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SUSCEPTIBILITY

Drug Susceptibility Profiling and Genetic Determinants of Drug Resistance in *Mycobacterium kansasii*

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ABSTRACT Very few studies have examined drug susceptibility of *Mycobacterium kansasii*, and they involve a limited number of strains. The purpose of this study was to determine drug susceptibility profiles of *M. kansasii* isolates representing a spectrum of species genotypes (subtypes) with two different methodologies, i.e., broth microdilution and Etest assays. To confirm drug resistance, drug target genes were sequenced. A collection of 85 *M. kansasii* isolates, including representatives of eight different subtypes (I to VI, I/II, and IIB) from eight countries, was used. Drug susceptibility against 13 and 8 antimycobacterial agents was tested by using broth microdilution and Etest, respectively. For drug-resistant or high-MIC isolates, eight structural genes (*rrl*, *katG*, *inhA*, *embB*, *rrs*, *rpsL*, *gyrA*, and *gyrB*) and one regulatory region (*embCA*) were PCR amplified and sequenced in the search for resistance-associated mutations. All isolates tested were susceptible to rifampin (RIF), amikacin (AMK), cotrimoxazole (SXT), rifabutin (RFB), moxifloxacin (MXF), and linezolid (LZD) according to the microdilution method. Resistance to ethambutol (EMB), ciprofloxacin (CIP), and clarithromycin (CLR) was found in 83 (97.7%), 17 (20%), and 1 (1.2%) isolate, respectively. The calculated concordance between the Etest and dilution method was 22.6% for AMK, 4.8% for streptomycin (STR), 3.2% for CLR, and 1.6% for RIF. For EMB, INH, and SXT, not even a single MIC value determined by one method equaled that by the second method. The only mutations disclosed were A2266C transversion at the *rrl* gene (CLR-resistant strain) and A128G transition at the *rpsL* gene (strain with STR MIC of >64 mg/liter). In conclusion, eight drugs, including RIF, CLR, AMK, SXT, RFB, MXF, LZD, and ethionamide (ETO), showed high *in vitro* activity against *M. kansasii* isolates. Discrepancies of the results between the reference microdilution method and Etest preclude the use of the latter for drug susceptibility determination in *M. kansasii*. Drug resistance in *M. kansasii* may have different genetic determinants than resistance to the same drugs in *M. tuberculosis*.

KEYWORDS *Mycobacterium kansasii*, drug resistance, molecular resistance, susceptibility testing

Mycobacterium kansasii, an opportunistic pathogen and causative agent of pulmonary and extrapulmonary infections, is one of the six most frequently isolated nontuberculous mycobacterial (NTM) species worldwide (1). To date, seven *M. kansasii* subtypes (I to VII), along with two intermediate (I/II) and atypical (IIb) types, have been identified (2, 3). Most of the disease-related strains belong to type I, while the others have usually been linked to environmental sources. Infections due to NTM, including *M. kansasii*, have been on the rise recently, which is due to increasing numbers of

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TABLE 1 MIC values obtained for different *M. kansasii* subtypes ($n = 23$) and clinical isolates from Poland ($n = 62$) with broth microdilution method[c]

