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RESEARCH ARTICLE

Clinical and Microbiological Characteristics of *Mycobacterium kansasii* Pulmonary Infections in China

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ABSTRACT *Mycobacterium kansasii*, an important opportunistic pathogen of humans, causes serious pulmonary disease. Sixty *M. kansasii* isolates were collected for investigating the clinical characteristics of patients with *M. kansasii* infections as well as drug susceptibility and genotypes of *M. kansasii*. More than 90% of the patients infected with *M. kansasii* were from eastern China. According to the internal transcribed spacers (ITS), *rpoB*, *hsp65*, and *tuf*, all *M. kansasii* isolates were classified as molecular type I, irrespective of the disease manifestation. Sixty *M. kansasii* isolates from China were diverse and separated into four branches. Pairwise average nucleotide identity (ANI) values for *M. kansasii* isolates affiliated with different genotypes were more than 85%. The earliest isolate was isolated from Jiangsu in 1983. Of the isolates, 78.3% (47/60) were isolated since 1999. All isolates were sensitive to rifabutin. All but one isolate was sensitive to clarithromycin. Sensitivity rates to rifampin, amikacin, moxifloxacin, and linezolid were 80.0%, 90.0%, 88.3%, and 91.7%, respectively. A high rate of resistance was noted for ciprofloxacin (44 isolates, 73.3%) and ethambutol (46 isolates, 76.7%). Compared with *M. tuberculosis* H37Rv, 12 mutations of *embCA* were observed in all *M. kansasii* isolates. All these 60 *M. kansasii* isolates shared identical sequences of *rpoB*, *inhA*, *katG*, *rrl*, *rrs*, *rpsL*, *gyrA*, and *gyrB*. In conclusion, *M. kansasii* isolates are exhibiting greater genetic diversity globally. The resistance mechanism of *M. kansasii* is not necessarily related to gene mutation.

IMPORTANCE *M. kansasii* type I is the main genotype spreading worldwide. The molecular history of the global spread of type I isolates remains largely unclear. We conducted a detailed analysis of genomic evolution of global *M. kansasii* isolates. Our results suggest that *M. kansasii* isolates exhibit greater genetic diversity globally.

KEYWORDS *Mycobacterium kansasii*, drug sensitivity, genotype

Mycobacterium kansasii, a slow-growing nontuberculous *mycobacterium* (NTM), is one of the most pathogenic and common NTMs isolated from humans (1). To date, seven genotypes (I–VII) have been identified, along with two intermediate (I/II) and atypical (IIb) types (2, 3). Of these, I and II are the most prevalent types that have been associated with NTM-pulmonary disease, while the others have usually been linked to environmental sources (3). There are three major methodologies used for the identification of *M. kansasii* subtypes, including sequence analysis of either *rpoB* or *hsp65* genes, 16S-23S rDNA internal transcribed spacers (ITS), and *tuf* typing (4).

The prevalence of *M. kansasii* diseases has varied widely by region and over time. In Slovakia, the United Kingdom, and Poland, a high prevalence of *M. kansasii* infections of all NTM isolates had been consistently reported (36%, 11%, and 35%, respectively) (5). Likewise, Brazil, Japan, and South Africa have reported high *M. kansasii* rates of infection of all NTM

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isolates (6). However, the prevalence of *M. kansasii* infections of all NTM isolates was low in Europe, with a mean isolation rate of 5% (5). *M. kansasii* infection is also more common in human immunodeficiency virus (HIV)-infected individuals. The annual incidence of *M. kansasii* infection in people living with HIV may be as high as 5.32%, compared with 0.005% in a survey of 44 U.S. states prior to the HIV epidemic (7–10). *M. kansasii* is also the second most frequent NTM found in HIV-infected patients.

M. kansasii pulmonary infection often presents with a clinical syndrome indistinguishable from that of *M. tuberculosis*; the most common presenting symptoms are cough, chest pain, dyspnea, and nonmassive hemoptysis (11, 12). In addition, common sites of *M. kansasii* extrapulmonary disease include the lymph nodes, skin, and musculoskeletal and genitourinary systems (1).

American Thoracic Society/Infectious Disease Society of America (ATS/IDSA) guidelines recommend daily therapy with isoniazid, rifampin, and ethambutol when the disease is fully sensitive to these drugs (11). For use only in the rare event of resistance to rifampin, alternative medications that are active against *M. kansasii* include streptomycin, clarithromycin, amikacin, ethionamide, sulfamethoxazole, rifabutin, linezolid, and fluoroquinolones. To date, *in vitro* drug susceptibility tests are standardized for only a few species of NTM. The resistance breakpoints of isoniazid and streptomycin to *M. kansasii* are not listed in the Clinical and Laboratory Standards Institute (CLSI) M24-A2 (13), and the genetic determinants of drug resistance in *M. kansasii* are virtually absent. In *Mycobacterium tuberculosis*, the genetic determinations of drug resistance are well characterized. The majority of drug resistance in clinical *Mycobacterium tuberculosis* strains is attributed to chromosomal mutations (14). Resistance related mutations could also exert certain fitness cost to the drug-resistant *Mycobacterium tuberculosis* strains, and growth fitness could be restored by the presence of compensatory mutations (14, 15).

The purpose of this study was to investigate the molecular identification and determination subtypes, clinical characteristics, geographic distribution, and drug susceptibility of clinical isolates of *M. kansasii* in China.

RESULTS

Demographic data and clinical characteristics. A total of 60 *M. kansasii* strains were isolated from patients. Fig. 1 shows the geographical distribution of these isolates. More than 90% of the patients were from eastern China. Characteristics of the study group are shown in Table 1. The mean age of patients was 43.7 $\pm$ 24.6 years, ranging from 17 to 85 years. Patients younger than 50 years accounted for 75% of the study population. Of the 60 patients, 44 (73.3%) were male. None of the medical records mentioned HIV/AIDS-positive status. The most common presenting symptom was cough, reported in 75.0% of patients. It was followed by sputum (61.7%) and hemoptysis (28.3%).

Associated lung diseases included COPD (9 patients, 15.0%), previous tuberculosis (13 patients, 21.7%), and bronchiectasis (5 patients, 8.3%). Radiological analysis revealed that pulmonary opacities and infiltrations were the dominant patterns in patients with *M. kansasii* infection (86.7% with opacities form and 63.3% with infiltrations form). Cavities and nodules were observed in approximately two-thirds of patients.

Genotypes. All 60 isolates tested were classified as *M. kansasii* type I based on *rpoB* and *hsp65* gene sequences (Fig. S1A and B in the supplemental material). Fifty-nine isolates exhibited 98.3% identity at both loci when compared with the *M. kansasii* genotype I reference strain (ATCC12478) based on ITS and *tuf* gene sequences (Fig. S1C and D). The polygenetic tree that was constructed based on the *tuf* and ITS grouped the *M. kansasii* subtypes incorrectly in some cases. *M. kansasii* isolate UM200925T0090 from this study was identified as *M. kansasii* genotype II based on the ITS sequence. *M. kansasii* isolate UM200925T0137 from this study was identified as *M. kansasii* genotype II based on the *tuf* gene. *M. kansasii* isolate UM200925T0080 has not been typed based on the *tuf* gene. A separate tree was created using the concatenated *rpoB*, *hsp65*, ITS, and *tuf* genes (Fig. 2). In addition, *M. kansasii* isolate GCA_002086895.1 isolated from the Netherlands was genotype II based on the ITS sequence, but genotype I according to the other method. Three isolates isolated from South

