Mixed Infections and Rifampin Heteroresistance among *Mycobacterium tuberculosis* Clinical Isolates

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Mixed infections and heteroresistance of *Mycobacterium tuberculosis* contribute to the difficulty of diagnosis, treatment, and control of tuberculosis. However, there is still no proper solution for these issues. This study aimed to investigate the potential relationship between mixed infections and heteroresistance and to determine the high-risk groups related to these factors. A total of 499 resistant and susceptible isolates were subjected to spoligotyping and 24-locus variable-number tandem repeat methods to analyze their genotypic lineages and the occurrence of mixed infections. Two hundred ninety-two randomly selected isolates were sequenced on their *rpoB* gene to examine mutations and heteroresistance. The results showed that 12 patients had mixed infections, and the corresponding isolates belonged to Manu2 ($n = 8$), Beijing ($n = 2$), T ($n = 1$), and unknown ($n = 1$) lineages. Manu2 was found to be significantly associated with mixed infections (odds ratio, 47.72; confidence interval, 9.68 to 235.23; $P < 0.01$). Four isolates (1.37%) were confirmed to be heteroresistant, which was caused by mixed infections in three (75%) isolates; these belonged to Manu2. Additionally, 3.8% of the rifampin-resistant isolates showing no mutation in the *rpoB* gene were significantly associated with mixed infections (χ^2, 56.78; $P < 0.01$). This study revealed for the first time that Manu2 was the predominant group in the cases of mixed infections, and this might be the main reason for heteroresistance and a possible mechanism for isolates without any mutation in the *rpoB* gene to become rifampin resistant. Further studies should focus on this lineage to clarify its relevance to mixed infections.

Mixed *Mycobacterium tuberculosis* infections were recognized as early as 1975 ([1]), and they continue to cause an increase in the false-negative rate of drug resistance testing obtained by both drug susceptibility tests (DST) and GeneXpert ([2–4]). Mixed infections can assist *M. tuberculosis* strains in acquiring additional mutations, facilitate the spread of drug-resistant strains, and boost the rate of treatment failure ([3, 5]). With the popularization of genetic analysis tools, the situation in which a single patient gets infected with more than one *M. tuberculosis* isolate was found to be more common than we previously expected ([6]). Currently, mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR)-based methods have been the most widely used to detect mixed infections ([5]). Mixed infections are defined by the presence of strains with different MIRU-VNTR patterns at two or more loci in the same sputum, lymph, or other sample while having clonal heterogeneity with a different MIRU-VNTR pattern at a single locus ([7–9]).

In some cases, quickly acquired drug resistance may be caused by misdiagnosis of mixed infections or heteroresistance ([3, 10, 11]). However, there are only very few publications on heteroresistance ([11]), which is defined as the coexistence of susceptible and resistant bacteria in the same sample ([12]). Heteroresistance, as another confounding factor in diagnosis and treatment, is more difficult to detect by the phenotypic DST method from the preliminary stage to full resistance ([11, 12]). In comparison, DNA sequencing, a robust method for analyzing sequence variants, with results showing dual peaks in the *rpoB* gene region, can be regarded as the gold standard to determine rifampin heteroresistance ([11]). There are two mechanisms for heteroresistance, one

being the mixed infection, and the other being the splitting of a single strain into susceptible and resistant clones due to evolution.

The diagnosis of mixed infection is expensive and labor-intensive, since it requires the lineages of single colonies to be identified individually from the original culture ([6]). In the cases of mixed infections, the usefulness of spoligotyping might be compromised, as a spoligotype pattern may reflect the cumulative spacers of all strains present or that of the dominant strain ([7]). However, if we could confirm certain spoligotypes whose existence was most probably related to mixed infection, it would be easier to investigate and control this situation ([6]). A computer program has been designed to investigate some "troublesome" spoligotype lineages by generating relative occurrences of one spoligotype being the combination of two known spoligotypes, but the results still need to be confirmed by MIRU-VNTR or other methods ([6]). To date,

only one study using an in-house PCR-based method to detect some defined lineages was able to determine whether certain strain lineages were prone to cause mixed infections (10). However, this method did not determine the extent of mixed infections with different strains of the same lineage or other undefined strain lineages whose primers were not included, such as Manu.

Mixed infections and heteroresistance may be particularly common in regions with a high rate of tuberculosis (TB), especially drug-resistant TB (5, 6, 11). Sichuan is the province with the second-largest number of TB cases in China, where the prevalence of drug-resistant TB is much higher than the average level observed in eastern China (13). Therefore, the objectives of this study were to map the prevalence of mixed infections and heteroresistance of *M. tuberculosis* in Sichuan and their potential correlation, and to investigate the group with a high risk of mixed infections and heteroresistance.

MATERIALS AND METHODS

Study design and isolates. A retrospective cross-section study was conducted between January 2008 and March 2011. A total of 5,090 *M. tuberculosis* clinical isolates, including 3,356 (65.9%) pansusceptible isolates and 1,734 (34.1%) drug-resistant isolates, were collected from 5,090 pulmonary tuberculosis patients coming mainly from Sichuan Province. These patients were diagnosed at the Chengdu Public Health Clinical Center, the only professional antituberculosis hospital in Sichuan. Among the 1,734 resistant isolates, 415 were randomly selected, 82 isolates from the 3,356 pansusceptible isolates were selected, and 2 isolates were without any clinical information were selected; in total, 499 isolates were selected, with one isolate per patient.

Clinical data, including treatment time, gender, age, and nationality, were obtained from the medical records of the subjects without collecting private information about the patients or disclosing information about the patients to any commercial agency. This study was approved by the Medical Ethics Committee of Chengdu Public Health Clinical Center. All patients included in this study provided written informed consent for the use of clinical samples. The results of this study did not influence patient treatment in any way.

