1] and Institute of Biomedical Sciences, Fudan University,[2] Shanghai, China; Departments of Pathology[3] and Medicine,[4] Vanderbilt University Medical Center, Nashville, Tennessee; and Ibis Biosciences, Inc., a subsidiary of Abbott Molecular, Inc., Carlsbad, California[5]

Received 14 February 2011/Returned for modification 25 March 2011/Accepted 21 April 2011

We used multilocus PCR and electrospray ionization mass spectrometry (PCR/ESI-MS) to determine the genotype and drug resistance profiles for 96 *Mycobacterium tuberculosis* isolates circulating in regions of high and low tuberculosis (TB) endemicity in China. The dominant principal genetic group (PGG) circulating in China was PGG1, and drug-resistant gene mutations were more diversified in the region of low rather than high TB endemicity.

Tuberculosis (TB) remains a significant public health problem worldwide. In China, multidrug-resistant tuberculosis (MDR-TB), which is defined as TB caused by organisms resistant to at least isoniazid (INH) and rifampin (RIF), is spreading, due mainly to the HIV epidemic as well as the lack of funding for health infrastructure. This, in turn, leads to incorrect or incomplete treatment, which increases the resistance rate (21). In some provinces of China, the prevalence of MDR-TB among new cases and previously treated cases ranges from 10% to 30% (1, 24). In addition, extensively drug-resistant TB (XDR-TB), which is defined as MDR-TB with additional resistance to any fluoroquinolone (FQ) and at least one second-line injectable drug, has been emerging in many countries, including China (9, 24, 27). The mortality rate of XDR-TB patients varies in different countries and depends on the study population and their HIV status (8, 14, 16, 22, 23, 25).

A rapid, sensitive method for detecting drug-resistant phenotypes of *Mycobacterium tuberculosis* is one of the more urgent requirements for effective treatment of tuberculosis patients. Molecular biology tools have been developed in order to provide a rapid susceptibility profile through the direct detection of drug resistance-related mutations in mycobacterial genomes (10, 13, 18, 26, 28). Several commercial assays are available, including the GenoType MTBDR*plus* (13, 17, 20), the InnoLiPA Rif.TB (11), and the GeneXpert MTB/RIF. The new GenoType MTBDRsl assay also covers FQ, amikacin-capreomycin, and ethambutol (EMB) resistance (12).

A new strategy for the molecular determination of TB drug resistance couples broad-range PCR to electrospray ionization mass spectrometry (PCR/ESI-MS). The technology was initially developed for the identification of microbes in samples where multiple pathogens may be present, primarily for biodefense applications (2, 4). It has since been successfully applied to the detection and identification of a variety of microorganisms present in cultured specimens or patient samples (3, 5, 6). Recently, a PCR/ESI-MS assay was developed for the primary characterization of MDR-TB (15). The assay also determines whether the main mutations associated with EMB and FQ resistance are present and identifies nontuberculous mycobacteria to the species level. The assay panel includes 16 primer pairs in 8 multiplexed reactions for multilocus PCR amplification. Following PCR amplification, the amplicons are analyzed by Plex-ID, an ESI-MS-based instrument (4, 15). In the present study, we used the PCR/ESI-MS methodology to detect and identify gene mutations associated with INH, RIF, EMB, and FQ resistance in 96 *M. tuberculosis* isolates circulating in regions of low and high TB endemicity in China, representing TB annual incidence above and below 107 and 100,000, respectively (7, 27).

***M. tuberculosis* isolates.** *M. tuberculosis* isolates were collected from Shanghai and Chongqing Pulmonary Disease Hospitals, the specialized hospitals serving TB patients locally in China, from 2005 to 2009. None of these patients from whom isolates were recovered were immunocompromised or HIV-1 infected. The identification of these isolates was confirmed by using an *M. tuberculosis*/nontuberculosis mycobacteria (MTB/NTM) ACE detection kit (Seegene Inc., Seoul, South Korea) according to the manufacturer's instructions. Among them, 88 were resistant phenotypically to at least one of the first-line antituberculosis drugs, and 8 were phenotypically susceptible as determined on Lowenstein-Jensen medium by the absolute-