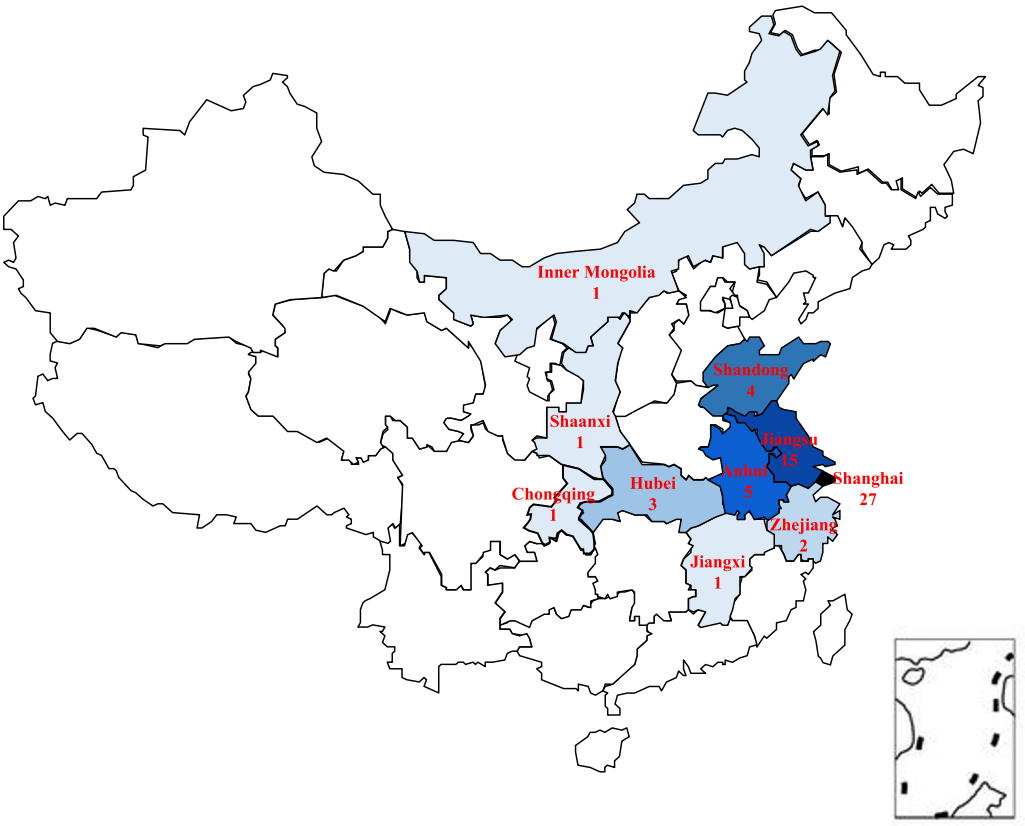

FIG 1 Map of China showing the distribution of 60 *M. kansasii* isolates included in this study (the numbers indicate the number of isolates in each region).

Korea (GCA_002705825.1, GCA_002705865.1, and GCA_002705785.1) were genotype II based on *hsp65* sequence, while classified as type I based on the ITS, *rpoB*, and *tuf*.

The ANI values for isolates of the same *M. kansasii* genotype were close to 100% (Fig. 3). Pairwise ANI values for *M. kansasii* isolates affiliated with different genotypes were more than 85%.

TABLE 1 Clinical characteristics of 60 patients with *M. kansasii* infection[a]

Characteristics	Data (*n*, %)
Age, yrs (mean ± SD)	43.7 ± 24.6
Sex	
Male	44 (73.3)
Female	16 (26.7)
Associated lung disease	
Previous tuberculosis	13 (21.7)
COPD	9 (15.0)
Bronchiectasis	5 (8.3)
Symptoms	
Chest pain	9 (15.0)
Cough	45 (75.0)
Sputum	37 (61.7)
Hemoptysis	17 (28.3)
Febrile sense	15 (25.0)
Radiologic findings	
Nodule	49 (81.7)
Cavity	40 (66.7)
Opacities	52 (86.7)
Infiltrations	38 (63.3)
Pleural thickening	21 (35.0)

[a]SD, standard deviation; COPD, chronic obstructive pulmonary disease.

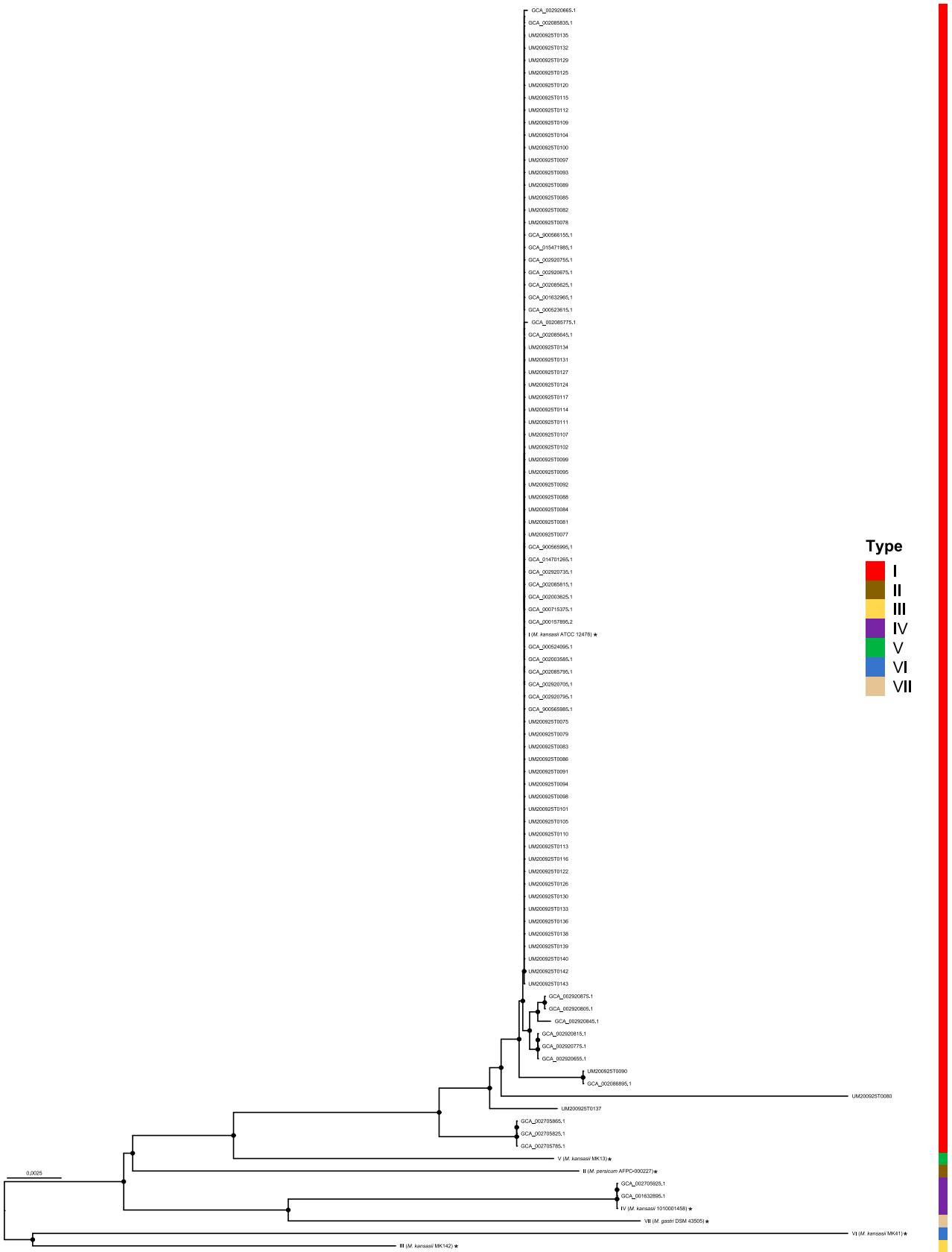

FIG 2 Phylogenetic tree based on *rpoB*, *hsp65*, internal transcribed spacers (ITS), and *tuf* genes sequences constructed using the neighbor-joining method. The bootstrap values were calculated from 1,000 replications.

