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Prevalence and Molecular Characteristics of Drug-Resistant *Mycobacterium tuberculosis* in Hunan, China

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To determine the prevalence and molecular characteristics of drug-resistant tuberculosis in Hunan province, drug susceptibility testing and spoligotyping methods were performed among 171 *M. tuberculosis* isolates. In addition, the mutated characteristics of 12 loci, including *katG*, *inhA*, *rpoB*, *rpsL*, nucleotides 388 to 1084 of the *rrs* gene [*rrs*(388–1084)], *embB*, *pncA*, *tlyA*, *eis*, nucleotides 1158 to 1674 of the *rrs* gene [*rrs*(1158–1674)], *gyrA*, and *gyrB*, among drug-resistant isolates were also analyzed by DNA sequencing. Our results indicated that the prevalences of isoniazid (INH), rifampin (RIF), streptomycin (SM), ethambutol (EMB), pyrazinamide (PZA), capreomycin (CAP), kanamycin (KAN), amikacin (AKM), and ofloxacin (OFX) resistance in Hunan province were 35.7%, 26.9%, 20.5%, 9.9% 15.2%, 2.3%, 1.8%, 1.2%, and 10.5%, respectively. The previously treated patients presented significantly increased risks for developing drug resistance. The majority of *M. tuberculosis* isolates belonged to the Beijing family. Almost all the drug resistance results demonstrated no association with genotype. The most frequent mutations of drug-resistant isolates were *katG* codon 315 ($katG_{315}$), *inhA15*, $rpoB_{531}$, $rpoB_{526}$, $rpoB_{516}$, $rpsL_{43}$, rrs_{514}, $embB_{306}$, $pncA_{96}$, rrs_{1401}, $gyrA_{94}$, and $gyrA_{90}$. These results contribute to the knowledge of the prevalence of drug resistance in Hunan province and also expand the molecular characteristics of drug resistance in China.

The emergence of drug-resistant tuberculosis (TB), particularly multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), has been identified as one of the major obstacles to effective TB control in many countries (1). In China, which is one of the 22 high-TB-burden countries, the prevalence of drug-resistant TB is also a serious problem. The latest data from the national baseline survey on TB indicate that the frequencies of drug-resistant TB, MDR-TB, and XDR-TB among pulmonary TB patients in China were 38.25%, 8.32%, and 0.68% (2), respectively. The magnitude and pattern of drug resistance varied greatly with the region because of the huge size of the country, the diverse population density, and the unbalanced economic development in China (3).

Drug-resistant TB is usually associated with inadequate anti-TB treatment or direct transmission of drug-resistant strains from one individual to another. Knowledge of the clinical characteristics and molecular characteristics of drug-resistant TB is very helpful in their rapid diagnosis and containment. However, previous studies demonstrated that such information varied in different geographical areas (1, 4–7).

Hunan province, with a total population of 69.251 million, is a high-TB-burden area in south-central China. According to recently updated epidemiological data, the prevalence of smear-positive pulmonary TB in this region was estimated at 94 cases per 100,000 inhabitants in 2010 (data not published), higher than the average level of China (66 cases per 100,000 inhabitants; http://www.gov.cn/gzdt/2011-03/21/content_1828718.htm). Unfortunately, thus far, the prevalence and molecular characteristics of drug-resistant TB of Hunan province remain unclear and should be explored to facilitate control of the TB epidemic in this region and throughout China.

Therefore, in the present study, we investigated the genotypes and current levels of resistance to the TB drugs isoniazid (INH), rifampin (RIF), streptomycin (SM), ethambutol (EMB), pyrazinamide (PZA), capreomycin (CAP), kanamycin (KAN), amikacin (AKM), and ofloxacin (OFX) among patients with pulmonary TB in Hunan. Moreover, we analyzed the association of 12 genetic loci, including *katG* and *inhA* (for resistance to INH), *rpoB* (RIF), *rpsL* and nucleotides 388 to 1084 of the *rrs* gene [*rrs*(388–1084)] (SM), *embB* (EMB), *pncA* (PZA), *tlyA* (CAP), *eis* (KAN or AKM), nucleotides 1158 to 1674 of the *rrs* gene [*rrs*(1158–1674)] (CAP, KAN, or AKM), and *gyrA* and *gyrB* (OFX), with drug resistance among drug-resistant isolates from Hunan.

MATERIALS AND METHODS

Ethical approval. The study obtained approval from the Ethics Committee of National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. The patients with TB were included in the present research only after we received informed written consent from themselves or from their parents/guardians in cases in which the patient was a child (<18 years of age). An assent was also obtained from participants between 14 and 18 years of age.