* Corresponding author. Mailing address for Wenhong Zhang: Department of Infectious Diseases, Huashan Hospital, 12 Wulumuqi Zhong Road, Shanghai 200040, China. Phone: 86 21 52888123. Fax: 86 21 62489015. E-mail: zhangwenhong@fudan.edu.cn. Mailing address for Yi-Wei Tang: Molecular Infectious Disease Laboratory, Vanderbilt University Hospital, 4605 TVC, Nashville, TN 37232-5310. Phone: (615) 322-2035. Fax: (615) 343-8420. E-mail: yiwei.tang@vanderbilt.edu.

▽ Published ahead of print on 27 April 2011.

TABLE 1. Resistance profiles determined by PCR/ESI-MS

Drug	No. of isolates				*P* value
	Resistant		Susceptible		
	Shanghai	Chongqing	Shanghai	Chongqing	
Isoniazid	39	39	10	8	0.671
Rifampin	42	42	7	5	0.589
Ethambutol	17	25	32	22	0.068
Fluoroquinolone	17	25	32	22	0.068

concentration method and interpreted according to the standards of the Clinical and Laboratory Standards Institute (19).

Nucleic acid extraction. Genomic DNA from these isolates was extracted using the DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. In brief, a loopful of the purified isolate colony was put into 1 ml of distilled water prior to the Qiagen extraction. Extracted nucleic acids were eluted into 200 μl of elution buffer AE (Qiagen) and used for PCR amplifications both for sequencing and ESI-MS analysis, as described below.

Multilocus PCR amplification and ESI-MS analysis. All PCRs were assembled in 40-μl reaction mixtures in the 96-well microtiter plate format using a Packard MPII liquid handling robotic platform and an Eppendorf Mastercycler Pro (Eppendorf, Hauppauge, NY). General methods and experimental conditions of PCR/ESI-MS analysis, including the primer sequences specific to the *M. tuberculosis* resistance assay, were as previously described (15).

A total of 96 *M. tuberculosis* isolates were collected for this study. Among them, 49 and 47 were collected from Shanghai and Chongqing areas representing regions of low and high TB endemicity, respectively. A basic phylogenetic classification of the isolates was provided by the determination of the principal genetic group (PGG), based on the presence or absence of lineage-dependent mutations at codons *katG463* and *gyrA95*. In both locations, PGG1 [*katG*(*L463wt*) plus *gyrA*(*T95wt*)] was largely predominant: 42/49 (85.7%) and 42/47 (89.4%) isolates from Shanghai and Chongqing, respectively. PGG2 [*katG*(*L463R*) plus *gyrA*(*T95wt*)] was seen in 6/49 (12.3%) and 4/47 (8.5%) isolates, while PGG3 [*katG*(*L463R*) plus *gyrA*(*T95S*)] was characterized in a single isolate in both locations. No significant differences in the distributions into PGG1, PGG2, and PGG3 were thus observed between the regions of high and low TB endemicity ($P > 0.05$).

The INH, RIF, EMB, and FQ resistance profiles determined by the PCR/ESI-MS assay on the 96 isolates are presented in Table 1. Overall, the proportions of MDR isolates seen within the Shanghai (38/49 or 78%) and Chongqing (39/47 or 83%) populations are similar ($P > 0.05$). However, further comparative analysis revealed that *M. tuberculosis* isolates circulating in regions of low and high TB endemicity in China possessed different mutation profiles: while no multiresistant profile was observed in more that two occurrences in the Shanghai isolates, the MDR isolates from Chongqing exhibited significant clustering (Fig. 1). In particular, a cluster of 11 PGG1 MDR isolates from the region of high TB endemicity (Chongqing) is characterized by a common string of INH, RIF, and EMB resistance mutations [*katG*(*S315T*) and *inhA* promoter C-15T, *rpoB*(*S531L*), *embB*(*M306I*)]. This mutation profile was not observed in the region of low TB endemicity (Shanghai) ($P = 0.0006$). In addition, a distinct cluster of 10 PGG1 MDR iso-