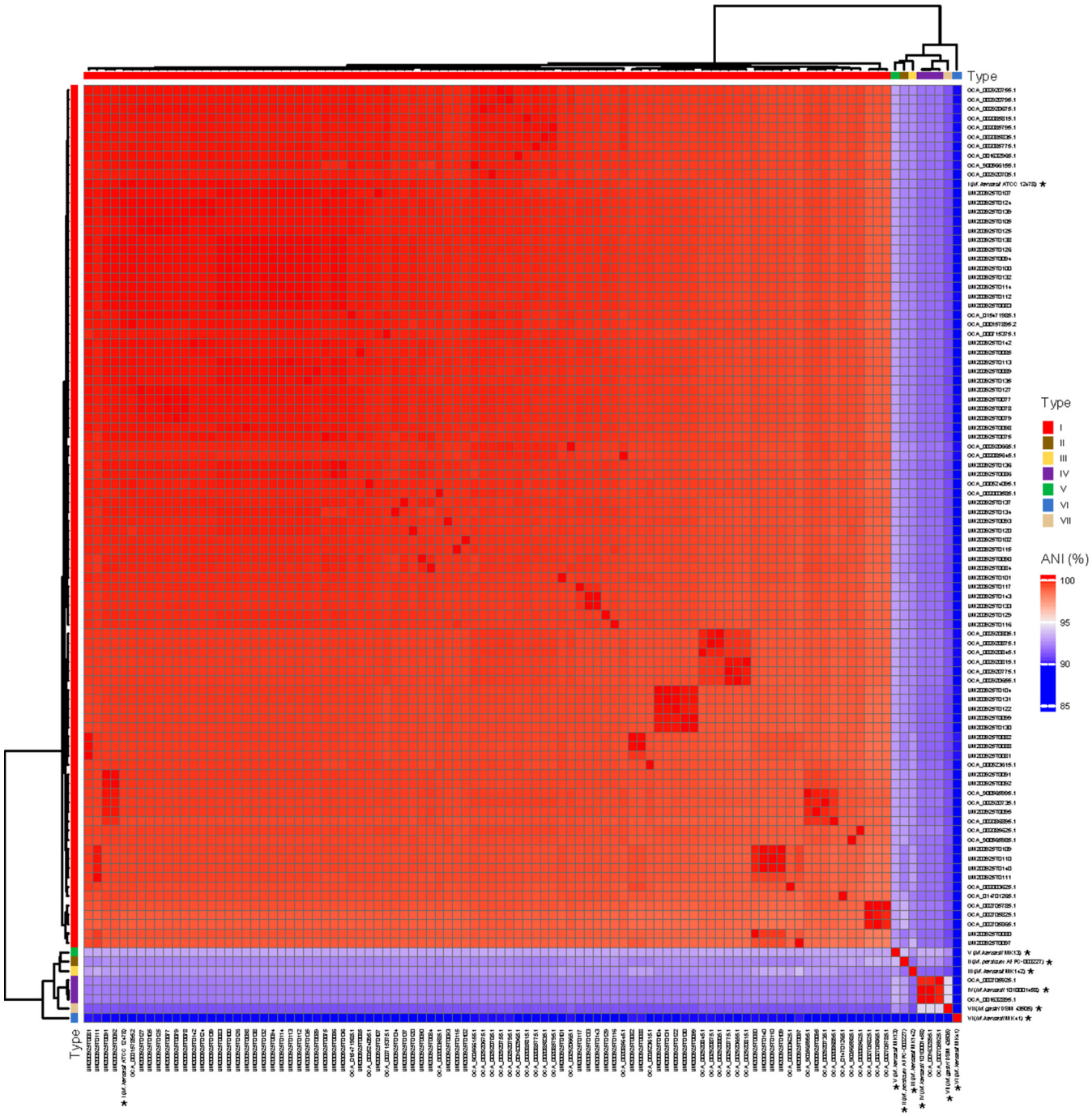

FIG 3 Pairwise comparisons of ANIs of *M. kansasii* subtypes I-VII.

Phylo-geographical context and comparative genomics of *M. kansasii*. To analyze the evolution of the *M. kansasii* from China, we determined the genome sequences of 60 *M. kansaii* isolates. The genomes of 36 *M. kansasii* isolates (supplemental file 2) previously reported by NCBI (https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/1793/) were included for analysis to gain a better understanding of the *M. kansasii* genome globally. Phylogenetic analysis and divergence time estimation among 60 *M. kansasii* isolates are shown in Fig. 4. The earliest isolate was from Jiangsu in 1983. Of the isolates, 78.3% (47/60) were isolated since 1999.

M. kansasii isolates exhibit greater genetic diversity globally (Fig. 5). Our analysis divided the 96 genomes into three main groups when compared to the genome of the ATCC isolate,

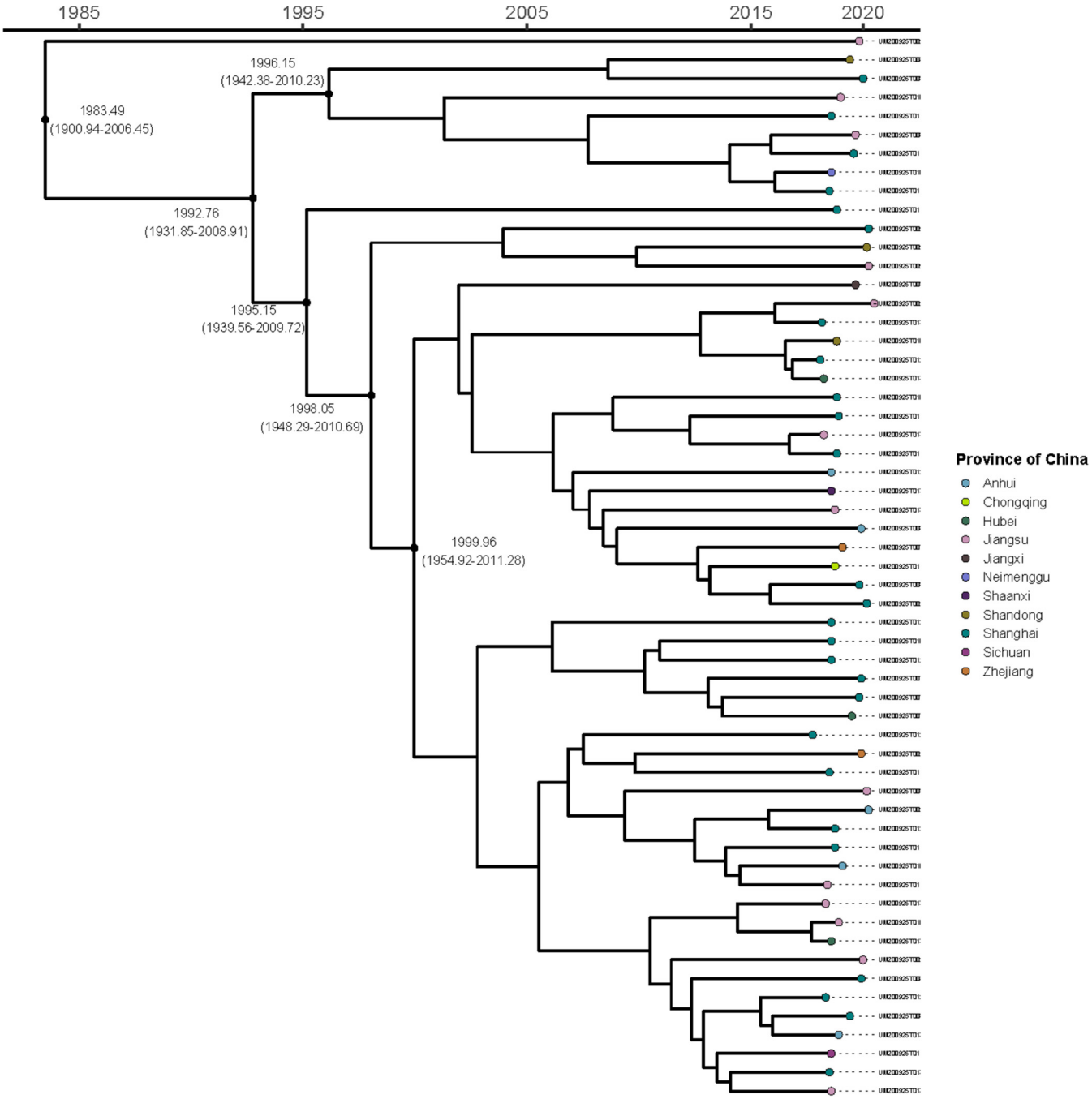

FIG 4 The output from Gubbins was used as the input for BactDating v1.0 to perform phylogenetic dating based on a Bayesian approach. The Markov chain Monte Carlo chain lengths were run for 100 million cycles to convergence; the effective sample size of the inferred parameters α, μ, and σ was >200.