Received 30 January 2014 Returned for modification 17 March 2014
Accepted 3 April 2014
Published ahead of print 14 April 2014
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.02426-14.
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doi:10.1128/AAC.02426-14

***Mycobacterium tuberculosis* isolates.** This study was carried out between December 2009 and August 2010 at the Hunan Chest Hospital located in the capital of Hunan province, which serves as the sole specialized TB hospital in Hunan. A total of 171 inpatients with clinically diagnosed pulmonary tuberculosis and positive cultures identified as *M. tuberculosis* complex (MTC) were interviewed and enrolled during the study period. Strain isolation and identification were performed at the provincial reference laboratory of Hunan Chest Hospital. Only one isolate per patient was collected and tested.

DST. The drug susceptibility testing (DST) was performed using a Bactec MGIT 960 system in a national tuberculosis reference laboratory (NTRL). The critical concentrations for the DST were 0.1 μg/ml for INH, 1.0 μg/ml for RIF, 1.0 μg/ml for SM, 5.0 μg/ml for EMB, 100.0 μg/ml for PZA, 2.5 μg/ml for CAP, 2.5 μg/ml for KAN, 1.0 μg/ml for AKM, and 2.0 μg/ml for OFX (8).

Data collection and definitions. The demographic and clinical information of enrolled patients, including gender, age, occupation, address, complication, and TB treatment history, was obtained from the inpatient's records.

MDR-TB was defined as resistance to the two first-line drugs, INH and RIF. XDR-TB was defined as resistance to INH and RIF plus OFX and at least one of the three injectable second-line drugs (CAP, KAN, or AKM). Pre-XDR TB was defined as TB with resistance to INH and RIF and either OFX or a second-line injectable agent but not both.

Migrants were defined as individuals from other provinces of China or another country who moved to Hunan. Residents were defined as persons with a registered permanent residence in Hunan. New or previously treated TB cases were defined as previously described (9).

DNA extraction. Genomic DNAs were prepared by the cetyltrimethylammonium bromide (CTAB) method as described previously (10) and stored at −20°C for further use.

Spoligotyping and data analysis. Spoligotyping was performed using 43 covalently bound oligonucleotides derived from the spacer sequences of *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG P3 as previously described by Kamerbeek et al. (11). The results were entered in binary format into an Excel spreadsheet and compared with data in the SpolDB4 spoligotyping database (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp).

PCR amplification and sequencing. Expected fragments were amplified using primers listed in Table S1 in the supplemental material. Each 30-μl PCR mixture contained 15 μl 2× *Taq* master mix (TaKaRa), 1 μl of the forward and reverse 5 μM primers, 12 μl distilled H_2O, and 1 μl of genomic DNA. The reaction conditions consisted of a denaturation step of 5 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C, and a final extension step of 5 min at 72°C. PCR products were sent for sequencing. All sequence data were manipulated with BioEdit version 7.05.3 and were compared to the published sequences (GenBank accession number NC_000962).

Resolution of discrepant results. For the results that were discrepant between the DST and DNA sequencing, retesting was performed twice using both methods. If the retesting results were in conflict with the original data, a third round of testing was completed, with the final value representing two of the three cycles.

Statistical analysis. The data were analyzed with the use of SPSS software, 16.0 version. Frequencies, percentages, ranges, and confidence intervals (CI) were calculated as appropriate. A chi-square test or Fisher's exact test was used for intergroup comparison. A two-sided P value < 0.05 was considered statistically significant.

RESULTS

Demographic information. Among the 171 isolates, 129 (75.4%) were from male patients and 42 (24.6%) from female patients. The ages of the patients ranged from 15 to 88 years (mean ± standard error [SE], 44.8 ± 1.3). The majority (96.5% [165/171]) of them were Hunan residents, and 56.7% (97/171) were newly diagnosed cases.

Drug susceptibility patterns. In this study, 40.9% (95% CI, 33.6% to 48.3%) of the TB cases were resistant to at least one of the five first-line drugs (INH, RIF, SM, EMB, and PZA); 11.1% (95% CI, 6.4% to 15.8%) were resistant to at least one of the four second-line drugs (CAP, KAN, AKM, and OFX) (Table 1). The proportions of MDR, pre-XDR, and XDR isolates were 25.2% (95% CI, 18.6% to 31.7%), 8.7% (95% CI, 4.5% to 13.0%), and 1.8%, respectively (Table 1). In addition, the proportions of drug-resistant TB among new cases and previously treated cases are summarized in Table 1.

Factors linked to drug-resistant tuberculosis. Table 2 shows the results of analysis of risk factors for MDR and drug-resistant (but not MDR) tuberculosis among all the 171 patients. The previously treated patients presented significantly increased risks for developing drug resistance (but not MDR), with an odds ratio (OR) of 3.35 (95% CI, 1.40 to 8.03; $P = 0.01$). Furthermore, the risk increased highly significantly in MDR cases, with an OR of 6.17 (95% CI, 1.98 to 19.23; $P = 0.00$).