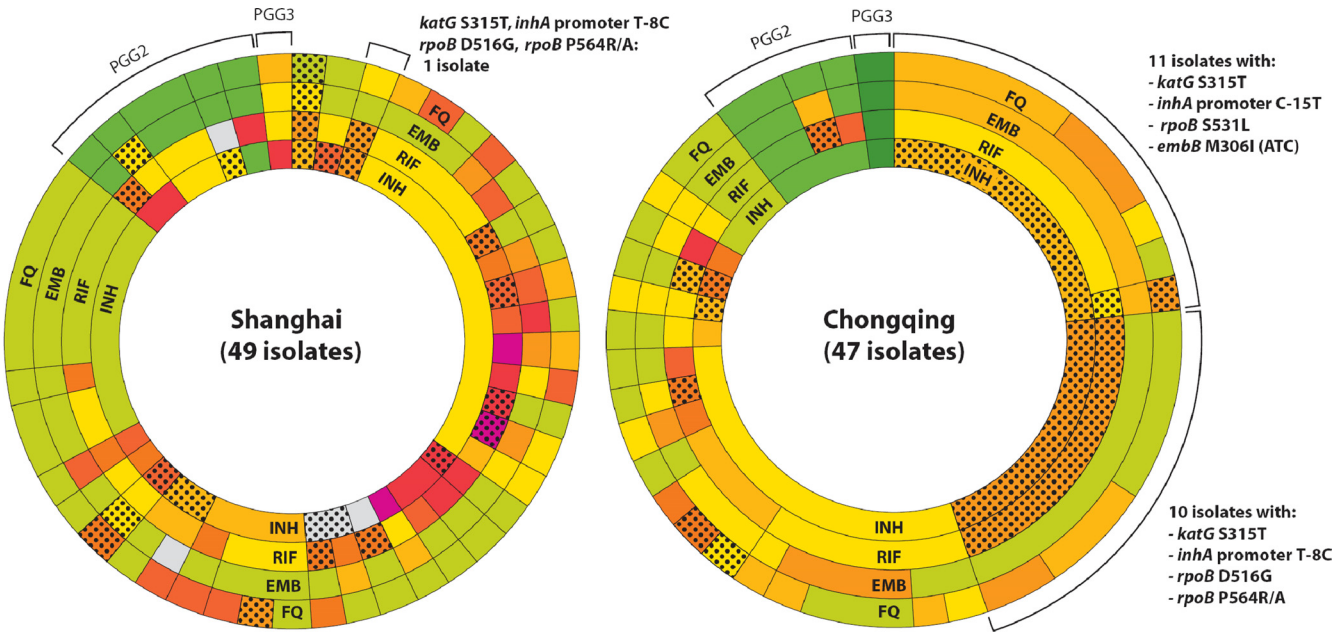

FIG. 1. Radial plots of the genotypic distribution of *M. tuberculosis* as determined by PCR/ESI-MS for 49 Shanghai isolates (left) and 47 Chongqing isolates (right). The area of the wedges corresponds to the number of isolates with the given genotypic signature. Wedges are primarily colored by principal genetic group (light, medium, and dark green backgrounds for PGG1, -2, and -3, respectively). The presence of mutations conferring resistance to isoniazid, rifampin, ethambutol, and fluoroquinolones is indicated by segments in various shades of yellow to magenta. Yellow segments correspond to the more common mutations [e.g., *katG*(*S315T*) for INH and *rpoB*(*S531L*) for RIF], while reddish tones indicate rarer mutations. Multiple mutations are indicated by stippling the corresponding segment.

lates [*katG*(*S315T*) and *inhA* promoter mutation T-8C, *rpoB*(*D516G*), and *rpoB* mutation P564(R/A)] was found in the region of high TB endemicity and was represented by a single isolate in the region of low TB endemicity ($P = 0.0049$).

