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Acquisition of second-line drug resistance and extensive drug resistance during recent transmission of *Mycobacterium tuberculosis* in rural China

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Abstract

Multidrug-resistant tuberculosis (MDR-TB) is prevalent in countries with a high TB burden, like China. As little is known about the emergence and spread of second-line drug (SLD) -resistant TB, we investigate the emergence and transmission of SLD-resistant *Mycobacterium tuberculosis* in rural China. In a multi-centre population-based study, we described the bacterial population structure and the transmission characteristics of SLD-resistant TB using Spoligotyping in combination with genotyping based on 24-locus MIRU-VNTR (mycobacterial interspersed repetitive unit-variable-number tandem repeat) plus four highly variable loci for the Beijing family, in four rural Chinese regions with diverse geographic and socio-demographic characteristics. Transmission networks among genotypically clustered patients were constructed using social network analysis. Of 1332 *M. tuberculosis* patient isolates recovered, the Beijing family represented 74.8% of all isolates and an association with MDR and simultaneous resistance between first-line drugs and SLDs. The genotyping analysis revealed that 189 isolates shared MIRU-VNTR patterns in 78 clusters with clustering rate and recent transmission rate of 14.2% and 8.3%, respectively. Fifty-three SLD-resistant isolates were observed in 31 clusters, 30 of which contained the strains with different drug susceptibility profiles and genetic mutations. In conjunction with molecular data, socio-network analysis indicated a key role of Central Township in the transmission across a highly interconnected network where SLD resistance accumulation occurred during transmission. SLD-resistant *M. tuberculosis* has been spreading in rural China with Beijing family being the dominant strains. Primary transmission of SLD-resistant strains in the population highlights the importance of routine drug susceptibility testing and effective anti-tuberculosis regimens for drug-resistant TB.

Keywords: Beijing family, *Mycobacterium tuberculosis*, rural China, second-line drug resistance transmission, transmission network
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Introduction

The global emergence of multidrug-resistant tuberculosis (MDR-TB; i.e. resistance to at least isoniazid and rifampin) has brought about formidable challenges to the progress of TB control programmes. Second-line drugs (SLDs) are essential for treating MDR-TB. More recently, the emergence of resistance to SLDs, and in particular extensively-drug-resistant (XDR) TB, has further exacerbated therapeutic outcomes and control efforts [1]. Although *Mycobacterium tuberculosis* isolates resistant to SLDs have been observed in populations with high burden of drug resistance, the extent and nature has not been well elucidated, particularly in MDR-TB hotspot areas [2].

China ranks second among the 22 countries with the highest TB burden [3] and probably harbours one of the largest reservoirs of individuals latently infected with MDR/XDR

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M. tuberculosis. According to the national baseline survey of drug-resistant TB conducted in 2010, the frequencies of MDR-TB among patients with pulmonary TB in China were 8.3% and 1.4% for XDR-TB [4], remaining at a high level. In that same report, the prevalence of drug-resistant TB including MDR-TB in the rural region of China was notably higher than the countrywide average [4]. Improper administration of anti-TB medications, readily accessible SLDs as well as a relatively poor case management system for patient follow up during treatment were cited as reasons for the high prevalence of drug resistance in rural China [4]. To better understand the underpinnings of drug-resistant TB, there is a need to examine *M. tuberculosis* isolates from a defined geographical region. Although there are molecular epidemiological TB studies from China, a detailed analysis of *M. tuberculosis* genetic diversity, covering both first-line drugs (FLDs) and SLDs, resistance in rural China is lacking.

To better understand the extent of MDR-TB and XDR-TB in rural China, we conducted a multi-centre population-based study in four rural counties of China with diverse geographic and socio-demographic characteristics. We combine molecular epidemiological and social network analysis to examine the *M. tuberculosis* genetic diversity of FLD-resistant and SLD-resistant TB and elucidate transmission patterns of MDR and XDR strains in rural China.

Materials and methods

Study design

A population-based study of patients with TB diagnosed at local TB dispensaries from four rural counties from eastern (Jiangsu province), middle-western (Sichuan province), southern (Guangdong Province) and east northern (Shandong Province) areas in China. All suspected pulmonary TB cases detected in general hospitals or community health centres were referred to TB dispensaries for diagnosis and treatment. All patients with TB notified in the County TB dispensaries from January 2011 to December 2013 were enrolled in the study. All protocols in this study were approved by the ethics committee of the School of Public Health, Fudan University and written informed consent was obtained from all the patients enrolled.

Drug susceptibility testing

The drug susceptibility testing (DST) for FLDs was determined with the proportion method [5], using LJ-based medium individually with the following drugs: rifampin (40 mg/L), isoniazid (0.2 mg/L), streptomycin (4.0 mg/L) and ethambutol (2.0 mg/L). The DST for SLDs was performed according to WHO recommendations. Critical SLD concentrations for the agar proportion method on 7H10 agar were: ofloxacin (2mg/L), levofloxacin (1mg/L), kanamycin (30 mg/L), capreomycin (40mg/L), and amikacin (40mg/L). To ensure reproducibility, the laboratory work underwent external quality assessment by the Shanghai municipal CDC laboratory.

Definitions

MDR-TB was defined as an isolate of *M. tuberculosis* that is resistant to at least isoniazid and rifampin. XDR was defined as *M. tuberculosis* being resistant to at least isoniazid, rifampin, a fluoroquinolone (FQ; ofloxacin or levofloxacin) and one of three injectable SLDs (capreomycin, kanamycin, or amikacin) [6]. Pre-XDR was defined as disease caused by the *M. tuberculosis* strain resistant to isoniazid and rifampin and either an FQ or an injectable drug, but not both [7]. Any drug resistance was defined as an isolate of *M. tuberculosis* that is resistant to at least one tested anti-tuberculosis drug. SLD resistance is defined as an isolates of *M. tuberculosis* that was resistant to at least one of the SLDs tested, including those with additional resistance to the FLDs. New cases were defined as TB patients who did not have any previous anti-TB treatment or who received anti-TB treatment for <30 days before the TB diagnosis. Previously treated cases were TB patients who reported having been treated for TB for at least 30 days.