Genotyping results. Among 171 TB isolates, 126 (73.7%) belonged to the Beijing family, while 45 (26.3%) belonged to the non-Beijing family (see Table S2 in the supplemental material). Isolates classified into the non-Beijing family included 34 isolates (19.9%) from the T family, 4 (2.3%) from the U family, 1 (0.6%) from the CAS1-DELHI family, and 1 (0.6%) from the MANU2 family, with the remaining 5 being from the Orphan family (2.9%).

Drug susceptibility profiles of different genotypes. The drug susceptibility profiles of isolates belonging to the Beijing family and the non-Beijing family are compared in Table S3 in the supplemental material. The distribution of resistances to any drug was observed more frequently among Beijing family members than among non-Beijing family members, but there was no significant difference except for SM ($P < 0.05$).

Detection of drug resistance-associated mutations by DNA sequencing. DNA sequencing identified 78.7% (48/61) INH-resistant isolates, 87.0% (40/46) RIF-resistant isolates, 88.6% (31/35) SM-resistant isolates, 76.5% (13/17) EMB-resistant isolates, 84.6% (22/26) PZA-resistant isolates, 75.0% (3/4) CAP-resistant isolates, 100.0% (3/3) KAN- or (2/2) AKM-resistant isolates, and 77.8% (14/18) OFX-resistant isolates. All the mutated characteristics of drug-resistant isolates are summarized in Table S4 to Table S10 in the supplemental material. The major mutations were *katG* codon 315 ($katG_{315}$) (67.2%), *inhA15* (9.8%), $rpoB_{531}$ (47.8%), $rpoB_{526}$ (21.7%) and $rpoB_{516}$ (8.7%), $rpsL_{43}$ (71.4%), rrs_{514} (11.5%), $embB_{306}$ (70.6%), $pncA_{96}$ (11.5%), $rrs514_{401}$ (75.0% or 100.0%), and $gyrA_{94}$ (55.6%) and $gyrA_{90}$ (22.2%) (Table 3). In addition, no mutations were found within the target regions of *tlyA*, *eis*, and *gyrB* among the corresponding drug-resistant isolates.

DISCUSSION

The present study demonstrated that 41.5% (71/171) of patients with pulmonary TB had drug-resistant disease, suggesting a serious epidemic of drug-resistant tuberculosis among patients with pulmonary TB in Hunan. The proportions of MDR- and XDR-TB among patients were 25.2% and 1.8%, nearly three times the proportions presented in the data from a national baseline survey in 2007 (2). The reason for this obvious difference was probably that

TABLE 1 Drug susceptibility patterns of 171 clinical *M. tuberculosis* isolates

Susceptibility or resistance category or drug(s)	Total patients ($n = 171$)		New patients ($n = 97$)		Previously treated patients ($n = 74$)	
	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)
Overall susceptibility to first-line drugs	101	59.1 (52.0–66.4)	78	80.4 (72.5–88.3)	23	31.1 (20.5–41.6)
Overall first-line drug resistance	70	40.9 (33.6–48.3)	19	19.6 (11.7–27.5)	51	68.9 (58.4–79.5)
INH	61	35.7 (28.5–42.9)	13	13.4 (6.6–20.2)	48	64.9 (54.0–75.7)
RIF	46	26.9 (20.3–33.6)	7	7.2 (2.1–12.4)	39	52.7 (41.3–64.1)
SM	35	20.5 (14.4–26.5)	13	13.4 (6.6–20.2)	22	29.7 (19.3–40.1)
EMB	17	9.9 (5.5–14.4)	2	2.1[a]	15	20.3 (11.1–29.4)
PZA	26	15.2 (9.8–20.6)	3	3.1[a]	23	31.1 (20.5–41.6)
Overall MDR	43	25.2 (18.6–31.7)	6	6.2 (1.4–11.0)	37	50.0 (38.6–61.4)
INH + RIF	10	5.9 (2.3–9.4)	1	1.0[a]	9	12.1 (4.7–19.6)
INH + RIF + SM	7	4.1 (1.1–7.1)	2	2.1[a]	5	6.8[a]
INH + RIF + EMB	1	0.6[a]	0	0	1	1.4[a]
INH + RIF + PZA	7	4.1 (1.1–7.1)	0	0	7	9.5 (2.8–16.1)
INH + RIF + SM + EMB	3	1.8[a]	0	0	3	4.1[a]
INH + RIF + SM + PZA	4	2.3[a]	1	1.0[a]	3	4.1[a]
INH + RIF + EMB + PZA	4	2.3[a]	0	0	4	2.3[a]
INH + RIF + SM + EMB + PZA	7	4.1 (1.1–7.1)	2	2.1[a]	5	5.4[a]
Susceptibility to all second-line drugs	152	88.9 (84.2–93.6)	95	98.0[a]	57	77.0 (67.4–86.6)
Overall second-line drug resistance	19	11.1 (6.4–15.8)	2	2.1[a]	17	22.9 (13.4–32.6)
CAP	4	2.3[a]	0	0	4	5.4[a]
KAN	3	1.8[a]	0	0	3	4.1[a]
AKM	2	1.2[a]	0	0	2	2.7[a]
OFX	18	10.5 (5.9–15.1)	2	2.1[a]	16	21.6 (12.2–31.0)
Overall pre-XDR	15	8.7 (4.5–13.0)	1	1.0[a]	14	14.9 (6.8–23.0)
CAP	4	2.3[a]	0	0	4	1.4[a]
KAN	3	1.8[a]	0	0	3	1.4[a]
AKM	2	1.2[a]	0	0	2	0
OFX	14	8.2 (4.1–12.3)	1	1.0[a]	13	13.5 (5.7–21.3)
Overall XDR	3	1.8[a]	0	0	3	4.1[a]
CAP + OFX	1	0.6[a]	0	0	1	1.4[a]
CAP + KAN + AKM + OFX	2	1.2[a]	0	0	2	2.7[a]