We report the use of a PCR/ESI-MS assay to determine drug resistance profiles among *M. tuberculosis* isolates circulating in regions of low and high TB endemicity in China. The PCR/ESI-MS assay employed 16 primer pairs targeting the genes associated with drug resistance to RIF, INH, EMB, and FQ. For each isolate, *M. tuberculosis* DNA was amplified in eight reaction mixtures, each containing two primer pairs on a 96-well plate. From specimen processing to result reporting, the PCR/ESI-MS assay can be completed within 8 h, providing another rapid and accurate laboratory diagnostic tool for first-line antituberculosis drug resistance determination. This new technique has the potential to facilitate rapid determination of MDR-TB in China, allowing timely guidance for individualized treatment.

The mutation profiles obtained by the PCR/ESI-MS assay indicated that the PGG1 profile (87.5%) was dominant across the isolates tested, a result that is consistent with the Beijing lineage being widespread in China (7). We further compared the genotypic distribution of TB drug resistance-related mutation profiles between *M. tuberculosis* isolates circulating in regions of low and high TB endemicity in China. It was revealed that a cluster of 11 isolates with *katG*(*S315T*) and *inhA* promoter C-15T, *rpoB*(*S531L*), and *embB*(*M306I*) was observed in Chongqing, the region of high TB endemicity, which was not observed in Shanghai, the region of low TB endemicity. In addition, another resistance mutation profile with *katG*(*S315T*) and *inhA* promoter T-8C, *rpoB*(*D516G*), and P564(R/A) was detected in 10 isolates in the region of high TB endemicity, in comparison to one isolate in the region of low TB endemicity. These data indicated that mutations conferring drug resistance were more diversified in the region of low TB endemicity than in the region of high TB endemicity.

We thank the patients, laboratorians, nurses, and physicians at Chongqing Pulmonary Disease Hospital and Shanghai Pulmonary Disease Hospital for their excellent assistance.

This study was supported in part by research grants from the Key Project of Science and Technology of Shanghai (10411955000), Shanghai Science and Technology Development Funds (10XD1400900, 10QA1401100), the Key Technologies Research and Development Program for Infectious Diseases of China (2008ZX10003003, 2009ZX10004-104), the National Natural Science Foundation of China (30901277), the U.S. Centers for Disease Control and Prevention (R44 AI078694-03), and the Vanderbilt CTSA grant from NCRR/National Institutes of Health (UL1 RR024975). Y.-W.T. is a consultant for Ibis Biosciences.