Drug susceptibility. Samples were collected and disposed of in accordance with WHO guidelines. Briefly, strains were cultured on Lowenstein-Jensen (LJ) slants at 37°C, and *M. tuberculosis* complex isolates were identified using standard biochemical methods, such as susceptibility to *p*-nitrobenzoic acid (PNB) and 2-thiopnene carboxylic acid hydrazide (TCH), pyrazinamidase activity (PZA), nitrate reduction, and niacin production. DST was done using a proportion method with 10 mg/ml streptomycin (STR), 0.2 mg/ml isoniazid (INH), 40 mg/ml rifampin (RIF), and 2 mg/ml ethambutol (EMB), as described elsewhere (2, 14, 15). The DST was repeated for all samples showing discordant results between phenotypic DST and mutation analysis.

DNA extract and genotyping. Genomic DNA from clinical isolates was extracted using the cetyltrimethylammonium bromide (CTAB) method (16). Spoligotyping was performed as described by Kamerbeek et al. (17). For MIRU-VNTRs, all 24 loci were amplified with the corresponding primers, as described by Supply et al. (18).

To avoid laboratory cross-contamination, standard precautions included physical separation of pre- and post-PCR areas, a separate environment control, and unidirectional workflow. Other precautionary measures included the use of dedicated consumables and equipment (including aerosol-resistant pipettes, use of the proper pipetting technique, and fresh gloves used in a PCR area), aseptic cleaning carried out periodically before and after PCR work, and periodic wipe tests. Lastly, only a limited number of samples were processed at one time, and DNA from *M. tuberculosis* strain H37Rv and deionized water was added as positive and negative controls, respectively, for each PCR. The PCR was repeated three

times if the sample showed more than one band at any MIRU-VNTR locus in order to confirm the result.

PCR of *rpoB* and sequencing. PCR procedures and two oligonucleotide primers for *rpoB* were used as described by Tang et al. (19). The amplicons were purified with QIAquick PCR purification kits (Qiagen, United Kingdom), according to the manufacturer's instructions. Direct sequencing of the PCR products was carried out with an ABI Prism 377 automated DNA sequencer (Applied Biosystems) and BigDye Terminator cycle sequencing kit (ABI Prism), according to the instructions provided by the manufacturer. The sequencing process was repeated three times to confirm the results from patients with mixed infections or heteroresistance.

Statistical analysis. The analysis of PCR fragment size and the assignment of the various VNTR alleles were achieved using the Quantity One (version 4.6.2) software (Bio-Rad Laboratories). The Hunter-Gaston discriminatory index (HGDI) was calculated as described previously (20). The spoligotypes and MIRU-VNTR patterns were compared using the SITVIT2 proprietary database of the Institut Pasteur de la Guadeloupe, which is an updated version of the previously released SITVIT WEB database (21) (see http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/).

Univariate logistic regression analysis was performed to identify potential influencing factors from patient and bacterial variables associated with mixed infections. Odds ratios (OR) and 95% confidence intervals (CI) were examined. A P value of <0.05 was considered statistically significant. Pearson's chi-square exact test (two-tailed) was used to compare the differences. The data were analyzed using the Stata statistical software (version 12; Stata Corporation, College Town, TX, USA).

The sequences were analyzed for the presence of single-nucleotide polymorphisms using the Phred/Phrap/PolyPhred/Consed software package (http://droog.gs.washington.edu/PolyPhred.html). The analytical results of *rpoB* sequencing were compared with *M. tuberculosis* H37Rv (GenBank accession no. NC_000962).

RESULTS

Diversity of *M. tuberculosis* by spoligotyping. Based on the spoligotyping results, 22 orphan patterns and 61 shared types (*n* = 477 isolates) were distributed in seven lineages from a total of 499 isolates of *M. tuberculosis* in Sichuan, China (Table 1). The Beijing family was the predominant group, representing 69.74% (*n* = 348) of all isolates, followed by the T family (*n* = 83 [16.63%]) and Manu2 (*n* = 37 [7.41%]). Eighteen (3.61%) isolates showed unknown patterns that were not assigned to any known major lineages in the SITVIT2 database, and 13 (2.61%) belonged to other minor lineages. Among the 22 orphan patterns, 20 (90.9%) belonged to unknown (*n* = 5 [33.33%]), T (*n* = 7 [8.43%]), and Manu2 (*n* = 8 [21.62%]) lineages.

Mixed infections and influencing factors. Twelve (2.4%) of the 499 isolates were identified as mixed infections by 17 relevant loci of the standard 24-locus set, and the DST profiles of the 12 corresponding isolates are shown in Table 2. Using spoligotyping, isolates with mixed infections appeared in four lineages, including Manu2 (8/37 [21.62%]), Beijing (2/348 [0.57%]), T (1/83 [1.2%]), and unknown (1/18 [5.56%]). Eight of 12 (66.67%) isolates from patients with mixed infections belonged to the Manu2 lineage. Univariate logistic regression analysis showed that mixed infections were only significantly associated with Manu2 (OR, 47.72; P < 0.01; Table 3). Although there were no other bacterial or host variables (drug susceptibility, gender, age, or nationality) that were statistically significant (Table 3), a higher rate of mixed infection was shown in samples from patients of Han nationality (2.57%) and in drug-resistant isolates (2.65%) than those from minority patients (1.45%) and in susceptible isolates (1.22%), respectively. The 12 patients with mixed infections consisted of one