[a] 95% CI was not determined.

the isolates of present study were obtained from the specialized hospital instead of from an epidemiological survey, resulting in a distinctly higher proportion of previously treated patients (43.3%) than in the data from the national baseline survey (22.7%). The proportions of drug-resistant TB in previously treated patients were considerably higher than those in new patients (2) and were confirmed by this study. A higher risk of drug resistance was also found among previously treated patients. Hence, some appropriate strategies must be implemented to increase continuity of treatment and reduce the rate of treatment default. Notably, a lower incidence of drug resistance in new patients was observed in this study than in the national baseline survey, implying that drug resistance in new cases varied greatly with the region and that the transmission of drug-resistant TB in Hunan is less extensive than in some other regions.

The percentages of second-line injectable drug resistance for CAP, KAN, and AKM were 2.3%, 1.8%, 1.2%, respectively, significantly lower than that of OFX (10.5%). These really low resistance levels were detected also in MDR-TB (9.3% for CAP, 7.0% for KAN, and 4.7% for AKM). All these results point out that the second-line injectable drug is excellent and represents the potential of a successful treatment against TB or MDR-TB. The high rate of OFX resistance in TB isolates, especially in MDR-TB isolates (32.6%), indicated that OFX has been used extensively in Hunan during the past years to treat drug-resistant TB patients and retreatment patients. In addition, among patients with MDR-TB, 34.9% (15/43) were pre-XDR TB, placing them only one step away from having XDR-TB.

The Beijing family was the most dominant genotype in Hunan province, which is in accordance with the findings from most areas of China (12–14). In addition to the Beijing family, other families, including T, H, MANU2, U, CAS1-DELHI, and Orphan, were also identified. Notably, only 1 strain belonged to the CAS1-DELHI family. This strain was isolated from a patient who was a student from Pakistan, a country with a predominance of examples of the CAS family (including CAS1, CAS subfamilies, and Orphan Pak clusters) (15).

Some publications showed that the Beijing family was associated with drug resistance (15, 16). However, lesser associations were reported also in other geographic settings (17, 18). Our study suggested that the association with drug resistance was significant only for SM, indicating that the Beijing family is

TABLE 2 Factors associated with drug-resistant tuberculosis[a]

Factor	No. (%) of isolates			Drug-resistant but not MDR TB vs pan-susceptible TB		MDR TB vs drug-resistant but not MDR TB	
	Pan-susceptible TB	Drug-resistant but not MDR TB	MDR TB	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Sex							
Male	76	20	33	Reference		Reference	
Female	24	8	10	1.27 (0.50–3.24)	0.62	0.76 (0.26–2.24)	0.62
Age group							
<30 yrs	27	8	6	Reference		Reference	
30–59 yrs	48	15	32	1.06 (0.40–2.81)	0.91	2.84 (0.84–9.67)	0.09
≥60 yrs	25	5	5	0.68 (0.20–2.34)	0.53	1.33 (0.26–681)	1.00
Occupation							
Farmer	53	19	29	Reference		Reference	
Others	47	9	14	0.53 (0.22–1.29)	0.16	1.02 (0.37–2.82)	0.97
Treatment							
New cases	77	14	6	Reference		Reference	
Previously treated cases	23	14	37	3.35 (1.40–8.03)	0.01*	6.17 (1.98–19.23)	0.00**

[a] CI, confidence interval. *, $P < 0.05$ (significant); **, $P < 0.01$ (highly significant).

less likely to be associated with the most drug-resistant strains in Hunan area.