one presenting less than 200 single nucleotide polymorphisms (SNPs; $n = 88$), a second group with either more than 1,500 SNPs and less than 2,000 SNPs ($n = 6$), and the third group with more than 10,000 SNPs ($n = 2$). SNPs are shown in supplemental file 3. The 60 *M. kansasii* isolates isolated from this study presented homogeneously distributed SNPs over the entire genome. Although genomic comparisons of 8 isolate from Brazil and Germany revealed greater heterogeneity, 60 isolates had fewer than 20 SNPs compared to the reference ATCC12478 isolate. A threshold of 4 SNPs will be best suited to fit the clustering from this study. SNP diversity among phylogenetically linked samples from this study ranged from 0 to 20 SNPs. Our analysis divided the 60 genomes into 4 main clusters, one presenting less than 4 SNPs, a second group

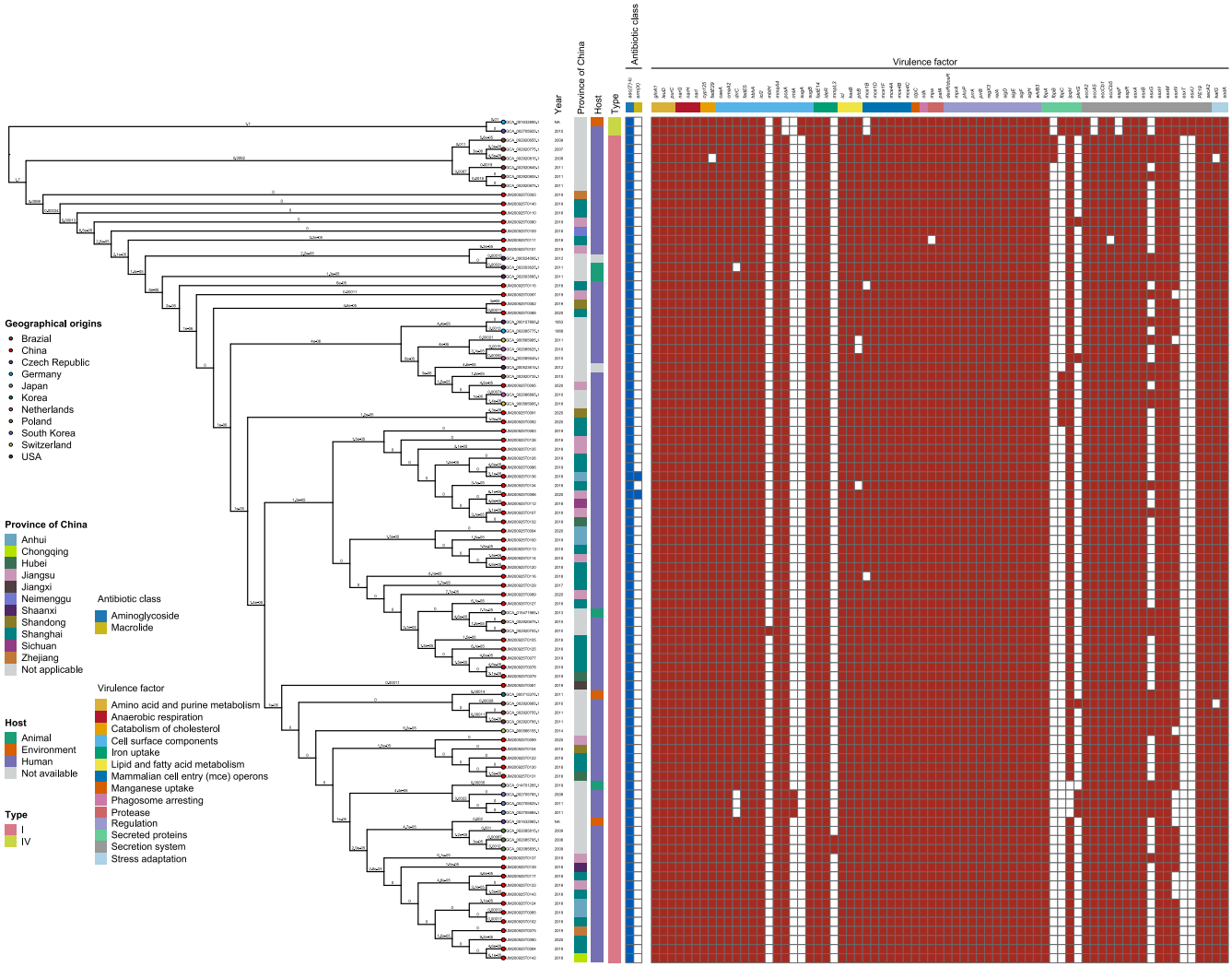

FIG 5 Phylogenetic analysis of 96 *M. kansasii* isolates. A phylogenetic tree was constructed using the SNPs outside of the recombination regions with RAxML using a GTR model and gamma correction. Colors in columns illustrate region of origin, mainland China provinces, host, type, antibiotic class, and virulence factor.

with less than 5 SNPs and more than 10 SNPs, a third group with more than 11 SNPs and less than 15 SNPs, and a fourth group with more than 15 SNPs.

Sixty *M. kansasii* isolates from China were diverse and separated into four branches. Among the 60 *M. kansasii* isolates, 45 isolates (75.0%) belonged to the same branch and were homologous to 45.7% (16/35) of *M. kansasii* isolates in the NCBI database. Twelve *M. kansasii* isolates from Brazil belonged to four branches, which was the same as in a previous study (16). *M. kansasii* isolates from Poland (3 isolates), South Korea (3 isolates), and the Czech Republic (1 isolate) were more closely related.

Virulence factor-encoding gene in *M. kansasii* genomes. All virulence genes that met the filter threshold were listed (the filter threshold refers to the reference virulence gene, and homologous genes should have a coverage of ≥85% and a similarity of ≥85%). Virulence factor-encoding genes are listed in Fig. 5. Compared with *M. kansasii* genotype I, *pcaA*, *mce1B*, *fbpB*, *eccA5*, and *espF* genes were missing in *M. kansasii* genotype IV isolates. The *sugA*, *fbpC*, *pknG*, *esxU*, and *esxT* genes were absent in *M. kansasii* genotype I isolates. The *esxG* gene was absent in 56 *M. kansasii* isolates isolated from this study.

Drug susceptibility pattern. MIC ranges, MICs, MIC_{50}, and MIC_{90} (MIC required to inhibit the growth of 50% and 90% of the strains, respectively) are summarized in Table 2. All isolates were sensitive to rifabutin (RFB), and all but one isolate was sensitive to clarithromycin (CLR). Sensitivity rates of 60 isolates tested to rifampin (RIF), amikacin (AMK),

TABLE 2 MIC ranges, MIC_{50}, MIC_{90}, and drug resistance in 60 clinical *M. kansasii* isolates from China

Antimicrobial agents	Tested concn ranges (μg/mL)	Breakpoints (μg/ml)	Resistance (%, *n*)	Susceptibility (%, *n*)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
Primary agents						
Clarithromycin	0.06–16	>16	1.67(1)	98.3(59)	0.25	2
Rifampin	0.12–8	>1	20.0(12)	80.0(48)	0.5	4
Secondary agents						
Amikacin	1–64	>32	10.0(6)	90.0(54)	2	16
Ciprofloxacin	0.12–64	>2	**73.3(44)**[c]	26.7(16)	2	16
Moxifloxacin	0.12–8	>2	11.7(7)	88.3(53)	0.125	2
Rifabutin	0.12–8	>2	0.0(0)	100.0(60)	0.25	0.5
Linezolid	1–64	>16	8.3(5)	91.7(55)	2	8
Streptomycin	0.5–64	-[a]			8	32
Ethambutol	0.5–16	>4	**76.7(46)**[c]	23.3(14)	8	16
Isoniazid	0.25–8	-[a]			2	8
SXT[b]	0.12–8	>2/38	31.7(19)	68.3(41)	0.5	8
Ethionamide	0.3–20	-[a]			0.6	2.5
Doxycycline	0.12–16	-[a]			16	16

[a]Only the MIC value reported, with no interpretation, for these drugs.
[b]SXT: trimethoprim-sulfamethoxazole.
[c]In boldface: resistance rate of ciprofloxacin and ethambutol to *M. kansasii* isolates was more than 70%.

moxifloxacin (MXF), and linezolid (LNZ) were 80.0%, 90.0%, 88.3%, and 91.7%, respectively. There were high resistance rates to ciprofloxacin (CIP; 73.3%, 44/60) and ethambutol (EMB; 76.7%,46/60). Four of the 13 antimicrobials tested, S, INH ETH, and DOX, have no cutoff point established by CLSI, so it was not possible to classify the samples as susceptible or resistant. MIC distributions of *M. kansasii* are shown in Fig. 6. The highest detected MIC for INH and S was >8 μg/mL and was found in 9 (15.0%) and 15 (25.0%) *M. kansasii* isolates, respectively. The MIC_{90} for S, INH, and DOX varied greatly (32 μg/mL, 8 μg/mL, and 16 μg/mL, respectively). However, ETH was highly active against *M. kansasii*, with MIC_{50} and MIC_{90} values of 0.6 μg/mL and 2.5 μg/mL, respectively.