TABLE 1 Distribution of mixed infections in 61 SITs (n = 477 isolates) and 22 orphan isolates with corresponding genotypes defined by spoligotyping[a]

SIT	Spoligotype description	Octal no.	Total no. (%) of isolates in this study	% in study vs database	No. (% in the SIT) of mixed infections in this study	Clade
1	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	000000000003771	324 (64.93)	3.11	2 (0.62)	Beijing
26	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	703777740003771	1 (0.2)	0.06	0 (0)	CAS1-Delhi
37	■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777377777760771	1 (0.2)	0.19	0 (0)	T3
42	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	777777607760771	1 (0.2)	0.03	0 (0)	LAM9
44	■■■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■□□□□■■■■■■■	777777757760771	1 (0.2)	0.46	0 (0)	T5
50	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	777777777720771	3 (0.6)	0.08	0 (0)	H3
52	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	777777777760731	10 (2)	1.05	0 (0)	T2
53	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777777760771	13 (2.61)	0.2	0 (0)	T1
54	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■	777777777763771	12 (2.4)	4.53	4 (33.33)	Manu2
118	■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777767777760771	1 (0.2)	0.61	0 (0)	T1
154	■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	757777777760771	1 (0.2)	0.88	0 (0)	T1
159	■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■■□□□□■■■■■■■	777740017760771	1 (0.2)	1.32	0 (0)	Unknown
189	■■■■■■■■■■■■■□□□□■■■■■■■■■■■■■■■□□□□■■■■■■■	777741777760771	1 (0.2)	7.69	0 (0)	T1
190	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■□■■■	000000000003731	4 (0.8)	2.17	0 (0)	Beijing
191	□□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	177777777760771	1 (0.2)	4.76	0 (0)	T1
196	■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	677777777760771	1 (0.2)	1.2	0 (0)	T1
250	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■	000000000000371	1 (0.2)	2.63	0 (0)	Beijing
265	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■□■■■■■■	000000000003371	1 (0.2)	1.23	0 (0)	Beijing
269	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■	000000000000771	1 (0.2)	1.08	0 (0)	Beijing
278	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■□■	777777777760761	1 (0.2)	8.33	0 (0)	T1
291	■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■□□□□■■■■■■■	777777677760771	1 (0.2)	1.11	0 (0)	T1
294	■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	577777777720771	3 (0.6)	9.09	0 (0)	H3
334	■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	577777777760771	18 (3.61)	16.22	0 (0)	T1
393	■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777757777760771	4 (0.8)	10	1 (25)	T1
462	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■■■□□□□■■■■■■■	777777777560771	1 (0.2)	1.72	0 (0)	T1
511	■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□■□□□□■■■■■■■	777777700020771	2 (0.4)	4.26	0 (0)	H3
583	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■	777737777763771	1 (0.2)	33.33	0 (0)	Manu2
616	■□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	400000000003771	1 (0.2)	9.09	0 (0)	Unknown
621	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■□■■■■■■■	000000000002771	3 (0.6)	8.33	0 (0)	Beijing
632	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■□■■■■■	000000000003571	6 (1.2)	20.69	0 (0)	Beijing
780	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□■■■■■■■	777777777600771	1 (0.2)	7.14	0 (0)	Unknown
796	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	000000000001771	1 (0.2)	9.09	0 (0)	Beijing
803	■■■■■■■■■■■■■□□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	777740007760771	1 (0.2)	2.94	0 (0)	LAM9
804	■□□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	477777777760771	1 (0.2)	3.85	0 (0)	T1
834	■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■□□□□□■■■■■■■	777767777740771	1 (0.2)	12.5	0 (0)	T1
848	■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	737777777760731	1 (0.2)	3.23	0 (0)	T2
1096	■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■	577777777763771	15 (3.01)	66.67	3 (20)	Manu2
1122	■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	767777777760771	1 (0.2)	1.67	0 (0)	T1
1168	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■□□■■■	000000000003631	1 (0.2)	14.29	0 (0)	Beijing
1302	■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	577777777760731	4 (0.8)	16.67	0 (0)	T2
1451	■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□□□□□□□□□□□□□□	777760000000000	1 (0.2)	7.14	0 (0)	Unknown
1475	■■■■■■■■■□■■■□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777357777760771	1 (0.2)	11.11	0 (0)	T1
1578	■■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	767777777760731	1 (0.2)	14.29	0 (0)	T2
1674	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■□■	000000000003761	1 (0.2)	14.29	0 (0)	Beijing
1690	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■□■■■■■■■	777777777762771	1 (0.2)	25	0 (0)	Manu2