REFERENCES

1. **Aziz, M. A., et al.** 2006. Epidemiology of antituberculosis drug resistance (the Global Project on Anti-Tuberculosis Drug Resistance Surveillance): an updated analysis. Lancet **368:**2142–2154.
2. **Ecker, D. J., et al.** 2005. Rapid identification and strain-typing of respiratory pathogens for epidemic surveillance. Proc. Natl. Acad. Sci. U. S. A. **102:** 8012–8017.
3. **Ecker, D. J., et al.** 2010. New technology for rapid molecular diagnosis of bloodstream infections. Expert Rev. Mol. Diagn. **10:**399–415.
4. **Ecker, D. J., et al.** 2008. Ibis T5000: a universal biosensor approach for microbiology. Nat. Rev. Microbiol. **6:**553–558.
5. **Ecker, J. A., et al.** 2006. Identification of *Acinetobacter* species and genotyping of *Acinetobacter baumannii* by multilocus PCR and mass spectrometry. J. Clin. Microbiol. **44:**2921–2932.
6. **Eshoo, M. W., et al.** 2010. Detection and identification of *Ehrlichia* species in blood by use of PCR and electrospray ionization mass spectrometry. J. Clin. Microbiol. **48:**472–478.
7. **Gagneux, S., and P. M. Small.** 2007. Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. Lancet Infect. Dis. **7:**328–337.
8. **Gandhi, N. R., et al.** 2006. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet **368:**1575–1580.
9. **Gandhi, N. R., et al.** 2010. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. Lancet **375:**1830–1843.
10. **Gegia, M., et al.** 2008. Prevalence of and molecular basis for tuberculosis drug resistance in the Republic of Georgia: validation of a QIAplex system for detection of drug resistance-related mutations. Antimicrob. Agents Chemother. **52:**725–729.
11. **Herrera, L., S. Jimenez, A. Valverde, M. A. Garcia-Aranda, and J. A. Saez-Nieto.** 2003. Molecular analysis of rifampicin-resistant *Mycobacterium tuberculosis* isolated in Spain (1996-2001). Description of new mutations in the *rpoB* gene and review of the literature. Int. J. Antimicrob. Agents **21:**403–408.
12. **Hillemann, D., S. Rusch-Gerdes, and E. Richter.** 2009. Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of *Mycobacterium tuberculosis* strains and clinical specimens. J. Clin. Microbiol. **47:**1767–1772.
13. **Hillemann, D., M. Weizenegger, T. Kubica, E. Richter, and S. Niemann.** 2005. Use of the Genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. J. Clin. Microbiol. **43:**3699–3703.
14. **Kim, H. R., et al.** 2007. Impact of extensive drug resistance on treatment outcomes in non-HIV-infected patients with multidrug-resistant tuberculosis. Clin. Infect. Dis. **45:**1290–1295.
15. **Massire, C., et al.** 2011. Simultaneous identification of mycobacterial isolates to the species level and determination of tuberculosis drug resistance by PCR followed by electrospray ionization mass spectrometry. J. Clin. Microbiol. **49:**908–917.
16. **Migliori, G. B., et al.** 2007. Extensively drug-resistant tuberculosis, Italy and Germany. Emerg. Infect. Dis. **13:**780–782.
17. **Miotto, P., F. Piana, D. M. Cirillo, and G. B. Migliori.** 2008. Genotype MTBDRplus: a further step toward rapid identification of drug-resistant *Mycobacterium tuberculosis*. J. Clin. Microbiol. **46:**393–394.
18. **Mokrousov, I., T. Otten, B. Vyshnevskiy, and O. Narvskaya.** 2002. Detection of *embB306* mutations in ethambutol-susceptible clinical isolates of *Mycobacterium tuberculosis* from Northwestern Russia: implications for genotypic resistance testing. J. Clin. Microbiol. **40:**3810–3813.
19. **NCCLS.** 2003. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes. Approved standard, 9th ed. National Committee for Clinical Laboratory Standards, Wayne, PA.
20. **Neonakis, I. K., et al.** 2009. Evaluation of GenoType mycobacteria direct assay in comparison with Gen-Probe *Mycobacterium tuberculosis* amplified direct test and GenoType MTBDRplus for direct detection of *Mycobacterium tuberculosis* complex in clinical samples. J. Clin. Microbiol. **47:**2601–2603.
21. **Nettleman, M. D.** 2005. Multidrug-resistant tuberculosis: news from the front. JAMA **293:**2788–2790.
22. **Shenjie, T., et al.** 2010. Extensively drug-resistant tuberculosis at a tuberculosis specialist hospital in Shanghai, China: clinical characteristics and treatment outcomes. Scand. J. Infect. Dis. **43:**280–285.
23. **Singh, J. A., R. Upshur, and N. Padayatchi.** 2007. XDR-TB in South Africa: no time for denial or complacency. PLoS Med. **4:**e50.
24. **Sun, Z., et al.** 2008. Characterization of extensively drug-resistant *Mycobacterium tuberculosis* clinical isolates in China. J. Clin. Microbiol. **46:**4075–4077.
25. **Sun, Z., et al.** 2010. Concomitant increases in spectrum and level of drug resistance in *Mycobacterium tuberculosis* isolates. Int. J. Tuberc. Lung Dis. **14:**1436–1441.
26. **Telenti, A., et al.** 1997. Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. J. Clin. Microbiol. **35:**719–723.
27. **Wright, A., et al.** 2009. Epidemiology of antituberculosis drug resistance 2002-07: an updated analysis of the Global Project on Anti-Tuberculosis Drug Resistance Surveillance. Lancet **373:**1861–1873.
28. **Yang, Z., et al.** 2005. Simultaneous detection of isoniazid, rifampin, and ethambutol resistance of *Mycobacterium tuberculosis* by a single multiplex allele-specific polymerase chain reaction (PCR) assay. Diagn. Microbiol. Infect. Dis. **53:**201–208.