Mutation profiling. The results of mutations are summarized in supplemental file 3. For EMB-resistant *M. kansasii* isolates and EMB-susceptible *M. kansasii* isolates, the sequences at the EMB resistance associated loci (*ebmB* and *embCA*) were identical. There were 304 amino acids different from those of *M. tuberculosis* H37Rv. All *M. kansasii* isolates had M306I and G406R substitution in the EMB resistance determining region, as it is referred to in *M. tuberculosis*. Compared with *M. tuberculosis* H37Rv, 12 mutations of *embCA* were observed in all *M. kansasii* isolates. All of these 60 *M. kansasii* isolates shared identical sequences of *rpoB*, *inhA*, *katG*, *rrl*, *rrs*, *rpsL*, *gyrA*, and *gyrB*.

DISCUSSION

M. kansasii is the second most common cause of NTM disease in some regions of the United States, England, Wales, and France (17). In China, *M. kansasii* has been isolated from pulmonary infections in many areas, but the incidence is highest in the highly urbanized eastern and southern coastal regions (18, 19). From 2008 to 2012 in Shanghai, it was responsible for nearly half of all NTM infections (20).

M. kansasii subtype I was responsible for most human infections in Europe, the United States, and Japan (21, 22). Zhang et al. have shown that all of these methods, RFLP analysis with the major polymorphic tandem repeat probe and the IS1652 probe, PFGE, amplified fragment length polymorphism analysis, and PCR restriction analysis (PRA) of the *hsp65* gene, showed excellent typeability and reproducibility (21). In this study, the results of *hsp65* gene analysis were highly similar to those obtained with the *tuf* gene. All genotype I strains were isolated from patients with *M. kansasii* infections who met the ATS case definition criteria. Interestingly, all *M. kansasii* isolates were classified as molecular type I irrespective of the disease manifestation. In Brazil, Edson Machado et al. showed that 12 clinical *M. kansasii* isolates from Brazilian patients with pulmonary disease belonged to genotype I, as determined by *hsp65* sequencing (16).

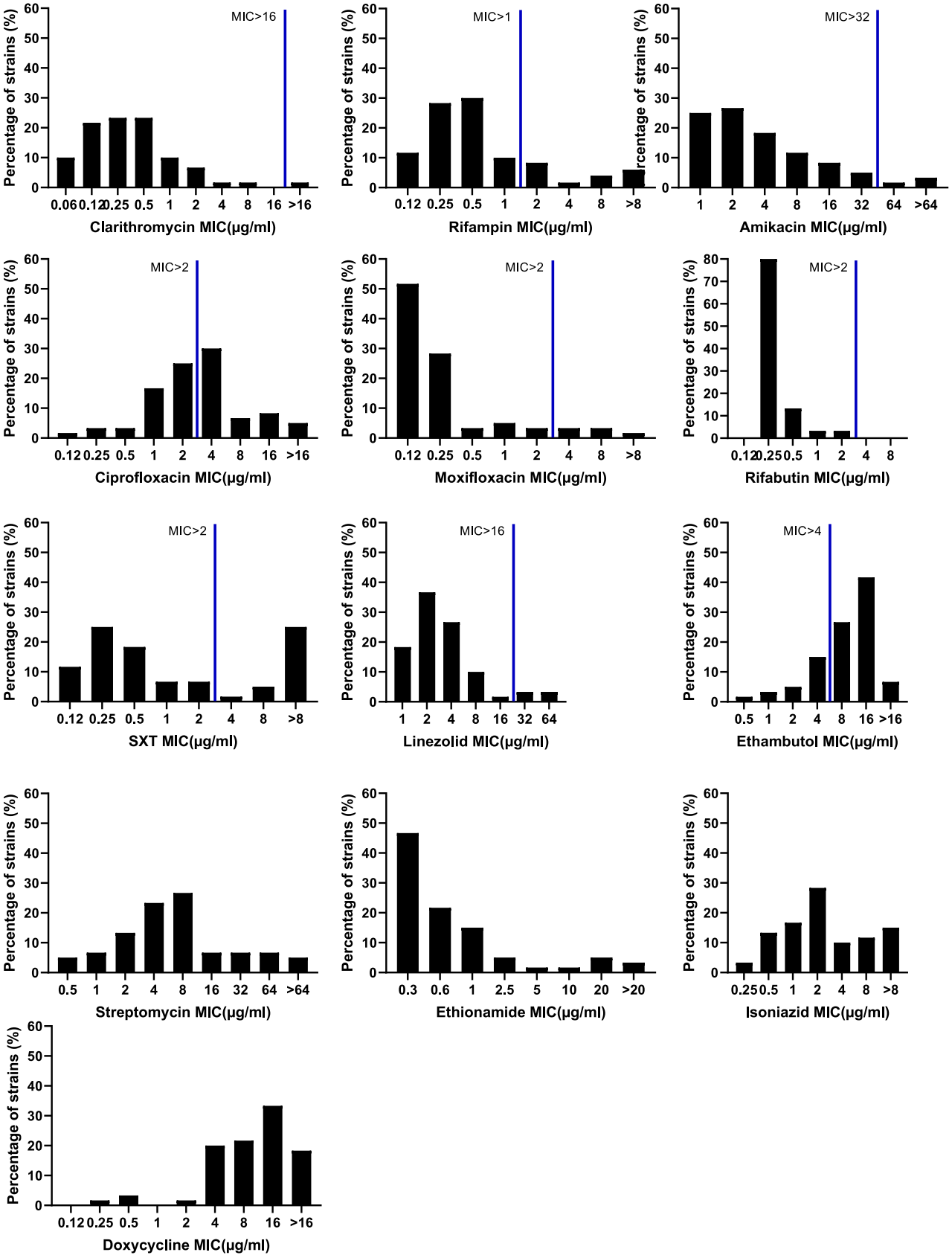

FIG 6 MIC distributions of 60 *M. kansasii* clinical isolates. Solid lines: tentative species-specific epidemiological cut-off (ECOFF) values (according to CLSI). No solid lines: no breakpoints were applicable.

M. kansasii genotype I, as defined by PCR restriction-enzyme pattern analysis, is the most common genotype associated with human disease in China. In the present study, 98.3% of *M. kansasii* isolates were highly homogenous. In other *Mycobacterium* species, such small differences would be suggestive of the patients being epidemiologically linked. It has been reported that the municipal water supply is the main source of *M. kansasii* infections (23). We speculated that *M. kansasii* in this study may also originate from bacteria in other places that spread through water sources and were derived from the same ancestor as previously reported. However, it is unclear how waterborne *M. kansasii* could have achieved dissemination.

A previous study reported that *M. kansasii* subtype I contains five ESX systems, and the overall arrangement of the five ESX systems is similar to that in *M. tuberculosis* H37Rv (24). ESX-3, which encodes the proteins EsxG and EsxH, is required for optimal growth of *M. tuberculosis* and has been associated with essential processes such as iron and zinc acquisition. The *esxG* gene was absent in 56 *M. kansasii* isolates in the present study.

In addition, a previous study reported that *esxG* and *esxH* were upregulated in a drug-resistant *M. tuberculosis* isolate (25), an effect of EsxG and EsxH expression in mycobacterial RIF and INH resistance. However, *esxG* and *esxH* had no association with drug resistance of *M. kansasii* isolates in the present study.