SIT	Spoligotype pattern	Octal code	n (%)	%	n (%)	Lineage
1890	(pattern)	7777777707760731	1 (0.2)	9.09	0 (0)	T2
2276	(pattern)	7777777777743771	2 (0.4)	50	1 (50)	Unknown
2979	(pattern)	0000000000000171	2 (0.4)	22.22	0 (0)	Beijing
3199	(pattern)	7717777777760731	1 (0.2)	33.33	0 (0)	T2
3233	(pattern)	7003777777760731	3 (0.6)	100	0 (0)	T2
3234	(pattern)	7777777777760371	2 (0.4)	50	0 (0)	Unknown
3235	(pattern)	6077777760060731	2 (0.4)	100	0 (0)	T2
3236	(pattern)	0000000000002031	1 (0.2)	50	0 (0)	Beijing
3237	(pattern)	7777777777400011	1 (0.2)	50	0 (0)	Unknown
3238	(pattern)	7777777777740300	2 (0.4)	100	0 (0)	T1
3239	(pattern)	7777600000000731	1 (0.2)	50	0 (0)	Unknown
3240	(pattern)	7616777777760731	1 (0.2)	50	0 (0)	T2
3241	(pattern)	0000000000003600	2 (0.4)	50	0 (0)	Beijing
3309*	(pattern)	7773777777760571	1 (0.2)	20	0 (0)	T2-Uganda
4011*	(pattern)	0000000200003771	2 (0.4)	100	0 (0)	Unknown
4012*	(pattern)	7777777770337771	1 (0.2)	50	0 (0)	Unknown
Orphan	(pattern)	7777777704303771	1 (0.2)	100	0 (0)	Unknown
Orphan	(pattern)	6000000000000011	1 (0.2)	100	0 (0)	Mungi
Orphan	(pattern)	7777400076060571	1 (0.2)	100	0 (0)	LAM9
Orphan	(pattern)	6677777776360771	1 (0.2)	100	0 (0)	T1
Orphan	(pattern)	7674000376060771	1 (0.2)	100	0 (0)	T1
Orphan	(pattern)	7775757777760531	1 (0.2)	100	0 (0)	T2
Orphan	(pattern)	4774777777760731	1 (0.2)	100	0 (0)	T2
Orphan	(pattern)	4777777777763771	1 (0.2)	100	1 (100)	Manu2
Orphan	(pattern)	5761717777760731	1 (0.2)	100	0 (0)	T2
Orphan	(pattern)	1777777777760700	1 (0.2)	100	0 (0)	T1
Orphan	(pattern)	5773777777763771	1 (0.2)	100	0 (0)	Manu2
Orphan	(pattern)	1423163777703771	1 (0.2)	100	0 (0)	Unknown
Orphan	(pattern)	0073770770370 3771	1 (0.2)	100	0 (0)	Unknown
Orphan	(pattern)	5770000000023771	1 (0.2)	100	0 (0)	Manu2
Orphan	(pattern)	0000000000017571	1 (0.2)	100	0 (0)	Unknown
Orphan	(pattern)	7777003003633771	1 (0.2)	100	0 (0)	Manu2
Orphan	(pattern)	1777003700003771	1 (0.2)	100	0 (0)	Unknown
Orphan	(pattern)	1777677777763771	1 (0.2)	100	0 (0)	Manu2
Orphan	(pattern)	5777777776363771	1 (0.2)	100	0 (0)	Manu2
Orphan	(pattern)	5777777777760401	1 (0.2)	100	0 (0)	T1
Orphan	(pattern)	4017777777763771	1 (0.2)	100	0 (0)	Manu2
Orphan	(pattern)	5776400077763771	1 (0.2)	100	0 (0)	Manu2

[a] SIT, spoligotype international shared type. A total of 58/61 SITs matched a preexisting shared type in the database, whereas 3/61 SITs were newly created either within the present study or after a match with an orphan in the database. A total of 22 SITs containing 438 isolates were clustered within this study (2 to 324 isolates per cluster), while 39 SIT plus 22 orphan (i.e., 61) isolates were unique. Note that SITs followed by an asterisk indicate a newly created shared type (n = 3 containing 4 isolates) due to ≥2 isolates belonging to an identical new pattern within this study or after a match with an orphan in the database. SIT 3309*—this study, n = 1; Colombia, n = 1; Ethiopia, n = 2. SIT 4011*—this study, n = 2. SIT 4012*—this study, n = 1; Brazil, n = 1.

TABLE 2 Genotyping and drug susceptibility test results of isolates from patients with mixed infections in the present study

| ID (no. of polymorphic alleles)[a] | DST result[b] | Spoligotype | VNTR locus (no. of isolates with polymorphic alleles; HGDI)[c] | | | | | | | | | | | | | | | | |
			T (5; 0.822)	U (4; 0.813)	F (2; 0.789)	O (5; 0.785)	H (3; 0.768)	N (3; 0.761)	I (2; 0.754)	V (2; 0.684)	C (5; 0.680)	D (2; 0.646)	J (6; 0.645)	G (1; 0.608)	K (3; 0.495)	Q (6; 0.408)	L (5; 0.260)	E (1; 0.221)	R (1; 0.113)
SC107 (2)	INH[r], RIF[r], STR[r]	Beijing	4	5, 6	7, 8	5	4	4	3	0	3	2	3	3	4	4	2	5	3
SC240 (2)	INH[r], STR[r]	Manu2	4	8	6	5	4	1, 3	3	1	3	3	3	3	4	2, 4	2	5	3
SSC0588 (2)	S	T1	0	9	4	1	3	3	2	ND	2	3	2	2	3, 4	2	2	5	3, 4
SC081 (2)	INH[r], RIF[r], EMB[r], STR[r]	Unknown	6	6, 8	8	6	3, 5	2	2	1	2	2	3	2	4	4	4	5	3
SSC0617 (3)	STR[r]	Beijing	6	2	6	4	3	4	3	ND	3	3	2, 3	3	4	2, 4	2	4, 6	3
SC023 (5)	INH[r], STR[r]	Manu2	4, 5	7, 8	7	7	4	3, 6	3	2	1, 3	3	5	2	3	2	1, 2	5	3
SSC0620 (5)	RIF[r]	Manu2	5, 6	5	7	4, 5	5	4	3	1, 2	3	2, 3	2, 4	2	4	4	2	5	3
SSCM034 (5)	INH[r], RIF[r], STR[r]	Manu2	5	ND	4, 7	5, 7	ND	1	2, 3	2	2	ND	2, 3	2, 3	4	4	1, 2	ND	3
SSC0619 (7)	INH[r]	Manu2	1, 3	4, 8	2	3	3	2	3	2	2, 4	1, 4	2, 4	2	2	2, 5	1, 2	5	3
SSC0407 (7)	INH[r], RIF[r], STR[r]	Manu2	3, 5	8	5	3, 6, 8	3	0	1	1	1, 2	2	3, 4	2	3, 4	1, 2	1, 2, 3	7	3
SC262 (7)	INH[r], RIF[r], EMB[r], STR[r]	Manu2	4, 6	8	5	3, 6	3, 5	2	2	1	1, 3	4	3	2	3, 4	2, 5	1, 2	5	3
SC187 (9)	RIF[r], EMB[r]	Manu2	5	7	4	2, 3	2, 5	1, 3	1, 2, 4	1, 3	1, 2, 3	2	3, 5	2	1	2, 4	1, 2, 3	5	3