DNA isolation and sequencing of loci

DNA from clinical isolates was extracted using the CTAB method [8]. The following nine resistance-determining loci were amplified by PCR: *rpoB* (rifampin), *katG* and *inhA* (isoniazid), *embB* (ethambutol), *gyrA* (FQ), *rrs* (kanamycin, capreomycin, and amikacin), *eis* (kanamycin) and *tlyA* (capreomycin). These previously reported drug resistance-determining regions were amplified using locus-specific primers [9]. Sequence data generated by an ABI 3130xl genetic analyser were reviewed for confidence levels using an ABI sequence scanner, and chromatograms were analysed for the presence or absence of mutations by comparison with published sequences of H37Rv using the SeqMan alignment application of the DNASTAR LASER gene (version 8.0) program.

Spoligotyping

Families of the isolates were determined by spoligotyping using a commercially available kit (Isogen Bioscience BV, Maarssen, the Netherlands) [10]. Spoligotyping profiles were compared using the SITVIT_WEB database at Institut Pasteur de Guadeloupe (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/). Major phylogenetic clades were assigned according to the signatures provided in the SpolDB4 database, which defined 62 discrete genetic lineages/sub-lineages [6].

Genotyping strategies

All the studied isolates were first genotyped by the 24-locus MIRU-VNTR (mycobacterial interspersed repetitive unit-variable-number tandem repeat) method, as proposed by Supply *et al.* [11]. Beijing family strains were further characterized using the four MIRU-VNTR (1982, 3232, 3820 and 4120), previously shown to have high discriminatory power within Beijing family [12]. The PCR products were detected by electrophoresis in 2.0% agarose gel using a 100-bp DNA ladder (Takara Bio Inc., Kusatsu, JAPAN). Size analysis of PCR fragments and assignment of the various MIRU-VNTR alleles were achieved using Quantity One (version 4.6.2) software (Bio-Rad Laboratories, Inc.,Hercules, CA, USA). The 24-locus MIRU-VNTR digital profiles were compared with MIRUVNTRplus (http://www.miru-vntrplus.org/MIRU/index.faces) for family and code assignment. MIRU-VNTR clusters were defined on the basis of identical allelic profiles (identical numbers of tandem repeat units at each MIRU/VNTR locus).

The investigation of epidemiological link

Questionnaire-based interviewing of clustered patients, defined by MIRU-VNTR genotyping, was performed to collect comprehensive epidemiological information including demographic characteristics, clinical history, predisposing risk factors and evidence of contact with patients with active disease. Information was also obtained on occupational, social and recreational history, and compliance with TB treatment. Chest radiographs of all patients were reviewed for the presence of cavitation.

Transmission network analysis

The transmission network was constructed under the following criteria: patients were included in a network if they were infected with clustered *M. tuberculosis* strain defined by a specific MIRU-VNTR genotype. Patients with earlier TB diagnosis were considered to have transmitted disease to patients who had a later diagnosis with a shared MIRU-VNTR profile. The transmission network was drawn with earlier cases on the nock and later cases on the arrowhead. Furthermore, the individual transmission networks were compared between central and peripheral townships in terms of the component size, network density and centrality [13].

Data analysis

Genotypic data for each isolate at a particular locus were recorded in a Microsoft Office Excel 2010 spreadsheet. Information on previous TB treatment was additionally verified through medical record review. Data were double-entered in EpiData and checked for errors. Analysis was performed using SPSS version 19 (IBM SPSS, Chicago, IL, USA). Descriptive statistics were computed for all variables. Means (± standard deviation) were calculated for continuous variables, whereas frequencies with percentages were calculated for categorical variables. To identify factors associated with the molecular clustering of SLD-resistant *M. tuberculosis*, univariate and multivariate logistic regression analyses were performed.

Results

Study population and drug susceptibility profile

A total of 1417 smear-positive TB patients were registered in the TB dispensaries during the study period. Twenty-four patients declined to participate and 28 did not meet the inclusion criteria. Among the 1365 patients recruited, specimens from 14 were culture-negative, and 19 failed in the DST. These 33 cases were excluded from the analysis (Fig. 1). As a result, a total of 1332 culture-positive patients with *M. tuberculosis* infection were included for analysis. The mean age of patients was 44 (±16.3) years (range 15–91 years), and 927 (69.6%) were male. Overall, 208 (15.6%) patients had previously been treated for TB.

Drug susceptibility testing indicated that *M. tuberculosis* isolates from a total of 623 (46.7%) patients were resistant to at least one of the anti-tuberculosis agents tested, including four FLDs (isoniazid, rifampin, streptomycin and ethambutol) and five SLDs (ofloxacin, levofloxacin, kanamycin, amikacin and capreomycin). In total 150 isolates (11.3%) were simultaneously resistant to isoniazid and rifampin and referred to MDR-TB. Among the 150 MDR-TB isolates, 45 (30.0%) were either resistant to FQs (31, 20.6%) or the second-line injectable drugs (14, 9.3%) and were referred to as pre-XDR. In addition, 15 MDR isolates (15/150 or 10.0%) were simultaneously resistant

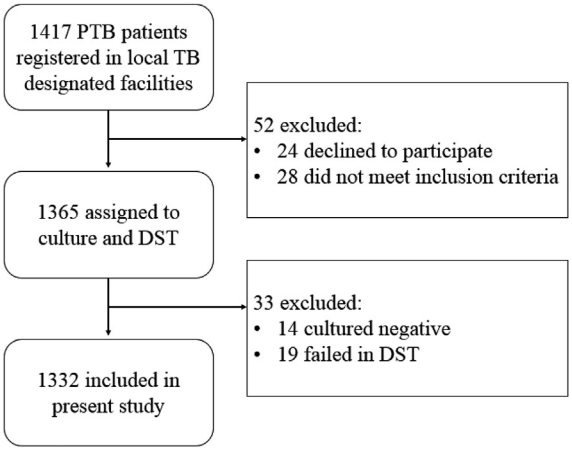


FIG. 1. Inclusion of the subjects.

to FQs and at least one of the injectable drugs and were referred to as XDR (Table 1).