Type	NTM disease	RIF			CLR			INH			EMB			STR			AMK		
		R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$
Individual types ($n = 23$)																			
I ($n = 5$)	Yes	0.25	0.25	0.25/0.25	0.12–>64	0.12	0.12/>64	2	2	2/2	16–>16	>16	>16/>16	4–>64	8	8/>64	1–4	2	2/4
I ($n = 1$)	No	0.25	0.25	0.25	0.12	0.12	0.12	2	2	2	16	16	16	8	8	8	2	2	2
I/II ($n = 1$)	Yes	0.5	0.5	0.5	0.25	0.25	0.12	2	2	2	>16	>16	>16	4	4	4	2	2	2
IIb ($n = 1$)	Yes	1	1	1	0.5	0.5	0.25	2	2	2	>16	>16	>16	8	8	8	4	4	4
IIb ($n = 1$)	No	0.5	0.5	0.5	0.25	0.25	0.5	1	1	1	>16	>16	>16	4	4	4	2	2	2
II ($n = 2$)	Yes	0.25–1	1.5	1/2	0.5–1	0.75	0.5/1	1–2	1.5	1/2	>16	>16	>16/>16	4–8	6	4/8	2–8	5	2/8
II ($n = 4$)	No	0.25–1	1	1/1	0.12–05	0.19	0.12/05	1	1	1/1	16–>16	>16	>16/>16	4–16	12	8/16	2–8	3	2/8
III ($n = 3$)	No	<0.12–0.5	0.12	0.12/0.5	<0.06–0.12	0.12	0.12/0.12	1–2	1	1/2	16–>16	>16	>16/>16	4–8	4	4/8	<1–4	2	2/4
IV ($n = 2$)	No	<0.12	<0.12	<0.12/<0.12	<0.06–0.06	>0.06	<0.06/0.06	1–2	1.5	1/2	16–>16	>16	16/>16	4	4	4/4	2–4	3	2/4
V ($n = 2$)	No	<0.12–0.5	0.31	<0.12/0.5	<0.06–0.12	0.09	<0.06/0.12	<0.25–2	1.1	<0.25/2	4	4	4/4	4	4	4/4	2	2	2/2
VI ($n = 1$)	No	0.25	0.25	0.25	0.12	0.12	0.12	2	2	2	16	16	16	8	8	8	1	1	1
Clinical isolates ($n = 62$)																			
I ($n = 39$)	Yes	<0.12–1	0.25	0.25/1	<0.6–0.5	0.12	0.12/0.5	1–4	2	2/4	8–>16	>16	>16/>16	2–>64	8	8/16	<1–8	2	2/4
I ($n = 22$)	No	0.25–1	0.25	0.25/1	0.12–0.5	0.25	0.25/0.25	1–4	2	2/2	8–>16	>16	>16/>16	1–64	8	8/16	<1–8	2	2/8
II ($n = 1$)	No	0.5	0.5	0.5	0.12	0.12	0.12	1	1	1	16	16	16	2	2	2	<1	<1	<1
Total		<0.12–1	0.25	0.25/1	<0.6–0.5	0.25	0.25/0.5	1–4	2	2/2	8–>16	>16	>16/>16	1–>64	8	8/16	<1–8	2	2/4
Total ($n = 85$)		<0.12–1	0.25	0.25/1	<0.06–>64	0.25	0.25/0.5	<0.25–4	2	2/2	4–>16	>16	>16/>16	1–>64	8	8/16	<1–8	2	2/4
No. (%) of resistant isolates ($n = 85$)				0 (0)			1 (1.2)			NA[a]			83 (97.6)			NA			0 (0)

[a]NA, not applicable. Critical drug concentration not available in CLSI guidelines.
[b]For SXT (ratio of 1:19 for trimethoprim to sulfamethoxazole), the concentration of trimethoprim is indicated.
[c]R, MIC ranges; M, median MIC.

susceptible hosts, progress in microbial identification, and greater awareness of NTM disease (4).

Studies on drug susceptibility of *M. kansasii* are still very sparse, and those few available have evaluated a limited number of strains (5–12). For drug susceptibility testing (DST) of *M. kansasii*, as of other mycobacteria, the Clinical and Laboratory Standards Institute (CLSI) recommends the microdilution method in Mueller-Hinton medium. Optionally, agar proportion assays can be performed (13).

Currently, only susceptibility tests for rifampin (RIF) and clarithromycin (CLR) are advocated by the CLSI for *M. kansasii*; only in the rare event of resistance to RIF is it advised that researchers determine susceptibility to isoniazid (INH), ethambutol (EMB), streptomycin (STR), amikacin (AMK), co-trimoxazole (SXT), rifabutin (RFB), moxifloxacin (MXF), linezolid (LZD), and ciprofloxacin (CIP) (13, 14). *M. kansasii* isolates are usually susceptible to those agents if the current CLSI breakpoints are applied (14, 15).