The recommended therapy for *M. kansasii* infections is isoniazid (INH), RIF, and EMB. Treatment failure is almost always associated with RIF resistance. Previously described levels of resistance to RIF, established with the microdilution method, varied widely from 1.9% to 56.4% for RIF. In this study, resistance to RIF (20%) was found in 12 *M. kansasii* isolates. However, very high rates of RIF resistance were found in Iran (50.0%) and Beijing, China (56.4%) (18, 26). These differences could be related to the prevailing susceptibility pattern of *M. kansasii* isolates found in a particular geographical area. In addition, RIF has been shown to be unstable in current media used in phenotypic drug susceptibility testing of SGM (27, 28). RIF concentration in Middlebrook 7H9 medium had decreased by 92% after 7 days, and the microbiological assay revealed decreases in RIF concentration of $\geq$75% after 14 days (27). Resistance to RFB was not detected among the isolates in this study. RFB may be an alternative to RIF. In addition, INH may be useful clinically, but breakpoints for susceptibility and resistance for NTM have not been established. The proportion of isolates resistant to INH by microdilution method, using 1 mg/L INH as breakpoint, was 100% (29). The frequencies of INH resistance were 2.9% in Spain (30) and 8% in Brazil (31), with a breakpoint set at 5 mg/L, lower than those detected by this study, 26.7%. Consequently, susceptibility testing of INH in *M. kansasii* isolates may use 5 mg/L as the critical concentrations (32).

Notably, we found a very high rate of resistance to CIP (73.35%) and EMB (76.7%). This is similar to a report from da Silva Telles et al., who observed a high ciprofloxacin resistance rate (66%) and a high EMB resistance rate (94%) in Brazil (31). Previously described levels of resistance to CIP and EMB, established with the microdilution method, varied widely from 15% to 66% for CIP (26, 29, 31–33) and 0% to 100% for EMB (18, 26, 29–33). The high drug resistance rate may be associated with the widespread use of antitubercular drugs.

More recently, clarithromycin showed excellent activity against these microorganisms (98.33%), similar to previous studies that showed high *in vitro* activity of CLR against *M. kansasii*, with less than 1% of isolates showing resistance. High resistance (20.5%) of the *M. kansasii* isolates to CLR has been reported only in Beijing, China (18).

Additionally, AMK, LNZ, S, and rimethoprim-sulfamethoxazole (SXT) have good activity against *M. kansasii in vitro*. Resistance to AMK has seldom been reported in *M. kansasii*, with 0–5.1% of isolates resistant (18, 26, 31, 33), while high resistance was reported only in the Netherlands (54%) (29). The reason may be application of a lower breakpoint (5 mg/L). In previous studies, resistance to LNZ was not detected among *M. kansasii* isolates. Only in one study from Beijing, China, 25 (32.1%) of the isolates were resistant to LNZ, MIC_{50}, and MIC_{90} values of 4 and 128 mg/L, respectively (18). However, our results (MIC_{50} = 2 mg/L and MIC_{90} = 8 mg/L) showed good activity, with 91.7% of isolates susceptible. LNZ may represent a

good therapeutic alternative in *M. kansasii* infections, but there have been significant toxicity issues with this drug. The newer oxazolidinones have fewer side effects than LNZ and also have good *in vitro* activity against *M. kansasii*. With a breakpoint of 10 mg/L, resistance to S was 23.3% in this study, 14% in Brazil (31), and 40% in Greece (34). The MIC values of S (MIC_{50} = 8 mg/L, MIC_{90} = 32 mg/L) were similar to that reported from Poland (MIC_{50} = 8 mg/L) (32), while different from that of the U.S. (MIC_{50} = 2 mg/L) (35). SXT resistance detected in this study (31.7%) was higher than that reported in Poland (0%) (32) and Taiwan (18.9%) (33) using the same criteria. The reason may be its widespread use for controlling other microorganisms.

This research has several limitations. First, there were a limited number of *M. kansasii* isolates covered in this study. Second, further studies are required to conduct analysis of *esxG* and *esxH* genes and their secretion system.

In conclusion, these are preliminary data on the variability within the *M. kansasii* genotype I isolates in China. We evidenced four clusters that are separated by the presence of large numbers of SNPs throughout the genome. We found high activity of RFB, CLR, RIF, AMK, MXF, and LNZ against *M. kansasii* isolates. The high resistance rates observed with CIP and EMB should be cause for concern. Drug resistance in *M. kansasii* may have different genetic determinants than resistance to the same drugs in *M. tuberculosis*.

MATERIALS AND METHODS

***M. kansasii* isolates and genotyping.** Sixty *M. kansasii* isolates isolated from 60 patients were included in this study. All *M. kansasii* isolates were collected from 2018 to 2020 at a tertiary hospital, Shanghai Pulmonary Hospital in Shanghai, eastern China. Each isolate was obtained from the sputum or bronchial lavage of patients with *M. kansasii* pulmonary infection, based on the ATS guidelines for the diagnosis of *M. kansasii* infection (11). The epidemiological and clinical characteristics were obtained from medical records.

M. kansasii isolates were grown in Middlebrook 7H9 broth with OADC (0.85% sodium chloride, 5% bovine albumin, 2% dextrose, 0.003% catalase) at 37°C. Traditional species identification with para nitro benzoic acid and thiophene-2-carboxylic acid hydrazide (TCH) was performed to distinguish NTM from *Mycobacterium tuberculosis* complex. In parallel, all isolates were identified as *M. kansasii* by whole genome sequencing (WGS). PRA for the *hsp65* and *tuf* genes was carried out as previously described (36).

Ethics statement. The research protocol was approved by the Ethics Committee of Shanghai Pulmonary Hospital. We confirm that all adult subjects provided informed consent. Written or oral informed consent was obtained.

Genome sequencing and assembly. For WGS, genomic DNA from all 60 *M. kansasii* strains was extracted using the cetyl-trimethyl-ammonium bromide method, as described elsewhere (37). Extracted genomic DNA was quantified by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Genomic DNA libraries were constructed using the Illumina TruSeq DNA Nano Library Prep Kit following the manufacturer's protocol. The libraries were sequenced on a HiSeq X or a NovaSeq instrument (Illumina, San Diego, CA, USA) at a read length of 2 × 150 bp. The genomes were assembled into contigs using SPAdes (https://github.com/ablab/spades) (38), and the complete assemblies were annotated by Prokka (https://github.com/tseemann/prokka) (39).

Genotyping and comparative genomics of *M. kansasii*. In addition to the genomes of 60 *M. kansasii* strains sequenced in this study, genomes of the other 36 *M. kansasii* genome sequences available on the NCBI genome database (http://www.ncbi.nlm.nih.gov/genbank/) were downloaded for analysis to gain a better understanding of the genome epidemiology of *M. kansasii*. We aligned 96 genome sequences (60 from our study, 12 from Brazil, 4 from the U.S., 4 from Korea, 3 from Poland, 3 from the Netherlands, 2 from Japan, 3 from Switzerland, 2 from the Czech Republic, 2 from Germany, and 1 from the American Type Culture Collection, ATCC12478). The genomic sequences of these strains' accession numbers are provided in supplemental file 2. For single-gene phylogenies, the respective sequences of each genetic locus were subjected to multiple alignment in MEGA X software (ClustalW algorithm). The resulting fragments were further trimmed to remove unnecessary gaps or regions using Trimal. These sequences were then used for evolutionary distance calculation according to the Jukes-Cantor model. Phylogenetic trees for each target locus sequence were built using the neighbor-joining method and midpoint rooted with the MEGA X software. Tree topologies were evaluated by bootstrap analysis based on 1,000 replications. The average nucleotide identity (ANI) was calculated by OrthoANI (40). The pairwise ANI values were determined from using pyani (https://github.com/widdowquinn/pyani) and visualized using the complex heatmap R package (41). Single nucleotide polymorphisms (SNPs) were identified using snippy (https://github.com/tseemann/snippy), and a pseudo-genome alignment was generated. Recombined regions were detected using Gubbins. A phylogenetic tree was constructed using the SNPs outside of the recombination regions with RAxML using a GTR model and gamma correction. The output from Gubbins was used as the input for BactDating v1.0 to perform phylogenetic dating based on a Bayesian approach. The Markov chain Monte Carlo chain lengths were run for 100 million cycles to convergence; the effective sample size of the inferred parameters α, μ, and σ was >200. Antimicrobial resistance genes were mined using AMRFinderPlus v3.9.8. Virulence genes were identified by ABRicate v1.01 (https://github.com/tseemann/abricate) using the VFDB database (http://www.mgc.ac.cn/VFs/main.htm) with 85% identity and 85% query coverage cutoffs.