Drug susceptibility profile within different *M. tuberculosis* families

Based on Spoligotyping analyses, Beijing family was the predominant group representing 74.8% (997 of 1332) of all isolates. Eighty-eight isolates were assigned to four other families: T family (11.8%), CAS (2.3%), Ural (1.1%), H family (0.9%) and MANU2 (0.8%). One hundred and eight (8.1%) could not be classified to previously described lineages and were referred to as 'Orphans'. The majority of drug-resistant isolates were within the Beijing and T families. Of the 997 Beijing family isolates, 136 (13.6%) were MDR, 273 (27.4%) were resistant to SLD, 42 (4.2%) met the definition for pre-XDR, and 15 (1.5%) were XDR. Within the T family, ten isolates (6.4%) were MDR and ten resistant to SLDs, whereas MDR isolates were also observed in the CAS and Ural lineages. No drug resistance was observed in strains labelled as MANU2 and LAM lineages. (Table 2)

Genetic characterization of drug-resistant TB

Genomic regions known to confer drug resistance to selected FLDs and SLDs are shown in the Supplementary material (Table S1). The mutations in *katG* were identified in 66.3% of isoniazid-resistant isolates, all harboured a mutation in codon 315. Mutations related to rifampin resistance occurred mostly in codon 531 (64.3%), 526 (22.2%) and 516 (7.0%) of the *rpoB* gene. Additionally, 73.4% of the FQ resistance was associated with mutations in *gyrA*. Of the 123 isolates resistant to kanamycin, amikacin and capreomycin, 97 (78.9%) contained mutations within *rrs* (75; 60.9%) or *eis* (27; 22.0%).

MIRU-VNTR genotyping and drug-resistant profile

Among 1332 *M. tuberculosis* strains characterized by VNTR-MIRU genotyping, 1202 VNTR genotypes were detected including 59 VNTR genotypes (clustered) shared by more than one isolate with the average cluster size of 2.4 (range: 2–4) and 1143 VNTR genotypes observed for only one isolate (unique). Therefore, the clustering rate and recent transmission rate were overall 14.2% and 9.8%, respectively, including 10.8% and 5.8% in drug-sensitive strains, 18.0% and 13.0% in drug-resistant strains.

Among 150 patients with MDR-TB, VNTR genotyping indicated 13 clusters in 27 patients; 123 patients had *M. tuberculosis* strains with unique VNTR pattern. The proportion of clustering and estimation of recent transmission equalled 18.0% and 8.7%, respectively. Furthermore, within 13 clusters containing MDR-TB isolates, nine clusters (comprising 27 isolates) contained MDR-TB and/or additional drug resistance to FQs or one of the injectable drugs (i.e. pre-XDR-TB). One XDR-TB isolate was in a cluster containing four isolates that were all resistant to isoniazid, whereas other XDR-TB isolates were in a cluster containing five isolates that were also all resistant to isoniazid. One cluster with two isolates had identical drug-resistance and associated mutation profile (Fig. 2). Based on Spoligotyping, 77 isolates in 28 clusters belonged to Beijing genotype.

Risk factors influencing the clustering proportion of drug-resistant *M. tuberculosis*

Clustering proportion among drug-resistant *M. tuberculosis* isolates was compared with the 703 drug-susceptible *M. tuberculosis* isolates collected simultaneously. Isolates of *M. tuberculosis* resistant to isoniazid (24.8% versus 10.8%; p 0.008; OR 2.64; 95% CI 1.816–4.021) and FQs (21.4% versus 10.8%, p 0.043; OR 2.21; 95% CI 1.421–3.487) were more likely to be clustered compared with drug-susceptible isolates (Table 3). The association between drug-resistance conferring genotype and clustering was investigated by comparing the clustering proportion between drug-resistant genotype and wild-type isolates. Among isoniazid-resistant strains, the alleles

TABLE 1. Baseline information of the subjects

	Total	No. (%) of isolates resistant to:		No. (%) of isolates resistant to:		No. (%) of isolates with:		
		INH	RIF	FQs	Injective	MDR	Pre-XDR	XDR
	1332	(n = 294)	(n = 171)	(n = 192)	(n = 123)	(n = 150)	(n = 45)	(n = 15)
Area								
East	266	49 (18.4)	27 (10.2)	51 (19.1)	34 (12.8)	21 (7.9)	8 (3.0)	2 (0.8)
South	306	60 (19.6)	37 (12.1)	58 (18.9)	36 (11.8)	28 (9.2)	9 (2.9)	3 (1.0)
North	361	81 (22.4)	48 (13.3)	40 (11.1)	32 (8.9)	44 (12.2)	11 (3.0)	4 (1.1)
West	399	104 (26.1)	59 (14.8)	43 (10.8)	21 (5.3)	57 (14.3)	17 (4.3)	6 (1.5)
Sex								
Male	927	201 (21.7)	118 (12.7)	122 (13.2)	79 (8.5)	102 (11.0)	28 (3.0)	10 (1.1)
Female	405	93 (23.0)	53 (13.1)	70 (17.3)	44 (10.9)	48 (11.8)	17 (4.2)	5 (1.2)
Age, years	44 ± 16.3	47 ± 16.1	50 ± 17.1	45 ± 19.5	50 ± 21.3	53 ± 15.1	55 ± 17.1	61 ± 15.3
Treatment history								
New	1124	199 (17.7)	122 (10.9)	151 (13.4)	98 (8.7)	101 (9.0)	35 (3.1)	8 (0.7)
Previously treated/New	208	95 (45.7)	49 (23.6)	41 (19.7)	25 (12.0)	49 (23.6)	10 (4.8)	7 (3.4)

Abbreviations: FQs, fluoroquinolones; INH, isoniazid; MDR, multidrug resistance; RIF, rifampin; XDR, extensively drug resistance.