Whereas studies on DST are lacking, those concerning genetic determinants of drug resistance in *M. kansasii* are virtually absent. So far, only three studies have addressed this issue, with a focus on RIF and CLR resistance. Similar to *M. tuberculosis*, mutations in *rpoB* and *rrl* genes were identified as a major mechanism behind resistance to these agents in *M. kansasii* (12, 16, 17).

The purpose of this study was to determine drug susceptibility profiles of *M. kansasii* representing a spectrum of species genotypes (subtypes) with broth microdilution and Etest methods. Furthermore, a panel of nine genetic loci associated with drug resistance in *M. tuberculosis* was screened for the presence of mutations in drug-resistant *M. kansasii* isolates.

RESULTS

Broth microdilution. MIC ranges, median MICs, MIC_{50}s, and MIC_{90}s are all summarized in Table 1. All of the isolates tested were susceptible to RIF, AMK, SXT, RFB, MXF, and LZD. Among drug-resistant isolates, 83 (97.6%) were resistant to EMB, 17 (20%) to CIP, and 1 (1.2%) to CLR. The highest detected MIC for INH was 4 mg/liter and was evidenced in five (5.9%) *M. kansasii* isolates. The MICs for STR and DOX varied widely

TABLE 1 (Continued)

SXT[b]			RFB			MXF			LZD			CIP			DOX			ETO		
R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$
<0.12–0.5	<0.12	<0.12/0.5	<0.25	<0.25	<0.25/<0.25	<0.12	<0.12	<0.12/<0.12	1–2	2	2/2	1–4	1.5	2/4	4–8	4	4/8	<0.3–0.6	<0.3	<0.3/0.6
<0.12	<0.12	<0.12	<0.25	<0.25	<0.25	<0.12	<0.12	<0.12	<1	<1	<1	1	1	1	4	4	4	<0.3	<0.3	<0.3
0.12	0.12	0.12	<0.25	<0.25	<0.25	<0.12	<0.12	<0.12	2	2	2	1	1	1	4	4	4	<0.3	<0.3	<0.3
0.12	0.12	0.12	<0.25	<0.25	<0.25	<0.12	<0.12	<0.12	2	2	2	1	1	1	8	8	8	<0.3	<0.3	<0.3
0.12	0.12	0.12	<0.25	<0.25	<0.25	<0.12	<0.12	<0.12	2	2	2	0.5	0.5	0.5	4	4	4	<0.3	<0.3	<0.3
0.12–2	1	0.12/0.2	<0.25	<0.25	<0.25/<0.25	<0.12–0.25	0.19	<0.12/0.25	2–4	3	2/4	1	1	1/1	8	8	8/8	<0.3–0.6	0.45	<0.3/0.6
<0.12	<0.12	<0.12/<0.12	<0.25	<0.25	<0.25/<0.25	<0.12	<0.12	<0.12/<0.12	2–4	2	2/4	0.5–2	1.5	1/2	4–16	8	8/16	<0.3	<0.3	<0.3/<0.3
<0.12–0.5	<0.12	<0.12/0.5	<0.25	<0.25	<0.25/<0.25	<0.12	<0.12	<0.12/<0.12	<1–2	2	2/2	0.5–2	1	1/2	1–4	2	2/4	<0.3	<0.3	<0.3/<0.3
0.12	0.12	0.12/0.12	<0.25	<0.25	<0.25/<0.25	<0.12	<0.12	<0.12/<0.12	<1–2	1.5	<1–2	0.5–2	1.25	0.5/2	2–4	3	2/4	<0.3	<0.3	<0.3/<0.3
0.12	0.12	0.12/0.12	<0.25	<0.25	<0.25/<0.25	<0.12–0.25	0.06	<0.12/0.25	2	2	2/2	1–2	1.5	1/2	4	4	4/4	<0.3	<0.3	<0.3/<0.3
<0.12	<0.12	<0.12	<0.25	<0.25	<0.25	<0.12	<0.12	<0.12	2	2	2	1	1	1	8	8	8	<0.3	<0.3	<0.3
<0.12–1	<0.12	<0.12/0.12	<0.25	<0.25	<0.25	<0.12–0.5	<0.12	<0.12/0.25	<1–8	2	2/4	0.25–8	1	1/4	1–>16	8	8/>16	<0.3–1.2	<0.3	<0.3/0.6
<0.12–0.5	<0.12	<0.12/0.25	<0.25	<0.25	<0.25	<0.12–0.5	<0.12	<0.12/0.25	<1–4	2	2/4	0.25–8	1	1/4	1–>16	8	8/16	<0.3–0.6	<0.3	<0.3/0.3
<0.12	<0.12	<0.12	<0.25	<0.25	<0.25	<0.12	<0.12	<0.12	<1	<1	<1	0.25	0.25	0.25	2	2	2	<0.3	<0.3	<0.3
<0.12–1	<0.12	<0.12/0.12	<0.25	<0.25	<0.25/<0.25	<0.12–0.5	<0.12	<0.12/0.25	<1–8	2	2/4	0.25–8	1	1/4	1–>16	8	8/>16	<0.3–1.2	<0.3	<0.3/0.6
<0.12–2	<0.12	<0.12/0.25 0 (0)	<0.25 0 (0)	<0.25	<0.25/<0.25	<0.12–0.5 0 (0)	<0.12	<0.12/0.25	<1–8 0 (0)	2	2/4	0.25–8 17 (20)	1	1/4	1–>16 NA	8	8/16	<0.3–1.2 NA	<0.3	<0.3/0.6