[a] ID, patient number.
[b] DST, drug susceptibility test; RIF[r], resistant to rifampin; INH[r], resistant to isoniazid; STR[r], resistant to streptomycin; S, pansusceptible; EMB[r], resistant to ethambutol.
[c] VNTR loci: C = 960, D = 1644, E = 2531, F = 2996, G = 3007, H = 3192, I = 4348, J = 802, K = 2165, L = 2461, N = 424, O = 1955, Q = 2401, R = 3171, T = 2163b, U = 4052, V = 4156. HGDI, Hunter-Gaston discriminatory index (the order of the VNTR locus was listed according to the HGDI from high to low); ND, not determined.

young patient (0.85%), nine adults (2.8%), and two older patients (3.45%).

Mutation analysis and heteroresistance. The randomly selected 292 isolates (78 RIF susceptible and 212 RIF resistant) from 499 isolates were sequenced on their *rpoB* gene, of which 218 isolates (74.66%) had mutations in their amplified region (see Table S1 in the supplemental material). When the phenotypic DST results were compared against the results of *rpoB* gene sequencing, 20 results were discordant (Table 4). Twelve (5.5%) of the 218 isolates were RIF susceptible, including two isolates of mixed infections (*M. tuberculosis* SC023 and SSC0588), and eight (10.81%) of the other 74 isolates with no mutation detected were RIF resistant, including four isolates of mixed infections (*M. tuberculosis* SSCM034, SC262, SSC0407, and SC187). Among the six isolates with mixed infections as described above, which had discordant results between their DST and mutation analysis, three were RIF heteroresistant and belonged to the Manu2 lineage, and their corresponding patients were infected with SC187, SC262, and SC023. Another RIF-heteroresistant isolate from a patient (SC222) had evidence of clonal heterogeneity (data not shown). In total, there were four isolates (1.37%) confirmed to be RIF heteroresistant, which had coexisting wild-type (WT) segments and mutations in the *rpoB* gene region, according to the sequencing results (Fig. 1).

DISCUSSION

The rates of mixed infections were first shown to be 3.4 to 14.1% by phage typing (1, 22) and were later confirmed to be 0.4 to 15.4% using DNA fingerprint analysis (23, 24) or PCR-based approaches in which primers were specific at the level of lineage only (3, 10). The rates of mixed infections ranged from 2.1 to 57.9% by 7 to 24 loci in MIRU-VNTR-based analysis in different regions (Table 5), and the rates of clonal heterogeneity were 1.3 to 9.3% (9, 25–27). One may speculate that the differences in the proportions of mixed infections are caused not only by differences in the methodology of detection, but they also might vary by geographic region. According to the available literature, there was no link between the detection rate of mixed infections and the discriminatory power of the loci (Table 5). Recently, 12% of mixed *M. tuberculosis* infections were identified by whole-genome sequencing (28); however, this method is limited by the cost, complexity of data analysis, and the short sequencing reads used to account for repeat regions (5). Mixed Beijing and non-Beijing strains caused the most pathogen-pathogen compatibility (10, 14, 29, 30); however, the mixed non-Beijing strains were also reported to be primary in some regions (12, 31). Besides, although Beijing strains were commonly present in mixed infections, their spoligotypes usually were masked by the spoligotypes of non-Beijing strains (14). Therefore, there might be some specific properties of the non-Beijing lineage that allow the stable coexistence of mixed strains of *M. tuberculosis* in a single host.

Similar to findings in previous studies (see Table 5), non-Beijing spoligotype strains were significantly associated with mixed infections to a greater extent than Beijing ones (6.62% versus 0.57%; χ^2, 16.41; $P < 0.01$) in Sichuan. Moreover, the prevalence of the non-Beijing family (30.26%) in this region was much higher than that in three provinces neighboring Sichuan to the north, including Tibet (9.62%), Gansu (12.5%), and Shanxi (20%) (32). Further statistical analysis revealed that Manu2 was the most likely lineage related to mixed infections in our study, and this lineage was also related to the orphan pattern (χ^2, 28.09; $P < 0.01$). These

TABLE 3 Potential factors associated with mixed infections by the univariate logistic analysis[a]

Variable	Total no. of cases	No. of mixed infections	% of mixed infections	Univariate analysis[b]		
				OR	95% CI	*P* value
Patient						
Gender						
Male	327	8	2.45	1.04	0.31–3.51	0.949
Female	170	4	2.35			
Nationality						
Han	428	11	2.57			
Minority	69	1	1.45	0.56	0.07–4.39	0.579
Age (yr)						
Young (≤25)	117	1	0.85			
Adult (25–55)	321	9	2.80	3.35	0.42–26.70	0.254
Old (≥55)	58	2	3.45	4.14	0.37–46.66	0.250
Bacterial						
Drug susceptibility						
Pansusceptible	82	1	1.22			
Resistant[c]	415	11	2.65	2.14	0.27–16.81	0.469
Spoligotype						
Beijing	348	2	0.57			
Manu2	37	8	21.62	47.72	9.68–235.23	0.000
Others	114	2	1.75	3.09	0.43–22.19	0.262