TABLE 2. Drug susceptibility patterns within different *M. tuberculosis* strain lineages

	Beijing	T	CAS	H	MANU2	LAM	Ural	Orphans
	n = 997	*n* = 157	*n* = 30	*n* = 12	*n* = 10	*n* = 3	*n* = 15	*n* = 108
First-line drug-resistant pattern								
INH	266 (26.7)	15 (9.5)	3 (10.0)	1 (8.3)	0 (0)	0 (0)	2 (13.3)	7 (6.5)
RIF	157 (15.7)	11 (7.0)	1 (3.3)	1 (8.3)	0 (0)	0 (0)	1 (6.7)	0 (0)
Second line drug-resistant pattern								0 (0)
FQs	178 (17.9)	11 (7.0)	2 (6.7)	1 (8.3)	0 (0)	0 (0)	0 (0)	0 (0)
KAN, CAP, AMK	112 (11.2)	6 (3.8)	3 (10.0)	2 (16.7)	0 (0)	0 (0)	0 (0)	0 (0)
First- and second-drug cross-resistant pattern								0 (0)
MDR	136 (13.6)	10 (6.4)	2 (6.7)	1 (8.3)	0 (0)	0 (0)	1 (6.7)	0 (0)
Pre-XDR-TB	42 (4.2)	3 (1.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
XDR-TB	15 (1.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Abbreviations: AMK, amikacin; CAP, capreomycin; INH, isoniazid; KAN, kanamycin; MDR, multidrug resistance; RIF, rifampin; TB, tuberculosis; XDR, extensively drug resistance.

with the mutation in *katG* (30.8% versus 11.8%; p 0.001; OR 3.39; 95% CI 2.312–4.897) were associated with clustering. No other alleles conferring resistance to FLDs or SLDs were associated with clustering.

In the multivariate analysis (Table 4), Beijing family strain had a significantly higher proportion of clustering than those from other strain families among SLD-resistant *M. tuberculosis* and accounted for all the clustered FLD- and SLD-resistant and pre-XDR isolates. Previous treatment with FLDs was independently associated with clustering among drug-resistant *M. tuberculosis* isolates (30.0% versus 15.1%; OR 2.41; 95% CI 1.349–4.341) and SLD resistance (41.9% versus 18.3%; OR 3.10; 95% CI 1.034–10.85). A history of TB contact significantly increased the risk of clustering among any drug-resistant *M. tuberculosis* isolates (38.3% versus 20.7%; OR 4.04; 95% CI 2.235–7.345) and SLD-resistant *M. tuberculosis* isolates (45.0% versus 17.9%; OR 3.94; 95% CI 1.014–9.650). Furthermore, among the SLD resistance, isolates from smear-positive patients were more likely to be clustered than smear-negative individuals (31.7% versus 11.0%; OR 3.58; 95% CI 1.086–13.66).

Epidemiological link and social network analysis

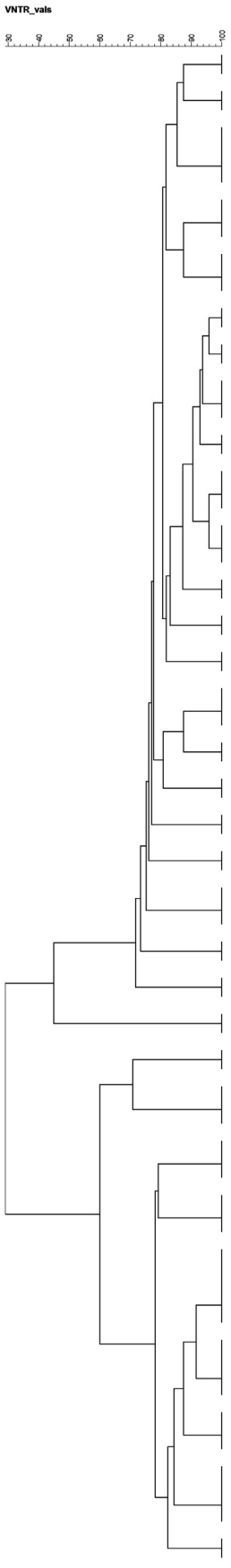
Epidemiological links within drug-resistant clusters were further analysed by contact investigation. Transmission networks were constructed between MIRU-VNTR-defined clusters containing drug-resistant TB cases (Fig. 3). Of the 31 clusters, 24 were considered inter-regional spread from the Centre Township to the Peripheral Township involving 20 MDR strains, 32 non-MDR isolates resistant to SLDs and 11 pre-XDR/XDR-TB strains. The Centre Township of the studied counties contained 30 clusters, involving 20 MDR-TB isolates, 19 isolates resistant to SLDs and nine pre-XDR and two XDR-TB isolates. Examining putative epidemiological links, 14 of 84 (16.7%) molecularly clustered isolates were recovered from family members (household contact), friends or colleagues (social contact). No epidemiological links or geographic correlations were established for 15 clustered drug-resistant isolates. For network parameters, central townships were the true centre of the TB transmission network, with size of this largest node up to five and highest ties up to 20. Additionally, the central township also had the higher average geodesic distance and betweenness compared with the peripheral township (see Supplementary material, Table S2).