(1 to >64 mg/liter and 1 to >16 mg/liter), whereas the ethionamide (ETO) MICs were within a narrow range of <0.3 to 1.2 mg/liter. Twelve (12/20; 60%) isolates with the highest MICs for DOX (≥16 mg/liter) were also resistant to CIP.

No statistically significant differences were observed between MICs of different drugs and presence or absence of *M. kansasii* disease (*P* value of >0.05). The only two exceptions were INH and ETO, whose MICs were significantly lower in patients without *M. kansasii* disease (*P* value of <0.03).

Etest. The Etest results of the 62 *M. kansasii* clinical isolates are presented in Table 2. All isolates tested had SXT MICs of >32/608 mg/liter (trimethoprim/sulfamethoxazole), i.e., at least 16 times that in the dilution method.

The median Etest MICs for RIF and EMB were 0.008 mg/liter and 0.5 mg/liter, 30 times lower than the median MICs for these two drugs according to the dilution method. Only two isolates had Etest MICs above the breakpoint level (4 mg/liter) defined for the dilution method.

The Etest MICs of CLR were within a broad range of values (<0.016 mg/liter to >256 mg/liter), quite similar to what was observed for the dilution method (MIC range, <0.06 to >64 mg/liter).

Higher activities of AMK and STR than KAN were evidenced by the MIC_{50} and MIC_{90} values (for AMK and STR, 1.5 and 4 mg/liter, respectively, as opposed to 2 and 12 mg/liter for KAN).

Overall, for all seven drugs tested with both microdilution and Etest methods, the MIC values determined with these two methods were significantly different (*P* value of ≤0.001) (Fig. 1).

Mutation profiling. The results of sequence analysis are summarized in Fig. S1 to S9 in the supplemental material. Since all but two (type V) isolates were identified as EMB resistant with the dilution method, sequence analysis of the EMB resistance-associated loci (*embB* and *embCA*) was limited to only 8 isolates, representing 8 distinct *M. kansasii* types. For *M. kansasii* type IV (EMB resistant) and V (EMB susceptible), the sequences at both loci were identical. All *M. kansasii* subtypes had a G918C nucleotide change, translated into an M306I substitution, in the EMB resistance-determining region (ERDR), as it is referred to in *M. tuberculosis*. This mutation was always coupled with G406P (GGC1216-1218CCG or GGC1216-1218CCC) and M423I (G1269C) alterations (Table S11).