DNA sequencing of the hot spot regions in genetic loci demonstrated that the frequency of mutations occurring among INH-resistant isolates was 78.7%, similar to the results reported in Jiangxi (19) but lower than the data from Fujian and Shanghai, China. For the RIF-resistant isolates in China, substantial proportions (90.2% to 97.2%) of mutations within the *rpoB* gene were observed (19–25). Our results showed that 87.0% of the RIF-resistant isolates carried mutations in the *rpoB* gene. We presumed it probable that this was due to the limited number of RIF-resistant isolates (46 isolates) analyzed in our study. Furthermore, the mutated profiles of *M. tuberculosis* isolates from different areas might have somewhat geographically related differences.

TABLE 3 Most frequently identified mutations within 12 drug-resistance-associated loci among drug-resistant *M. tuberculosis* isolates

Drug (no. of drug-resistant isolates)	Locus	Mutated position	No. (%) of isolates	Relative frequency (%)[a]
INH (61)	*katG*	315	41	67.2
	inhA	−15	6	9.8
RIF (46)	*rpoB*	531	22	47.8
		526	10	21.7
		516	4	8.7
SM (35)	*rpsL*	43	25	71.4
	rrs(388–1084)	514	4	11.5
EMB (17)	*embB*	306	12	70.6
PZA (26)	*pncA*	96	3	11.5
CAP (4)	*rrs*(1158–1674)	1401	3	75.0
	tlyA	None	NA[b]	NA
KAN (3)	*rrs*(1158–1674)	1401	3	100.0
	eis	None	NA	NA
AKM (2)	*rrs*(1158–1674)	1401	2	100.0
	eis	None	NA	NA
OFX (18)	*gyrA*	94	10	55.6
		90	4	22.2

[a] Relative-frequency data represent comparisons to the total number of isolates resistant to the drug of interest.
[b] NA, not applicable.

The mutation frequencies in SM-, EMB-, PZA-, and OFX-resistant isolates were 88.6%, 76.5%, 84.6%, and 77.8%, respectively, which were in agreement with the findings from many investigations (19, 26–28). However, the mutation frequencies in CAP- and KAN/AKM-resistant isolates (75.0% and 100.0%) were inconsistent with previous reports (19, 27–30). One possible reason was that the number of CAP- and KAN/AKM-resistant isolates investigated in our study was very small.

There were still some resistant isolates harboring no mutation within the sequenced regions. Although retesting was performed using DST and DNA sequencing, the retesting results were unchanged. This implied that these isolates probably harbored mutations outside the sequenced area or that the resistance may be caused by other mechanisms, such as efflux pumps (31). In addition, the TB cultures might also have comprised a mixed population of resistant and susceptible bacteria (32), and thus, ordinary PCR-based DNA sequencing could not detect the low proportion of drug-resistant TB strains among the predominant wild-type TB strains, leading to a falsely low detection rate.

The most common mutations among these drug-resistant isolates were $katG_{315}$, *inhA15*, $rpoB_{531}$, $rpoB_{526}$ and $rpoB_{516}$, $rpsL_{43}$, rrs_{514}, $embB_{306}$, rrs_{1401}, and $gyrA_{94}$ and $gyrA_{90}$, as previously reported (19, 27–30, 33). For the *pncA* gene, although codon 96 was the location of major mutation, it was found in only 3 PZA-resistant isolates (11.5%). Next were codons 120, 141, 142, and 177, which were identified in 2 PZA-resistant isolates each. Other mutations consisted of 11 unique alterations distributed throughout

the whole gene. These results supported the hypothesis that the mutations harbored by *pncA* are tremendously diverse and scattered along the whole gene (27, 34, 35). There was no mutation found within *tlyA*, *eis*, and *gyrB* conferring drug resistance in the present study, which was probably due to the limited number of resistant isolates corresponding to CAP, KAN/AKM, and OFX.

The limitations of the current study should be addressed. First, this study was carried out in only one specialized TB hospital in Hunan. Although this hospital is the largest specialized hospital for TB in the region, the results based on inpatients from only one hospital might not reflect the overall situation in the region. Then, the specialized hospital might have higher rates of serious TB cases than other hospitals in the region, probably leading to an overestimation of the incidence of drug-resistant tuberculosis. Next, the number of second-line drug-resistant isolates, especially for CAP-resistant ($n = 4$), KAN-resistant ($n = 3$), and AKM-resistant ($n = 2$) isolates, was relatively small in our research. This might have limited the detection of the variety of gene variations. Moreover, since the sequencing data for drug-susceptible isolates were not included in our study, the specificities of different resistance-related mutations were not evaluated. Therefore, additional studies that include a substantial panel of drug-resistant and -susceptible isolates will be required in the future.