Discussion

China is a country with considerable TB incidence and prevalence. Of growing concern is the high burden of MDR-TB cases in rural areas. This study reports on the extent and genetic diversity of the circulating bacillary population that is resistant to FLDs and SLDs in eastern rural China, an area with a population of over 656 million (The Sixth National Population Census of China, 2010). We report that SLD resistance is considerable among both MDR (40%) and non-MDR (14.8%) TB populations.

Consistent with previous reports, genotyping results indicate that the Beijing family of strains are over-represented in this population [14–16]. Although other strain families were represented (i.e. T1, H, MANU2 and LAM), MDR-TB and pre-XDR-TB were only found within Beijing, CAS, H, Ural and T1 families. The distribution of non-MDR and MDR-TB clinical isolates within the lineages varied. Whereas Beijing strains represented over 90.7% of MDR isolates, this percentage increased to around 95% among pre-XDR-TB and XDR clinical isolates. Monitoring Beijing strains and their drug susceptibilities may inform MDR-TB and XDR-TB control strategies.

To study the emergence of SLD resistance, we examined the drug susceptibility profiles and genetic correlates of resistance within MIRU-VNTR-defined clusters. Among 31 clusters with SLD resistance, 30 contained isolates with different drug susceptibility profiles whereas only one cluster had isolates with the same drug-resistant pattern. The high proportion of isolates with different genotypic patterns and drug-resistance profiles



INH	RIF	OFL	LEV	KAN	AMK	CAP	
R	S	S	S	S	S	S	katG315Thr
R	S	R	S	S	S	S	katG315Thr
S	S	S	S	R	R	S	
S	S	S	S	R	R	R	rrs1401G
R	S	S	S	S	S	S	katG315Thr
R	S	S	S	S	S	S	katG315Thr
R	R	S	S	S	S	S	katG315Thr, rpoB531Ile
R	R	R	R	R	R	S	katG315Thr,rpoB531Ile,gyrA94Gly
S	S	R	R	S	S	S	gyrA94Gly
S	S	R	R	S	S	S	gyrA94Gly
S	S	R	R	S	S	S	gyrA94Gly
S	S	S	S	S	S	S	
S	S	R	S	S	S	S	gyrA90Val
S	S	S	S	S	S	S	
S	S	R	S	S	S	S	
S	S	S	S	R	R	R	rrs1401G
S	S	S	R	S	S	S	
S	S	S	S	S	S	S	
S	S	S	S	R	S	S	eis(-10)T
S	S	S	S	S	S	S	
S	S	R	R	S	S	S	gyrA90Val
R	S	S	S	S	S	S	katG315Thr
R	S	R	S	S	S	S	katG315Thr,gyrA94Asn
S	S	S	S	S	S	S	
S	S	S	R	S	S	S	gyrA91Pro
S	S	S	S	S	S	S	
S	S	S	S	R	R	R	rrs1401G
S	S	S	S	S	S	S	
S	S	S	S	S	S	S	
R	R	R	R	S	S	S	katG315Thr,rpoB516Val,gyrA94Gly
R	S	S	S	S	S	S	katG315Thr
S	S	S	S	S	S	S	
S	S	R	S	R	S	S	gyrA94Gly
R	S	R	R	S	S	S	katG315Thr
R	S	R	R	S	S	S	katG315Thr, gyrA94Asn
R	S	S	S	S	S	S	katG315Thr
R	R	R	S	S	S	S	katG315Thr,rpoB526Arg,gyrA94Gly
R	S	S	S	S	S	S	katG315Thr
S	S	S	S	R	R	R	rrs1401G
S	S	S	S	S	S	S	
S	S	S	R	S	S	S	
S	S	S	S	R	S	S	eis(-10)T
R	S	S	S	S	S	S	katG315Thr
R	S	R	R	S	S	S	katG315Thr
S	S	S	S	S	S	S	
S	S	R	R	S	S	S	gyrA91Pro
R	S	S	S	S	S	S	katG315Thr
R	S	R	S	S	S	S	katG315Thr,gyrA94Asn
R	S	S	S	S	S	S	katG315Thr
R	S	R	R	S	S	S	katG315Thr,gyrA94Gly
R	S	S	S	S	S	S	katG315Thr
R	S	S	S	R	R	S	katG315Thr,rrs1401G
R	R	S	S	S	S	S	katG315Thr,rpoB526Tyr
R	S	R	S	S	S	S	katG315Thr
R	S	R	R	S	S	S	katG315Thr,gyrA90Val
R	R	S	S	R	R	S	katG315Thr,rrs1401G
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R	S	S	S	S	S	S	katG315Thr
R	R	S	S	R	R	R	katG315Thr,rpoB531Leu,rrs1401G
R	R	R	R	S	S	S	katG315Thr,rpoB531Leu,gyrA94Asn
S	S	R	S	S	S	S	WT
S	S	R	S	S	S	S	WT
S	S	R	S	R	S	S	rrs1401G
R	R	S	S	S	S	S	katG315Thr
R	R	R	R	S	S	S	katG315Thr,rpoB531Leu
R	S	R	R	S	S	S	katG315Thr,gyrA94Gly
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R	S	R	R	S	S	S	katG315Thr
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R	R	S	S	S	S	S	WT
R	S	S	S	S	S	R	WT
R	S	R	R	S	S	R	katG315Thr，gyrA91Pro
R	S	R	R	R	S	S	katG315Thr,eis(-10)T
R	S	R	S	S	S	S	katG315Thr
R	S	R	R	S	S	R	katG315Thr，gyrA91Pro
S	S	S	S	S	S	S	
S	S	S	S	S	S	S	
S	S	S	S	R	R	S	rrs1401G
R	S	R	S	S	S	S	katG315Thr
R	R	R	S	S	S	S	katG315Thr
R	S	R	R	S	R	S	katG315Thr
R	R	R	S	S	S	S	katG315Thr
S	S	R	R	S	S	S	gyrA94Gly
S	S	R	R	S	S	S	gyrA94Gly

FIG. 2. The drug-resistance profile and genetic mutation of the clustered *Mycobacterium tuberculosis* strain.