TABLE 2 MIC values obtained for clinical isolates from Poland ($n = 62$) with Etest method[b]

Type (clinical isolates; $n = 62$)	NTM disease	RIF			CLR			INH		
		R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$
I ($n = 39$)	Yes	0.002–0.047	0.008	0.008/0.023	<0.016–>256	0.047	0.047/0.47	<0.016–>256	>256	>256/>256
I ($n = 22$)	No	<0.002–0.023	0.01	0.012/0.016	<0.016–0.25	0.064	0.064/0.125	<0.016–>256	>256	>256/>256
II ($n = 1$)	No	0.047	0.047	0.047	0.064	0.064	0.064	>256	>256	>256
Total		<0.002–0.047	0.008	0.008/0.023	<0.016–>256	0.047	0.047/0.125	<0.016–>256	>256	>256/>256

[a]For SXT (ratio of 1:19 for trimethoprim to sulfamethoxazole), the concentration of trimethoprim is indicated.
[b]R, MIC ranges; M, median MIC.

The 17 CIP-resistant isolates (type I) had no mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* and *gyrB* loci compared with CIP-susceptible isolates (i.e., with the lowest CIP MICs).

The only detected CLR-resistant isolate (type I) differed from its susceptible counterparts by a single mutation (A2266C) in the *rrl* gene.

Five isolates (type I) with the highest STR MICs (≥16 mg/liter) had their *rrs* and *rpsL* gene sequences determined. Upon comparison with the corresponding sequences of the low-MIC isolates (≤2 mg/liter), a single polymorphism in the *rpsL* gene (A128G and K42R) was detected in one of the high-MIC isolates.

Five isolates (type I) with the highest INH MIC (4 mg/liter) were screened for mutations in the *inhA* and partial *katG* gene, with the corresponding sequences of the five low-MIC (1 mg/liter) isolates as a reference. All 10 of these isolates shared identical sequences.

DISCUSSION

The scant knowledge about drug susceptibility in *M. kansasii* and molecular determinants of drug resistance in this species were behind the undertaking of this study. The data reported on drug resistance in *M. kansasii* in the available literature were summarized in Table 3.

Drug susceptibility profiles established with broth microdilution. The four drugs which currently form the core of treatment schemes for *M. kansasii* infections are RIF, EMB, INH, and CLR (13, 15).

In this study, resistance to RIF was not detected among the isolates, whereas all of the isolates collected in Poland were resistant to EMB (two isolates susceptible to EMB were collected either in Germany or in the Netherlands). Previously described levels of resistance to those drugs, established with the microdilution method, varied widely from 1.9% to 56.4% for RIF and 0% to 94% for EMB (7, 8, 10, 12, 18).

Although there is no consensus on the INH breakpoint for *M. kansasii*, the most commonly used value has been 5 mg/liter. The frequency of INH resistance with this breakpoint was 2.9% in Spain (8) and 8% in Brazil (7). However, in a study from the Netherlands, a breakpoint set at 1 mg/liter was used, yielding all *M. kansasii* isolates INH resistant (10). The highest MIC of INH, detected in 5 (5/85; 5.9%) isolates in this study, was 4 mg/liter. According to the CLSI, INH MICs for untreated *M. kansasii* isolates are between 0.5 and 5 mg/liter, thus a concentration of 1 mg/liter for testing strains of *M. tuberculosis* should not be applied (13).

In patients with RIF-resistant *M. kansasii* disease a three-drug regimen should be administered, with a macrolide (CLR or azithromycin), fluoroquinolone (FQ; MXF), and SXT or STR (15, 19). In the case of resistance to any of these drugs, alternative agents should be considered, including aminoglycosides other than STR, tetracyclines, LZD or ETO, and RFB. Exclusion of INH and EMB in RIF-resistant cases is motivated by the observed increase in INH and EMB MICs in isolates with acquired RIF resistance (19).

All Polish isolates were found to be susceptible to MXF, SXT, RFB, CLR, and LZD. The frequencies of resistance to these drugs greatly varied in previous studies, ranging from 0 to 40.5% for MXF, 8 to 18.9% for SXT, and 0 to 50% for RFB (7–10, 12, 18, 20).