[a] The analysis was performed on 496 isolates of the total 499, as the clinical information for two patients was lost, and the age of one patient was unknown.
[b] OR, odds ratio; 95% CI, 95% confidence interval.
[c] Resistant to one or more drugs.

two links supported the recent hypothesis that the misassignments or some undefined patterns in the SITVIT2 database might primarily be caused by mixed infections (6). Manu2 was identified as the lineage with the highest probability of being the admixture of two individual patterns in Sichuan. The similar situation also appeared in Vietnam, where the most isolates of mixed infections were found to belong to the East African-Indian (EAI) lineage (14). Compared with Beijing strains, EAI strains showed not only less virulence but also a 100-fold lower replication level in lung infections (33). It is noteworthy that the Manu and EAI lineages are believed to be closely related or even derived from a latest common ancestor (34).

Although a small number of resistant strains might not be detected by molecular methods because of the overgrowth of sensitive strains in drug-free medium, a majority of resistant strains in cases of mixed infections can be detected in drug selection medium. Isolates with mixed infections or heteroresistance usually show a contradiction between their phenotypic DST data and mutation analysis results (4, 11). Heteroresistance is a reason for the change in drug susceptibility patterns, and it was described for INH, RIF, EMB, STR, and fluoroquinolones by DST (35), line

probe assays (11), and simultaneous detection of WT and mutated sequences by using PCR-based techniques or sequencing (11, 36–39). In our study, a low heteroresistance pattern rate was observed in the *rpoB* gene (1.37%), and similar rates have also been found in other regions, such as 1.4% in Italy (40), 1.9% in Finland and Russia (41), and 1.9% in Pakistan (42); however, other studies have reported significantly higher rates, such as 14.3% in Uzbekistan (12) and 28.8% in India (11). This discrepancy was reported to be related to differences in sample collection, detection method, or the prevalence of drug-resistant TB (12). Previous investigations reported that heteroresistance is primarily caused by mixed *M. tuberculosis* infections (12); similarly, in our study, 75% of the RIF heteroresistant isolates came from patients with mixed *M. tuberculosis* infections, and all belonged to Manu2 lineage. Therefore, the rate of heteroresistance may also vary with the level of mixed infections in a particular region.

Until now, about 5% of RIF-resistant isolates have had no mutation detected in the *rpoB* gene (19). In our study, 3.8% of the RIF-resistant isolates without any mutation detected were significantly associated with mixed infections (χ^2, 56.78; $P < 0.01$), which was possibly because the mutations of the RIF-resistant

TABLE 4 Isolates identified as mixed infections by phenotypic drug susceptibility test for rifampin versus mutation analysis in *rpoB* gene[a]

Mutation category (no. of isolates)	No. (%) of isolates[b]		ID of mixed infections (ID with secondary peak)	
	RIF[r]	RIF[s]	RIF[r]	RIF[s]
None (74)	8 (10.81)	66 (89.19)	SSCM034, SC262, SC0407, SC187	SSC0617, SSC0619, SC240, (SC023)
Single (177)	165 (93.22)	10 (5.65)	SC107, SSC0620, (SC187, SC262)	SSC0588, SC023
Multiple (41)	39 (95.12)	2 (4.88)	SC081	