TABLE 3. Clustering proportion in *Mycobacterium tuberculosis* isolates by drug-resistant patterns and genetic mutations

	No. of isolates		Unadjusted		Adjusted	
	Total	Clustered (%)	OR	95% CI	OR[a]	95% CI[a]
Isolates resistant to:						
Drug susceptible	703	76 (10.8)	1			
INH	294	73 (24.8)	2.73	1.877–3.949*	2.64	1.816–4.021*
RIF	171	27 (15.8)	1.55	0.923–2.530	1.57	0.902–2.631
STR	255	16 (6.3)	0.55	0.295–0.980	0.56	0.291–0.991
EMB	136	19 (14.0)	1.34	0.736–2.340	1.39	0.721–2.386
FQs	192	41 (21.4)	2.24	1.432–3.465*	2.21	1.421–3.487*
CAP, AMK, KAN	123	19 (15.4)	1.51	0.825–2.664	1.52	0.745–2.591
MDR-TB	150	27 (18.0)	1.81	1.075–2.978*	1.73	1.061–2.993*
Pre-XDR/XDR-TB	60	12 (20.0)	2.06	1.022–4.156*	2.04	1.013–5.123*
Drug-resistant isolates with mutation in:						
WT	910	107 (11.8)				
katG	195	60 (30.8)	3.37	2.322–4.862*	3.39	2.312–4.897*
rpoB	163	27 (16.6)	1.49	0.903–2.392	1.53	0.889–2.413
rpsL	160	13 (8.1)	0.66	0.333–1.224	0.45	0.312–1.235
embB	81	12 (14.8)	1.31	0.622–2.529	1.23	0.612–2.632
gyrA	141	24 (17.0)	1.54	0.906–2.533	1.49	0.893–2.564
rrs	75	10 (13.3)	1.15	0.513–2.350	1.13	0.498–2.381
eis	27	3 (11.1)	0.94	0.178–3.169	0.89	0.173–3.253

Abbreviations: AMK, amikacin; CAP, capreomycin; EMB, ethambutol; FQs, fluoroquinolones; INH, isoniazid; KAN, kanamycin; MDR, multidrug resistance; RIF, rifampin; STR, streptomycin; TB, tuberculosis; WT, wild-type; XDR, extensively drug resistance.
*$p < 0.05$.
[a]OR and 95% CI were adjusted by age, sex and county of subjects in binary logistic regression model.

TABLE 4. Factors associated with the clustering of second-line drug-resistant *Mycobacterium tuberculosis* strains

	Total	Clustered (%)	OR (95% CI)	Adjusted OR(95% CI)
Total drug resistance				
City/rural	122/300	13.4/20.4	0.59 (0.304–1.099)	0.57 (0.291–1.154)
Female/Male	285/137	19.8/15.6	1.35 (0.761–2.466)	1.37 (0.754–2.507)
Age(years)	47 ± 19.9 vs 47 ± 20.5		1.02 (0.981–1.029)	1.03 (0.982–1.107)
Previously treated/New	94/328	30.0/15.1	2.41 (1.352–4.252)[a]	2.41 (1.349–4.341)[a]
BMI(values)	20 ± 12.3 vs 19 ± 4.4		0.98 (0.936–2.875)	0.99 (0.927–1.084)
Cavity/No	77/345	20.9/14.1	1.61 (0.911–2.936)	1.65 (0.832–2.999)
Smear-positive/negative	261/161	18.3/12.3	1.03 (0.599–1.776)	1.03 (0.537–1.795)
TB contacts/no	80/342	38.3/20.7	4.07 (2.257–7.263)	4.04 (2.235–7.345)[a]
Beijing genotype/no	328/94	18.7/7.4	1.11 (0.593–2.190)	1.09 (0.587–2.223)
Second-line drug resistance				
City/rural	53/182	19.0/23.6	0.75 (0.310–1.685)	0.76 (0.165–2.803)
Female/Male	152/83	25.0/18.1	1.51 (0.745–3.180)	1.2 (0.378–3.809)*
Age(years)	49 ± 18.2 vs 49 ± 21.2		1.00 (0.983–1.025)	1.0 (0.977–1.030)
Previously treated/New	43/192	41.9/18.3	3.23 (1.480–6.916)[a]	3.10 (1.034–10.85)*
BMI(values)	18 ± 4.9 vs 19 ± 14.6		0.98 (0.953–1.034)	0.99 (0.930–1.054)
Cavity/No	91/144	27.8/19.3	1.64 (0.838–3.201)	1.61 (0.531–4.796)
Smear positive/negative	126/109	31.7/11.0	3.33 (1.604–7.225)[a]	3.58 (1.086–13.66)*
TB contacts/no	40/195	45/17.9	3.74 (1.688–8.156)[a]	3.94 (1.014–9.650)*
Beijing genotype/no	171/64	28.1/7.8	4.60 (1.796–15.52)[a]	4.46 (1.050–12.17)*
First- and second-line drugs cross-resistance				
City/rural	30/67	30.0/31.3	0.94 (0.322–2.599)	0.91 (0.205–3.159)
Female/Male	24/73	25.0/32.9	0.68 (0.196–2.101)	0.62 (0.147–2.848)
Age(years)	49 ± 5.5 vs 51 ± 5.0		0.99 (0.955–1.039)	0.97 (0.913–1.042)
Retreatment/New	27/70	33.3/30.0	1.17 (0.394–3.287)	1.17 (0.342–3.939)
BMI(values)	18 ± 5.0 vs 21 ± 5.5		1.09 (0.934–1.276)	1.09 (0.931–1.278)
Cavity/No	36/61	33.3/18	1.19 (0.443–3.150)	1.12 (0.374–3.963)
Smear positive/negative	76/21	36.8/9.5	5.54 (1.173–11.89)[a]	5.58 (1.134–12.93)[a]
TB contacts/no	18/79	66.7/22.8	6.78 (1.972–24.77)[a]	6.57 (1.806–28.50)[a]
Beijing genotype/no	84/13	32.1/23.1	1.58 (0.364–9.604)	1.54 (0.336–10.95)
Pre-XDR/XDR-TB				
City/rural	17/43	11.7/23.3	0.44 (0.042–2.491)	0.43 (0.027–2.943)
Female/Male	22/38	18.1/21.0	0.78 (0.154–3.377)	0.53 (0.134–4.436)
Age(years)	50 ± 8.3 vs 51 ± 8.8			0.97 (0.919–1.031)
Retreatment/New	16/43	31.3/15.6	1.90 (0.343–8.958)	1.11 (0.015–12.94)
BMI(values)	17 ± 7.8 vs 19 ± 7.3		1.06 (0.864–1.235)	1.05 (0.851–1.293)
Cavity/No	25/35	28.0/14.3	2.33 (0.536–10.69)	2.36 (0.486–11.34)
Smear positive/negative	53/7	22.2/0		–
TB contacts/no	13/47	61.5/8.5	3.57 (0.690–17.03	3.50 (0.558–18.51)
Beijing genotype/no	54/6	22.2/0	–	–