In summary, our results indicated that the prevalences of INH, RIF, SM, EMB, PZA, CAP, KAN, AKM, and OFX resistance in Hunan province were 35.7%, 26.9%, 20.5%, 9.9%, 15.2%, 2.3%, 1.8%, 1.2%, and 10.5%, respectively. The majority of *M. tuberculosis* isolates belonged to the Beijing family. Almost all the drug resistance demonstrated no association with genotype. The most frequent mutations were $katG_{315}$, *inhA15*, $rpoB_{531}$, $rpoB_{526}$ and $rpoB_{516}$, $rpsL_{43}$, rrs_{514}, $embB_{306}$, $pncA_{96}$, rrs_{1401}, and $gyrA_{94}$ and $gyrA_{90}$. These results were very helpful to generate the appropriate TB control policy and establish the rapid molecular diagnostic methods which will be implemented in Hunan province and throughout China.

ACKNOWLEDGMENTS

We declare that we have no conflicts of interest.

This study was supported by the projects of National Natural Science Foundation of China (grant no. 81201348) and the National Key Program of Mega Infectious Diseases (grant no. 2013ZX10003002-001). The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. **Gandhi NR, Nunn P, Dheda K, Schaaf HS, Zignol M, van Soolingen D, Jensen P, Bayona J.** 2010. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. Lancet **375:**1830–1843. http://dx.doi.org/10.1016/S0140-6736(10)60410-2.
2. **Zhao Y, Xu S, Wang L, Chin DP, Wang S, Jiang G, Xia H, Zhou Y, Li Q, Ou X, Pang Y, Song Y, Zhao B, Zhang H, He G, Guo J, Wang Y.** 2012. National survey of drug-resistant tuberculosis in China. N. Engl. J. Med. **366:**2161–2170. http://dx.doi.org/10.1056/NEJMoa1108789.
3. **He GX, Zhao YL, Jiang GL, Liu YH, Xia H, Wang SF, Wang LX, Borgdorff MW, van der Werf MJ, van den Hof S.** 2008. Prevalence of tuberculosis drug resistance in 10 provinces of China. BMC Infect. Dis. **8:**166. http://dx.doi.org/10.1186/1471-2334-8-166.
4. **Afanas'ev MV, Ikryannikova LN, Il'ina EN, Sidorenko SV, Kuz'min AV, Larionova EE, Smirnova TG, Chernousova LN, Kamaev EY, Skorniakov SN, Kinsht VN, Cherednichenko AG, Govorun VM.** 2007. Molecular characteristics of rifampicin- and isoniazid-resistant Mycobacterium tuberculosis isolates from the Russian Federation. J. Antimicrob. Chemother. **59:**1057–1064. http://dx.doi.org/10.1093/jac/dkm086.
5. **Filliol I, Driscoll JR, Van Soolingen D, Kreiswirth BN, Kremer K, Valetudie G, Anh DD, Barlow R, Banerjee D, Bifani PJ, Brudey K, Cataldi A, Cooksey RC, Cousins DV, Dale JW, Dellagostin OA, Drobniewski F, Engelmann G, Ferdinand S, Gascoyne-Binzi D, Gordon M, Gutierrez MC, Haas WH, Heersma H, Kallenius G, Kassa-Kelembho E, Koivula T, Ly HM, Makristathis A, Mammina C, Martin G, Mostrom P, Mokrousov I, Narbonne V, Narvskaya O, Nastasi A, Niobe-Eyangoh SN, Pape JW, Rasolofo-Razanamparany V, Ridell M, Rossetti ML, Stauffer F, Suffys PN, Takiff H, Texier-Maugein J, Vincent V, De Waard JH, Sola C, Rastogi N.** 2002. Global distribution of Mycobacterium tuberculosis spoligotypes. Emerg. Infect. Dis. **8:**1347–1349. http://dx.doi.org/10.3201/eid0811.020125.
6. **Ahmed N, Alam M, Rao KR, Kauser F, Kumar NA, Qazi NN, Sangal V, Sharma VD, Das R, Katoch VM, Murthy KJ, Suneetha S, Sharma SK, Sechi LA, Gilman RH, Hasnain SE.** 2004. Molecular genotyping of a large, multicentric collection of tubercle bacilli indicates geographical partitioning of strain variation and has implications for global epidemiology of Mycobacterium tuberculosis. J. Clin. Microbiol. **42:**3240–3247. http://dx.doi.org/10.1128/JCM.42.7.3240-3247.2004.