Antimycobacterial drug susceptibility testing. Antimicrobial susceptibility testing was performed following the guidelines of the CLSI (13). A panel of 13 antimicrobials using Sensititre SLOMYCO plates (Thermo Fisher Scientific, USA) were assessed according to the manufacturer's instructions. The antimicrobial agents and the concentrations tested were as follows: amikacin (AMK), 1–64 μg/mL; ciprofloxacin (CIP), 0.12–16 μg/m; clarithromycin (CLA), 0.06–64 μg/mL; moxifloxacin (MXF), 0.12-8 μg/mL; trimethoprim-sulfamethoxazole (SXT), 0.12–8 μg/mL; linezolid (LZD), 1–64 μg/mL; doxycline (DOX), 0.12–16 μg/mL; streptomycin (S), 0.5–64 μg/mL; rifampin (RIF), 0.25–8 μg/mL; rifabutin (RFB), 0.12–8 μg/mL; isoniazid (INH), 0.25–8 μg/mL; ethambutol (EMB), 0.5–16 μg/mL; and ethionamide (ETH), 0.3–20 μg/mL (Table 1). *M. kansasii* strain ATCC 12478 was used as a reference strain for MIC testing. MIC_{50} and MIC_{90} values were derived from MIC distribution.

Statistical analysis. All statistical analyses were performed using SPSS software, version 24 (IBM, USA). The MIC distributions were analyzed using GraphPad Prism software (version 7.00, La Jolla, CA, USA).

Data availability. The Illumina sequences of the 60 *M. kansasii* isolates in this study are available in the Sequence Read Archive (BioProject ID: PRJNA780966). The accession numbers are listed in supplemental file 1.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, XLS file, 0.03 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 4, XLS file, 0.03 MB.

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REFERENCES

1. Johnston JC, Chiang L, Elwood K. 2017. *Mycobacterium kansasii*. Microbiol Spectr 5:21.
2. Taillard C, Greub G, Weber R, Pfyffer GE, Bodmer T, Zimmerli S, Frei R, Bassetti S, Rohner P, Piffaretti JC, Bernasconi E, Bille J, Telenti A, Prod'hom G. 2003. Clinical implications of *Mycobacterium kansasii* species heterogeneity: Swiss National Survey. J Clin Microbiol 41:1240–1244. https://doi.org/10.1128/JCM.41.3.1240-1244.2003.
3. Iwamoto T, Saito H. 2006. Comparative study of two typing methods, *hsp65* PRA and ITS sequencing, revealed a possible evolutionary link between *Mycobacterium kansasii* type I and II isolates. FEMS Microbiol Lett 254:129–133. https://doi.org/10.1111/j.1574-6968.2005.00013.x.
4. Bakula Z, Modrzejewska M, Safianowska A, van Ingen J, Proboszcz M, Bielecki J, Jagielski T. 2016. Proposal of a new method for subtyping of *Mycobacterium kansasii* based upon PCR restriction enzyme analysis of the *tuf* gene. Diagn Microbiol Infect Dis 84:318–321. https://doi.org/10.1016/j.diagmicrobio.2015.12.009.
5. Hoefsloot W, van Ingen J, Andrejak C, Angeby K, Bauriaud R, Bemer P, Beylis N, Boeree MJ, Cacho J, Chihota V, Chimara E, Churchyard G, Cias R, Daza R, Daley CL, Dekhuijzen PN, Domingo D, Drobniewski F, Esteban J, Fauville-Dufaux M, Folkvardsen DB, Gibbons N, Gomez-Mampaso E, Gonzalez R, Hoffmann H, Hsueh PR, Indra A, Jagielski T, Jamieson F, Jankovic M, Jong E, Keane J, Koh WJ, Lange B, Leao S, Macedo R, Mannsaker T, Marras TK, Maugein J, Milburn HJ, Mlinko T, Morcillo N, Morimoto K, Papaventsis D, Palenque E, Paez-Pena M, Piersimoni C, Polanova M, Rastogi N, Richter E, et al. 2013. The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples: an NTM-NET collaborative study. Eur Respir J 42:1604–1613. https://doi.org/10.1183/09031936.00149212.
6. Martin-Casabona N, Bahrmand AR, Bennedsen J, Thomsen VO, Curcio M, Fauville-Dufaux M, Feldman K, Havelkova M, Katila ML, Koksalan K, Pereira MF, Rodrigues F, Pfyffer GE, Portaels F, Urgell JR, Rusch-Gerdes S, Tortoli E, Vincent V, Watt B, Spanish Group for Non-Tuberculosis Mycobacteria. 2004. Non-tuberculous mycobacteria: patterns of isolation. A multi-country retrospective survey. Int J Tuber Lung Dis 8:1186–1193. https://www.ncbi.nlm.nih.gov/pubmed/15527150.
7. Bamberger DM, Driks MR, Gupta MR, O'Connor MC, Jost PM, Neihart RE, McKinsey DS, Moore LA, Kansas City AIDS Research Consortium. 1994. *Mycobacterium kansasii* among patients infected with human immunodeficiency virus in Kansas City. Clin Infect Dis 18:395–400. https://doi.org/10.1093/clinids/18.3.395.
8. Bloch KC, Zwerling L, Pletcher MJ, Hahn JA, Gerberding JL, Ostroff SM, Vugia DJ, Reingold AL. 1998. Incidence and clinical implications of isolation of *Mycobacterium kansasii*: results of a 5-year, population-based study. Ann Intern Med 129:698–704. https://doi.org/10.7326/0003-4819-129-9-199811010-00004.
9. Carpenter JL, Parks JM. 1991. *Mycobacterium kansasii* infections in patients positive for human immunodeficiency virus. Rev Infect Dis 13:789–796. https://doi.org/10.1093/clinids/13.5.789.
10. Good RC. 1980. Isolation of nontuberculous mycobacteria in the United States, 1979. Infect Dis 142:779–783. https://doi.org/10.1093/infdis/142.5.779.
11. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, Holland SM, Horsburgh R, Huitt G, Iademarco MF, Iseman M, Olivier K, Ruoss S, von Reyn CF, Wallace RJ, Jr, Winthrop K, ATS Mycobacterial Diseases Subcommittee, American Thoracic Society, Infectious Disease Society of America. 2007. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med 175:367–416. https://doi.org/10.1164/rccm.200604-571ST.
12. Arend SM, Cerda de Palou E, de Haas P, Janssen R, Hoeve MA, Verhard EM, Ottenhoff TH, van Soolingen D, van Dissel JT. 2004. Pneumonia caused by *Mycobacterium kansasii* in a series of patients without recognised immune defect. Clin Microbiol Infect 10:738–748. https://doi.org/10.1111/j.1469-0691.2004.00898.x.
13. Clinical Laboratory Standards Institute. 2011. Susceptibility testing of Mycobacteria, Nocardiae, and other aerobic actinomycetes, 2nd ed. CLSI, Wayne, PA.
14. Gygli SM, Borrell S, Trauner A, Gagneux S. 2017. Antimicrobial resistance in *Mycobacterium tuberculosis*: mechanistic and evolutionary perspectives. FEMS Microbiol Rev 41:354–373. https://doi.org/10.1093/femsre/fux011.
15. Miotto P, Zhang Y, Cirillo DM, Yam WC. 2018. Drug resistance mechanisms and drug susceptibility testing for tuberculosis. Respirology 23:1098–1113. https://doi.org/10.1111/resp.13393.