Abbreviations: WT, wild type; MDR-TB, multidrug-resistant tuberculosis; XDR, extensively drug resistant.
*$p < 0.05$.
[a]OR and 95% CI were adjusted by age, sex and county of subjects in binary logistic regression model.

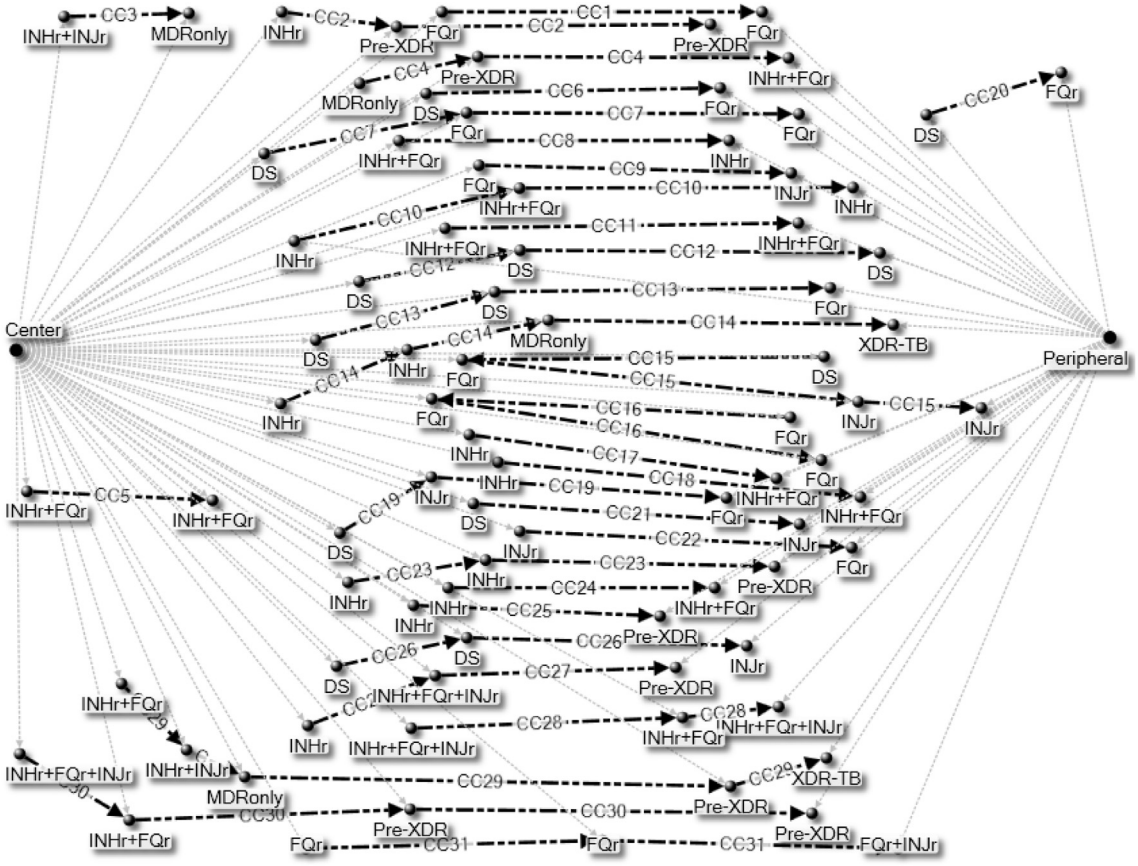

FIG. 3. The network analysis of the clusters containg the drug-resistant *Mycobacterium tuberculosis* isolates Each symbol refers to individual clustered patients and each number refers to the cluster number. Abbreviations: DS, drug sensitive; MDR-TB, multidrug-resistant tuberculosis; XDR, extensively drug resistant.

suggests that *de novo* or acquired resistance rather than primary transmission of drug resistance is driving SLD-resistant TB in rural China. Importantly, 22.6% of the analysed strains in these clusters were pre-XDR-TB. These data indicate that appropriate means to prevent emergence of SLD resistance among MDR-TB patients are not in place.