[a] A total of 292 isolates that were sequenced on the *rpoB* gene were included in this statistical analysis. Two isolates for which clinical information was lost had a single mutation. The isolates of patients whose IDs are shown in parentheses were RIF heteroresistant.
[b] RIF[r], resistant to RIF; RIF[s], susceptible to RIF.
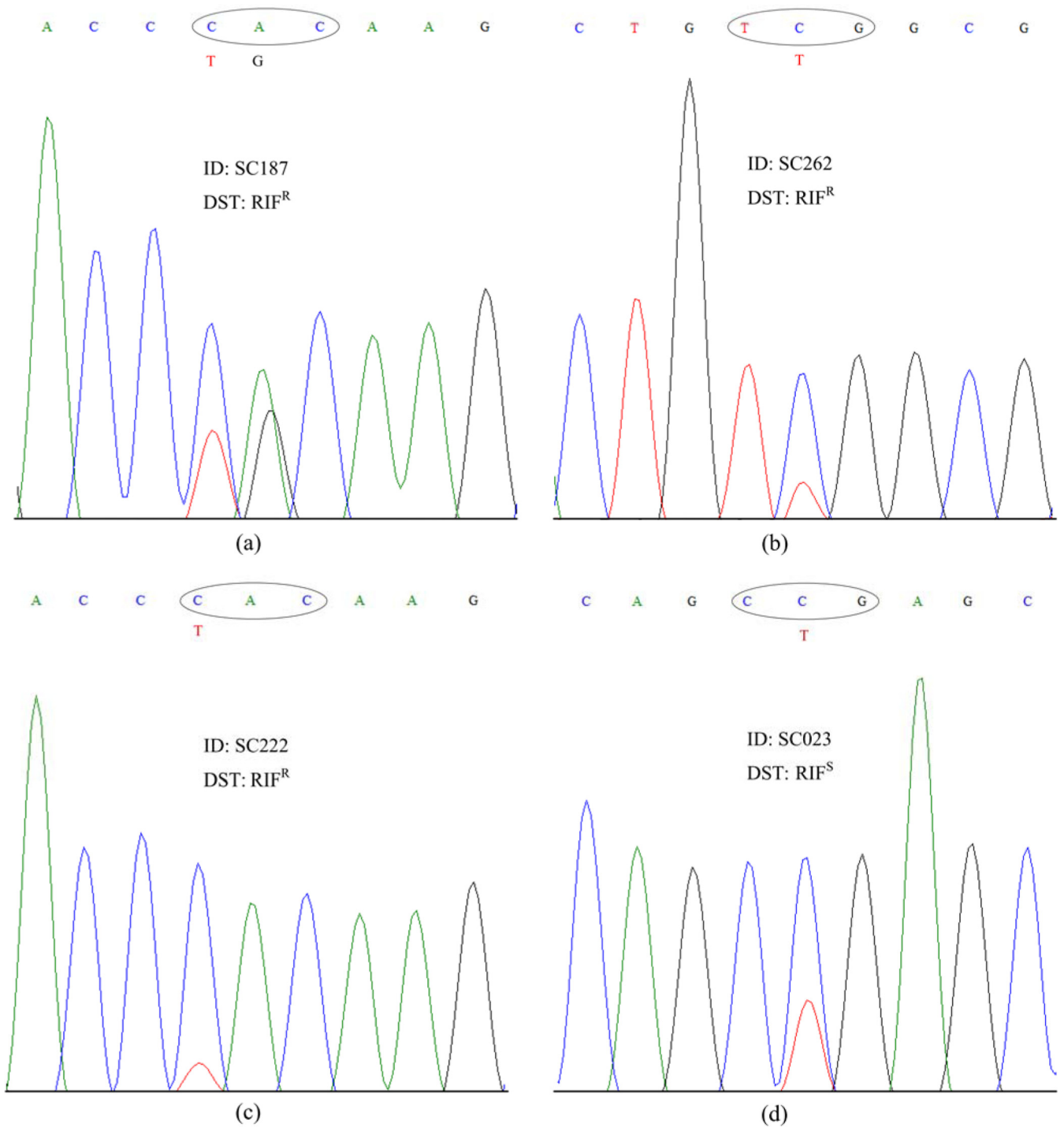
FIG 1 The four RIF heteroresistant isolates detected by DNA sequencing showed dual peaks of wild type (WT) and mutant bases at certain codons (circled). The phenotypic DST for RIF: RIF[r] (resistant to RIF) and RIF[s] (susceptible to RIF). (a to c) WT and secondary mutation bands at codons 526 (TGC), 531 (TTG), and 526 (TAC), respectively. (d) Main peak with mutant base (CCG) and WT (CTG) at codon 511.

isolates with mixed infections might be missed by DNA sequencing (4). It was reported that mixed infections might also be an important mechanism underlying the change in drug susceptibility patterns through the presence or absence of antibiotic pressure, which determined the dominant growth of strains of mixed infections (3). As the previous study suggested that there may be other

genes or mechanisms conferring RIF resistance in TB (19), our data identified mixed infections as a possible reason for RIF-resistant isolates with no mutation in the *rpoB* gene region. Furthermore, with twice-reliable DST, we had already confirmed an extensively drug-resistant TB (XDR-TB) isolate, which was a combination of two different resistant strains, according to the

TABLE 5 Mixed *M. tuberculosis* infections revealed by MIRU-VNTR-based methods across the literature

Geographic region[a]	Total no. of cases	No. (%) of mixed infections[b]	Corresponding SIT, lineage (*n*, %) of mixed infections	No. of loci (order of relevant loci by the no. of mixed infections detected)[c]	Reference or source
AFR					
Rwanda[d]	22	1 (4.3)	SIT52, T2 (1, 100)	12 (J = H)	43
South Africa[e]	54	10 (18.5)	SIT53, T1 (10, 100)	15 (**T** = O > C > V > M = Q = S > **K** = F > J = D = H > N)	9
Uganda (Kampala)	113	8 (7.1)	No description	15 (**J** > **F** > **Q** > **D** = H = M = O > **T** > K = C = B > S = U = V)	8
Malawi (Karonga)	72	2 (2.8)[f]	LAM11_ZWE (1, 50); T1 (1, 50)	24 (no report)	31
South Africa (KwaZulu-Natal)	56	5 (9)	SIT34, S (1, 20); SIT52, T2 (1, 20); SIT54, Manu2 (1, 20); SIT1196, undesignated (1, 20); no SIT (1, 20)	24 (M > J = K = E = L > F = C = H = N = Q = S = O = I = G = P = R)	7
Uganda (Mubende)	72	8 (11.1)	SIT53/137,[g] T1/X2[g] (1, 12.5); SIT52, T2-Uganda I (1, 12.5); SIT420, T2-Uganda II (1, 12.5); others not reported	15 (T > U > K = C = F > D > J > V = S = Q = N > B = H > M)	46
Botswana	370	18 (4.9)	No description	24 (no report)	44
EUR					
Belgium (Brussels)	2	1	No description	12 (A = C = H = I)	47
Spain (Almería)	780	11 (1.4)	No description	24 (not clear)	27
MECA					
Bangladesh (Mymensingh)	97	2 (2.1)[f]	Non-Beijing (2, 100)	13 (**H** = B > **M** = **J** = **D** = **F** = **S** = N = C = V = X)	15
Georgia (near Tbilisi)[h]	199	26 (13.1)[f]	No description	15 (N > Q > C = F = H > M = J = D = K = U > V > S > O > T > B)	2
Kyrgyzstan (near Bishkek)	56	3 (5.4)	SIT264, T5-RUS1 (1, 33.3); SIT53, T1 (1, 33.3); one not reported	12 (**F** > A = **C** = H = I > D)	30
Uzbekistan (Tashkent)[h]	7	4 (57.9)	SIT53, T1 (1, 25); SIT262, H3 (1, 25); SIT1196, undesignated (1, 25); unknown (1, 25)	24 (T > J = O = K = Q = F > D = H = S > N = M = A = C = I)	12
FEA					
China (Shanghai)[i]	249	2 (0.8)	Non-Beijing (1, 50); non-Beijing and Beijing (1, 50)	7 (O > U = W)	25
Vietnam (Rural South)[h]	1,248	60 (4.8)	EAI (33, 55); Manu2 (2, 3.3); Beijing (2, 3.3); CAS1-DELHI (1, 1.7); non-Beijing (1, 1.7); others not reported	15 (no report)	14
China (Sichuan)	499	12 (2.4)	SIT54, Manu2 (4, 33.3); SIT1096, Manu2 (3, 25); SIT1, Beijing (2, 16.7); orphan, Manu2 (1, 8.3); SIT2276, unknown (1, 8.3); SIT393, T1 (1, 8.3)	24 (J = Q > T = **O** = **C** = **L** > U > H = N = K > F = **I** = V = D > G = E = R)	This study