16. Machado E, Vasconcellos SEG, Cerdeira C, Gomes LL, Junqueira R, Carvalho LD, Ramos JP, Redner P, Campos CED, Caldas PCS, Gomes A, Goldenberg T, Montes FF, Mello FCQ, Mussi VO, Lasunskaia E, Soolingen DV, Miranda AB, Rigouts L, Jong BC, Meehan CJ, Catanho M, Suffys PN. 2018. Whole genome sequence *of Mycobacterium kansasii* isolates of the genotype 1 from Brazilian patients with pulmonary disease demonstrates considerable heterogeneity. Mem Inst Oswaldo Cruz 113:e180085. https://doi.org/10.1590/0074-02760180085.
17. Cook JL. 2010. Nontuberculous mycobacteria: opportunistic environmental pathogens for predisposed hosts. Br Med Bull 96:45–59. https://doi.org/10.1093/bmb/ldq035.
18. Li Y, Pang Y, Tong X, Zheng H, Zhao Y, Wang C. 2016. *Mycobacterium kansasii* subtype i is associated with clarithromycin resistance in China. Front Microbiol 7:2097. https://doi.org/10.3389/fmicb.2016.02097.
19. Yu X, Liu P, Liu G, Zhao L, Hu Y, Wei G, Luo J, Huang H. 2016. The prevalence of non-tuberculous mycobacterial infections in mainland China: systematic review and meta-analysis. J Infect 73:558–567. https://doi.org/10.1016/j.jinf.2016.08.020.
20. Wu J, Zhang Y, Li J, Lin S, Wang L, Jiang Y, Pan Q, Shen X. 2014. Increase in nontuberculous mycobacteria isolated in Shanghai, China: results from a population-based study. PLoS One 9:e109736. https://doi.org/10.1371/journal.pone.0109736.
21. Zhang Y, Mann LB, Wilson RW, Brown-Elliott BA, Vincent V, Iinuma Y, Wallace RJ Jr. 2004. Molecular analysis of *Mycobacterium kansasii* isolates from the United States. J Clin Microbiol 42:119–125. https://doi.org/10.1128/JCM.42.1.119-125.2004.
22. Picardeau M, Prod'Hom G, Raskine L, LePennec MP, Vincent V. 1997. Genotypic characterization of five subspecies of *Mycobacterium kansasii*. J Clin Microbiol 35:25–32. https://doi.org/10.1128/jcm.35.1.25-32.1997. https://www.ncbi.nlm.nih.gov/pubmed/8968876
23. Luo T, Xu P, Zhang Y, Porter JL, Ghanem M, Liu Q, Jiang Y, Li J, Miao Q, Hu B, Howden BP, Fyfe JAM, Globan M, He W, He P, Wang Y, Liu H, Takiff HE, Zhao Y, Chen X, Pan Q, Behr MA, Stinear TP, Gao Q. 2021. Population genomics provides insights into the evolution and adaptation to humans of the waterborne pathogen Mycobacterium kansasii. Nat Commun 12:2491. https://doi.org/10.1038/s41467-021-22760-6.
24. Guan Q, Ummels R, Ben-Rached F, Alzahid Y, Amini MS, Adroub SA, van Ingen J, Bitter W, Abdallah AM, Pain A. 2020. Comparative genomic and transcriptomic analyses of *Mycobacterium kansasii* subtypes provide new insights into their pathogenicity and taxonomy. Front Cell Infect Microbiol 10:122. https://doi.org/10.3389/fcimb.2020.00122.
25. Penuelas-Urquides K, Gonzalez-Escalante L, Villarreal-Trevino L, Silva-Ramirez B, Gutierrez-Fuentes DJ, Mojica-Espinosa R, Rangel-Escareno C, Uribe-Figueroa L, Molina-Salinas GM, Davila-Velderrain J, Castorena-Torres F, Bermudez de Leon M, Said-Fernandez S. 2013. Comparison of gene expression profiles between pansensitive and multidrug-resistant strains of *Mycobacterium tuberculosis*. Curr Microbiol 67:362–371. https://doi.org/10.1007/s00284-013-0376-8.
26. Heidarieh P, Mirsaeidi M, Hashemzadeh M, Feizabadi MM, Bostanabad SZ, Nobar MG, Hashemi Shahraki A. 2016. *In vitro* antimicrobial susceptibility of nontuberculous mycobacteria in Iran. Microb Drug Resist 22:172–178. https://doi.org/10.1089/mdr.2015.0134.
27. Schoutrop ELM, Brouwer MAE, Jenniskens JCA, Ferro BE, Mouton JW, Aarnoutse RE, van Ingen J. 2018. The stability of antimycobacterial drugs in media used for drug susceptibility testing. Diagn Microbiol Infect Dis 92:305–308. https://doi.org/10.1016/j.diagmicrobio.2018.06.01.5.
28. Wetzstein N, Kohl TA, Andres S, Schultze TG, Geil A, Kim E, Biciusca T, Hugel C, Hogardt M, Lehn A, Vehreschild M, Wolf T, Niemann S, Maurer FP, Wichelhaus TA. 2020. Comparative analysis of phenotypic and genotypic antibiotic susceptibility patterns in *Mycobacterium avium* complex. Int J Infect Dis 93:320–328. https://doi.org/10.1016/j.ijid.2020.02.059.
29. van Ingen J, van der Laan T, Dekhuijzen R, Boeree M, van Soolingen D. 2010. *In vitro* drug susceptibility of 2275 clinical non-tuberculous *Mycobacterium* isolates of 49 species in The Netherlands. Int J Antimicrob Agents 35:169–173. https://doi.org/10.1016/j.ijantimicag.2009.09.023.
30. Guna R, Munoz C, Dominguez V, Garcia-Garcia A, Galvez J, de Julian-Ortiz JV, Borras R. 2005. *In vitro* activity of linezolid, clarithromycin and moxifloxacin against clinical isolates of *Mycobacterium kansasii*. J Antimicrob Chemother 55:950–953. https://doi.org/10.1093/jac/dki111.
31. da Silva Telles MA, Chimara E, Ferrazoli L, Riley LW. 2005. *Mycobacterium kansasii*: antibiotic susceptibility and PCR-restriction analysis of clinical isolates. J Med Microbiol 54:975–979. https://doi.org/10.1099/jmm.0.45965-0.
32. Bakuła Z, Modrzejewska M, Pennings L, Proboszcz M, Safianowska A, Bielecki J, van Ingen J, Jagielski T. 2018. Drug susceptibility profiling and genetic determinants of drug resistance in *Mycobacterium kansasii*. Antimicrob Agents Chemother 62:e01788-17.
33. Wu TS, Leu HS, Chiu CH, Lee MH, Chiang PC, Wu TL, Chia JH, Su LH, Kuo AJ, Lai HC. 2009. Clinical manifestations, antibiotic susceptibility and molecular analysis of *Mycobacterium kansasii* isolates from a university hospital in Taiwan. J Antimicrob Chemother 64:511–514. https://doi.org/10.1093/jac/dkp238.
34. Gitti Z, Mantadakis E, Maraki S, Samonis G. 2011. Clinical significance and antibiotic susceptibilities of nontuberculous mycobacteria from patients in Crete, Greece. Future Microbiol 6:1099–1109. https://doi.org/10.2217/fmb.11.91.
35. Wallace RJ, Jr, Nash DR, Steele LC, Steingrube V. 1986. Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 broth. J Clin Microbiol 24:976–981. https://doi.org/10.1128/jcm.24.6.976-981.1986.
36. Tagini F, Aeby S, Bertelli C, Droz S, Casanova C, Prod'hom G, Jaton K, Greub G. 2019. Phylogenomics reveal that *Mycobacterium kansasii* subtypes are species-level lineages: description of *Mycobacterium pseudokansasii* sp. nov., *Mycobacterium innocens* sp. nov. and *Mycobacterium attenuatum* sp. nov. Int J Syst Evol Microbiol 69:1696–1704. https://doi.org/10.1099/ijsem.0.003378.
37. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, Hermans P, Martin C, McAdam R, Shinnick TM. 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol 31:406–409. https://doi.org/10.1128/jcm.31.2.406-409.1993.
38. Nurk S, Bankevich A, Antipov D, Gurevich AA, Korobeynikov A, Lapidus A, Prjibelski AD, Pyshkin A, Sirotkin A, Sirotkin Y, Stepanauskas R, Clingenpeel SR, Woyke T, McLean JS, Lasken R, Tesler G, Alekseyev MA, Pevzner PA. 2013. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. J Comput Biol 20:714–737. https://doi.org/10.1089/cmb.2013.0084.
39. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
40. Lee I, Ouk Kim Y, Park SC, Chun J. 2016. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. Int J Syst Evol Microbiol 66:1100–1103. https://doi.org/10.1099/ijsem.0.000760.
41. Chen H, Boutros PC. 2011. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. BMC Bioinformatics 12:35. https://doi.org/10.1186/1471-2105-12-35.