When comparing the drug resistance-related genotypes within clusters containing SLD-resistant isolates, we found that mutations occurring in *katG* and *gyrA* genes were cluster associated. The mutation *katG* 315Thr occurred exclusively in 17 clusters and *gyrA* D94G were exclusive to two clusters. Furthermore, 45 of 47 isoniazid-resistant isolates in 16 clusters had the *katG* 315Thr mutation, which was strongly associated with the mutations in *rpoB* and *gyrA* mutation, respectively. Additionally, eight isolates had the *gyrA* D94G mutation that was associated with the *rrs* A1401G mutation. These data illustrate possible instances of primary transmission of drug-resistant strains, although no patient data were available to establish epidemiological linkage. Given the high number of MDR-TB strains with FQ resistance and the high clustering proportion of FQ-resistant strains with *gyrA* mutation, rapid molecular diagnostics to evaluate *gyrA* before treatment with SLD may be warranted.

In the present study, a number of patient factors were associated with molecular clustering. We found that sputum smear positivity and having a recent contact with a TB patient were associated with clustering, consistent with previous reports [17–19]. Higher bacillary loads among clustered cases may indicate intrinsic strain-specific properties; but it is more likely to be related to delayed health-seeking behaviour. Contact with a patient who had TB diagnosed was an important indicator for clustering with drug-resistant strains. Additionally, previously treated subjects were more likely to be clustered, especially when they are infected with drug-resistant or SLD-resistant *M. tuberculosis*. This might point to the fact that previously treated TB patients may be at increased risk of re-infection rather than reactivation [20]. Paramount is the improvement of case management of drug-resistant patients to ensure favourable treatment outcomes and prevent primary transmission in the community. If effective measures are not

put in place to avoid the transmission of these strains, the inherently high treatment costs for MDR-TB and XDR-TB will place undue burden for national/local tuberculosis control programmes [21,22].

In this study, we examined the role of recent transmission among patients harbouring SLD-resistant and/or XDR-TB in rural China. We found that the majority of SLD resistant patients in clusters live in a Central Township and/or experienced contact with a TB patient. Furthermore, social network analysis indicated that, rather than a point source outbreak from a single patient; there was a high degree of interconnectedness that allowed multiple transmission events of drug-resistant *M. tuberculosis* strains over time. Drug-resistant TB transmission is probably occurring in the Central Township, where the local TB dispensary is located. The congregation of TB patients may allow nosocomial transmission of drug-resistant organisms. In addition, the long delay in diagnosing MDR/XDR-TB probably contributes to community transmission. Although infectiousness of TB patients can be highly variable, studies have consistently shown that patients receiving inadequate treatment regimens, including those for drug-resistant TB, account for the majority of transmission events [19].

Around 80% of MDR and pre-XDR-TB strains are not clustered, suggesting that MDR, pre-XDR and XDR strains are being built (acquired) and not transmitted in rural China. However, clustering of drug-resistant *M. tuberculosis* can be observed in 10% of individuals with no previous history of TB, as well as in 15% of individuals with a history of previous TB treatment. A recent meta-analysis also showed that primary drug resistance was associated with poor treatment outcomes if treatment regimens were not based on drug susceptibility test results [23,24]. In communities with a high rate of primary drug resistance, approaches that assign treatment regimens based solely on the patient's history of previous treatment may amplify drug resistance. Patients with MDR and XDR TB in China are often poor and cannot afford the SLDs, making it difficult to achieve treatment success. Patients with MDR and XDR TB without adequate treatment regimens will continue to be sources of infection in their communities. Therefore, control strategies must include efforts to improve cure rates for susceptible and MDR-TB cases, including infection control programmes to prevent transmission of drug-resistant *M. tuberculosis* strains. The success of infection control programmes hinges on their ability to promptly identify and separate XDR-TB patients from other patients [25]. Hence, in addition to well-recognized administrative, environmental and personal protection measures, implementation of rapid diagnostic testing for TB drug resistance [26] and redesign of healthcare facilities to minimize spaces where people congregate (e.g. wards and outpatient waiting areas) are critical elements of infection control programmes. Although financial and human resources are limited in developing countries, implementing such strategies has proven cost-effective when compared with the large public health and social costs of an escalating epidemic.

This study is subject to the usual limitations in survey design and data collection. Although restricted to the local TB dispensaries in rural China, these four dispensaries were responsible for diagnosing and treating approximately 85% of TB cases in study regions. Previous TB treatment was verified through medical record review when available, but it is possible for patients not to report TB history so as to either receive free treatment or avoid receiving daily streptomycin injections and longer treatment course. Although such a bias could lower our estimate of new MDR-TB and pre-XDR/XDR-TB prevalence, most of the cases were unclustered in the present study, which could suggest that these biases might not play a role and a substantial number of drug-resistant TB cases would be diagnosed if all TB cases were to be tested.

In conclusion, we report substantial SLD resistance among both MDR and non-MDR groups. The detailed molecular analysis coupled with treatment history indicates that SLD resistance is acquired (*de novo*) most probably due to inappropriate use of SLD rather than primary transmission. This study highlights the utmost importance of strict regulation in the prescription of SLDs and careful patient management. In addition, our study underscores the needs for drug susceptibility testing and rapid diagnostics to effectively control drug-resistant TB in China.

Authors' contributions

BX, YH, QZ and WW were involved in the conception and design of the study. Data were acquired by YH and WL. Analysis of data was performed by YH, BM and LC. The first draft of manuscript was designed by YH. It was critically revised by BX, BM and BK. The final version for submission was approved by all co-authors.

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Transparency declaration

No financial support was received for this study. The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2015.08.023.

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