7. **Ramazanzadeh R, Farnia P, Amirmozafari N, Ghazi F, Ghadertotonchi Z, Kamran J, Mohammadi F, Mirsaedi M, Masjedi M.** 2006. Comparison between molecular epidemiology, geographical regions and drug resistance in Mycobacterium tuberculosis strains isolated from Iranian and Afghan patients. Chemotherapy **52:**316–320. http://dx.doi.org/10.1159/000095971.
8. **WHO.** 2008. Policy guidance on TB drug susceptibility testing (DST) of second-line drugs. WHO/HTM/TB/2008392. WHO, Geneva, Switzerland.
9. **WHO Geneva/IUATLD Paris. International Union Against Tuberculosis and Lung Disease.** 1998. Guidelines for surveillance of drug resistance in tuberculosis. Int. J. Tuberc. Lung Dis. **2:**72–89.
10. **Somerville W, Thibert L, Schwartzman K, Behr MA.** 2005. Extraction of Mycobacterium tuberculosis DNA: a question of containment. J. Clin. Microbiol. **43:**2996–2997. http://dx.doi.org/10.1128/JCM.43.6.2996-2997.2005.
11. **Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M, van Embden J.** 1997. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J. Clin. Microbiol. **35:**907–914.
12. **Dong H, Liu Z, Lv B, Zhang Y, Liu J, Zhao X, Wan K.** 2010. Spoligotypes of Mycobacterium tuberculosis from different provinces of China. J. Clin. Microbiol. **48:**4102–4106. http://dx.doi.org/10.1128/JCM.00549-10.
13. **Liu Q, Yang D, Xu W, Wang J, Lv B, Shao Y, Song H, Li G, Dong H, Wan K, Wang H.** 2011. Molecular typing of Mycobacterium tuberculosis isolates circulating in Jiangsu province, China. BMC Infect. Dis. **11:**288. http://dx.doi.org/10.1186/1471-2334-11-288.
14. **Pang Y, Zhou Y, Zhao B, Liu G, Jiang G, Xia H, Song Y, Shang Y, Wang S, Zhao YL.** 2012. Spoligotyping and drug resistance analysis of Mycobacterium tuberculosis strains from national survey in China. PLoS One **7:**e32976. http://dx.doi.org/10.1371/journal.pone.0032976.
15. **Tanveer M, Hasan Z, Siddiqui AR, Ali A, Kanji A, Ghebremicheal S, Hasan R.** 2008. Genotyping and drug resistance patterns of M. tuberculosis strains in Pakistan. BMC Infect. Dis. **8:**171. http://dx.doi.org/10.1186/1471-2334-8-171.
16. **Ghebremichael S, Groenheit R, Pennhag A, Koivula T, Andersson E, Bruchfeld J, Hoffner S, Romanus V, Kallenius G.** 2010. Drug resistant Mycobacterium tuberculosis of the Beijing genotype does not spread in Sweden. PLoS One **5:**e10893. http://dx.doi.org/10.1371/journal.pone.0010893.
17. **Wang J, Liu Y, Zhang CL, Ji BY, Zhang LZ, Shao YZ, Jiang SL, Suzuki Y, Nakajima C, Fan CL, Ma YP, Tian GW, Hattori T, Ling H.** 2011. Genotypes and characteristics of clustering and drug susceptibility of Mycobacterium tuberculosis isolates collected in Heilongjiang Province, China. J. Clin. Microbiol. **49:**1354–1362. http://dx.doi.org/10.1128/JCM.02274-10.
18. **Alonso M, Alonso Rodriguez N, Garzelli C, Martinez Lirola M, Herranz M, Samper S, Ruiz Serrano MJ, Bouza E, Garcia de Viedma D.** 2010. Characterization of Mycobacterium tuberculosis Beijing isolates from the Mediterranean area. BMC Microbiol. **10:**151. http://dx.doi.org/10.1186/1471-2180-10-151.
19. **Yuan X, Zhang T, Kawakami K, Zhu J, Li H, Lei J, Tu S.** 2012. Molecular characterization of multidrug- and extensively drug-resistant Mycobacte-