[a] AFR, Africa; EUR, Europe; MECA, Middle-East and central Asia; FEA, Far-East Asia.

[b] The proportion of mixed infections in some studies may not be representative of the underlying population, since patients in special circumstances, such as those from a hospital or prison or with an age in a specific range, were selected by the corresponding researchers.

[c] A = 154, B = 580, C = 960, D = 1644, E = 2531, F = 2996, G = 3007, H = 3192, I = 4348, J = 802, K = 2165, L = 2461, M = 577, N = 424, O = 1955, P = 2347, Q = 2401, R = 3171, S = 3690, T = 2163b, U = 4052, V = 4156, W = VNTR3820, X = QUB1982, and the bold capital loci indicate three alleles.

[d] The patients in Rwanda were from four provinces, Kigali, Butare, Ruhengeri, and Rwamagana.

[e] The patients were from eight of the nine provinces of South Africa, including Eastern Cape, Limpopo, North West, Free State, Mpumalanga, Gauteng, KwaZulu-Natal, and Western Cape.

[f] Mixed infections were detected by spoligotyping, IS6110-restriction fragment length polymorphism (RFLP), or PCR amplification using different primers and confirmed with the MIRU-VNTR method.

[g] Corresponding SIT/lineage; the SIT53/T1 and SIT137/X2 isolates were from sputum and lymph node samples, respectively.

[h] The number of mixed infections was different between the IS6110-RFLP method and the MIRU-VNTR method in this publication, while in our study, it was in accordance with the definition of the MIRU-VNTR method mentioned in the introduction in the text.

[i] The isolates belonged to Beijing/non-Beijing based on deletion-targeted multiplex-PCR (DTM-PCR) genotyping, not spoligotyping.

sequencing result (data not shown), and this supports the idea that mixed infections increase the difficulty of diagnosis and treatment.

Nationality was considered for the first time as a factor influ-

encing the occurrence of mixed infections; however, it was not significantly associated with mixed infections in our study, an observation that is similar to that with many other previously reported factors (Table 3) (7, 8, 43). From our data, there was a trend

that the rate of mixed infections increased with age (0.85% to 2.8% to 3.45% in the young, adult, and older groups, respectively; Table 3). Older people, who normally have a poorer immune response, and those with immunosuppressive conditions, such as HIV infection, were considered to be associated with mixed infections to a higher extent (8, 44). The sensitivity of the Xpert MTB/RIF assay for RIF resistance detection was reduced in the cases of mixed infections and in HIV-infected patients who may suffer mixed infections (4). Our data suggest that Manu2 is the group proposing the highest risk of mixed infection in Sichuan. Besides, Manu lineage strains were recently reported to be associated with HIV infection (45), and Manu and EAI lineages were shown to be closely related. In a mouse model study, mice infected with EAI strains showed better survival than those infected with Beijing strains (33), which was similar to the phenomenon that patients with mixed infections had less extensive pulmonary pathology and increased immunological tolerance compared with those with single infections (14). Thus, the unique properties of the Manu2 lineage need further investigations in order to clarify its relevance to mixed infections.

It is realized that the minimal clinical information taken from the clinical records could not provide a highly concrete conclusion regarding the possible link between the occurrence of mixed infections, patient HIV status, and/or history of TB treatment. Indeed, the frequencies of mixed infections and RIF heteroresistance might be higher than those reported in the present study, since not all mixed infections were accurately identified by the MIRU-VNTR method that was reported to detect two mixed strains at a ratio of 1:99 (26); also, only one sample was taken from each patient. Other limitations include a lack of clear distinction between clonal heterogeneity and mixed infections (5), and Sanger sequencing may reduce sensitivity in the cases of mixed infection and heteroresistance compared to that with deep sequencing (4, 36).

In conclusion, this is the first study to describe situations of mixed infection and heteroresistance in Sichuan. The RIF heteroresistance pattern in this region was primarily caused by mixed infections, and the Manu2 group was the predominant lineage in the cases of mixed infections and RIF heteroresistance, which were greatly capable of superinfecting, cooperating with, or affecting the host immune response to coinfection. This is regarded as a possible part of the mechanisms conferring RIF resistance to isolates without any mutation in the *rpoB* gene region. Based on the present study, further studies are needed to clarify pathogen-pathogen compatibility, explain the characteristics of special lineages, such as Manu2 (described in our study), and establish a fast and effective method to detect, treat, and control both mixed and single infections of *M. tuberculosis*.

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