rium tuberculosis strains in Jiangxi, China. J. Clin. Microbiol. **50:**2404–2413. http://dx.doi.org/10.1128/JCM.06860-11.

20. **Tang K, Sun H, Zhao Y, Guo J, Zhang C, Feng Q, He Y, Luo M, Li Y, Sun Q.** 2013. Characterization of rifampin-resistant isolates of Mycobacterium tuberculosis from Sichuan in China. Tuberculosis (Edinb) **93:**89–95. http://dx.doi.org/10.1016/j.tube.2012.10.009.
21. **Yue J, Shi W, Xie J, Li Y, Zeng E, Wang H.** 2003. Mutations in the rpoB gene of multidrug-resistant Mycobacterium tuberculosis isolates from China. J. Clin. Microbiol. **41:**2209–2212. http://dx.doi.org/10.1128/JCM.41.5.2209-2212.2003.
22. **Zhao LL, Chen Y, Liu HC, Xia Q, Wu XC, Sun Q, Zhao XQ, Li GL, Liu ZG, Wan KL.** 2014. Molecular characterization of multidrug-resistant Mycobacterium tuberculosis isolates from China. Antimicrob. Agents Chemother. **58:**1997–2005. http://dx.doi.org/10.1128/AAC.01792-13.
23. **Yu XL, Wen ZL, Chen GZ, Li R, Ding BB, Yao YF, Li Y, Wu H, Guo XK, Wang HH, Zhang SL.** 11 December 2013. Molecular characterization of multidrug-resistant Mycobacterium tuberculosis isolated from South-central in China. J. Antibiot. (Tokyo) http://dx.doi.org/10.1038/ja.2013.133.
24. **Luo T, Zhao M, Li X, Xu P, Gui X, Pickerill S, DeRiemer K, Mei J, Gao Q.** 2010. Selection of mutations to detect multidrug-resistant Mycobacterium tuberculosis strains in Shanghai, China. Antimicrob. Agents Chemother. **54:**1075–1081. http://dx.doi.org/10.1128/AAC.00964-09.
25. **Chan RC, Hui M, Chan EW, Au TK, Chin ML, Yip CK, AuYeang CK, Yeung CY, Kam KM, Yip PC, Cheng AF.** 2007. Genetic and phenotypic characterization of drug-resistant Mycobacterium tuberculosis isolates in Hong Kong. J. Antimicrob. Chemother. **59:**866–873. http://dx.doi.org/10.1093/jac/dkm054.
26. **Zhu C, Zhang Y, Shen Y, Siu GK, Wu W, Qian X, Deng G, Xu Y, Lau R, Fan X, Zhang W, Lu H, Yam WC.** 2012. Molecular characterization of fluoroquinolone-resistant Mycobacterium tuberculosis clinical isolates from Shanghai, China. Diagn. Microbiol. Infect. Dis. **73:**260–263. http://dx.doi.org/10.1016/j.diagmicrobio.2012.03.025.
27. **Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, Hooks DP, Cowan LS, Plikaytis BB, Posey JE.** 2011. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **55:**2032–2041. http://dx.doi.org/10.1128/AAC.01550-10.
28. **Jnawali HN, Hwang SC, Park YK, Kim H, Lee YS, Chung GT, Choe KH, Ryoo S.** 2013. Characterization of mutations in multi- and extensive drug resistance among strains of Mycobacterium tuberculosis clinical isolates in Republic of Korea. Diagn. Microbiol. Infect. Dis. **76:**187–196. http://dx.doi.org/10.1016/j.diagmicrobio.2013.02.035.
29. **Du Q, Dai G, Long Q, Yu X, Dong L, Huang H, Xie J.** 2013. Mycobacterium tuberculosis rrs A1401G mutation correlates with high-level resistance to kanamycin, amikacin, and capreomycin in clinical isolates from mainland China. Diagn. Microbiol. Infect. Dis. **77:**138–142. http://dx.doi.org/10.1016/j.diagmicrobio.2013.06.031.
30. **Hu Y, Hoffner S, Wu L, Zhao Q, Jiang W, Xu B.** 2013. Prevalence and genetic characterization of second-line drug-resistant and extensively drug-resistant Mycobacterium tuberculosis in rural China. Antimicrob. Agents Chemother. **57:**3857–3863. http://dx.doi.org/10.1128/AAC.00102-13.
31. **da Silva PE, Von Groll A, Martin A, Palomino JC.** 2011. Efflux as a mechanism for drug resistance in Mycobacterium tuberculosis. FEMS Immunol. Med. Microbiol. **63:**1–9. http://dx.doi.org/10.1111/j.1574-695X.2011.00831.x.
32. **Baldeviano-Vidalón GC, Quispe-Torres N, Bonilla-Asalde C, Gastiaburú-Rodriguez D, Pro-Cuba JE, Llanos-Zavalaga F.** 2005. Multiple infection with resistant and sensitive M. tuberculosis strains during treatment of pulmonary tuberculosis patients. Int. J. Tuberc. Lung Dis. **9:**1155–1160.
33. **Almeida Da Silva PE, Palomino JC.** 2011. Molecular basis and mechanisms of drug resistance in Mycobacterium tuberculosis: classical and new drugs. J. Antimicrob. Chemother. **66:**1417–1430. http://dx.doi.org/10.1093/jac/dkr173.
34. **Barco P, Cardoso RF, Hirata RD, Leite CQ, Pandolfi JR, Sato DN, Shikama ML, de Melo FF, Mamizuka EM, Campanerut PA, Hirata MH.** 2006. pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis clinical isolates from the southeast region of Brazil. J. Antimicrob. Chemother. **58:**930–935. http://dx.doi.org/10.1093/jac/dkl363.
35. **Mphahlele M, Syre H, Valvatne H, Stavrum R, Mannsaker T, Muthivhi T, Weyer K, Fourie PB, Grewal HM.** 2008. Pyrazinamide resistance among South African multidrug-resistant Mycobacterium tuberculosis isolates. J. Clin. Microbiol. **46:**3459–3464. http://dx.doi.org/10.1128/JCM.00973-08.