TABLE 2 (Continued)

EMB			STR			AMK			SXT[a]			KAN		
R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$	R	M	$MIC_{50/90}$
0.125–12	0.5	0.5/1.5	0.38–6	1.5	1.5/4	0.25–12	1.5	1.5/4	>32	>32	>32	0.125–24	2	2/12
0.19–8	0.69	0.64/1.5	0.125–>1,024	2.5	2/4	0.25–6	1.75	1.5/6	>32	>32	>32	0.38–24	2.5	1/12
0.38	0.38	0.38	2	2	2	0.75	0.75	0.75	>32	>32	>32	2	2	2
0.125–12	0.5	0.5/1.5	0.125–>1,024	1.5	1.5/4	0.25–12	1.5	1.5/4	>32	>32	>32	0.125–24	2	2/12

Several authors have shown high *in vitro* activity of CLR and LZD against *M. kansasii*, with less than 1% of isolates being resistant (7–10, 15, 18, 21). In one study from China, a third (32.1%) and a fifth (20.5%) of the *M. kansasii* isolates were described as LZD and CLR resistant, respectively (12).

High activity of ETO was evidenced in our study, with the highest detected MIC being 1.2 mg/liter. This is in line with a study by da Silva Telles et al., where only 5% of the isolates were ETO resistant, with an assumed breakpoint value of 4 mg/liter (7).

A quarter (25.8%) of our samples showed resistance to CIP. Similar or higher rates of CIP resistance (15 to 66%) had been reported earlier (7, 9, 10, 18).

Interestingly, more than two-thirds (70%) of our CIP-resistant isolates showed high DOX MICs (≥16 mg/liter). The cross-resistance between quinolones (CIP) and tetracyclines (DOX) had previously been described for several bacterial species (e.g., *Escherichia coli* or *Enterobacter aerogenes*) but, to our knowledge, not for mycobacteria. A hypothesized mechanism for this association is the broad substrate specificity of the FQ efflux systems, capable of extruding various drugs, including tetracyclines (22). Efflux pumps are important drivers of drug resistance in NTM (23).

As for aminoglycosides, resistance to AMK has seldom been reported in *M. kansasii*, with the percentage of resistant isolates not exceeding 6% (not detected in our study) (7, 9, 12, 18). The high (54%) frequency of AMK resistance was reported only in the Netherlands, and this might have been attributed to the lower breakpoint applied (5 mg/liter versus 32 mg/liter) (10). According to previous studies, STR showed much less activity than AMK against *M. kansasii*. With a breakpoint of 10 mg/liter, resistance to STR was 14% in Brazil (7) and 23% in the United States (5). In our study, nearly one-fourth (23%) of the isolates had STR MICs of >10 mg/liter.

Genotype-specific differences in phenotypes. Exposure to drug selection pressure may predispose human disease-associated *M. kansasii* types (I and II) to a drug-resistant phenotype. Li et al. showed CLR resistance to be associated with *M. kansasii* type I (12). An interesting observation from our study was that only type V *M. kansasii* isolates were susceptible to EMB. This difference may relate to different cell wall compositions, rendering the bacilli more sensitive to EMB. Apart from that, and as demonstrated by others, no significant differences were found between drug susceptibility profiles and disease-related *M. kansasii* subtypes except for those for ETO (7). A subtle correlation between INH and ETO and *M. kansasii* disease was found in our study. The median MICs of both of these drugs were significantly higher in patients with *M. kansasii* disease (*P* value of <0.03).

Etest in susceptibility profiling. Due to good correlation between drug susceptibility results and clinical response, microdilution in Mueller-Hinton medium is the CLSI-recommended drug susceptibility platform for five of the most frequently isolated and clinically relevant NTM species, including *M. kansasii*, *M. avium*, *M. xenopi*, *M. fortuitum*, and *M. abscessus* (13, 14). Since the Etest has only sporadically been used for NTM, its laboratory and clinical usefulness is doubtful.

An important purpose of this study was to explore the congruence of the results obtained with the reference dilution and Etest methods. Those few studies which addressed this issue in *M. abscessus*, *M. fortuitum*, and *M. chelonae* produced conflicting conclusions. Whereas Woods et al. (24) observed a high concordance, at a rate of 80% or 90%, between the MIC values of CLR, AMK, and CIP obtained with the Etest and

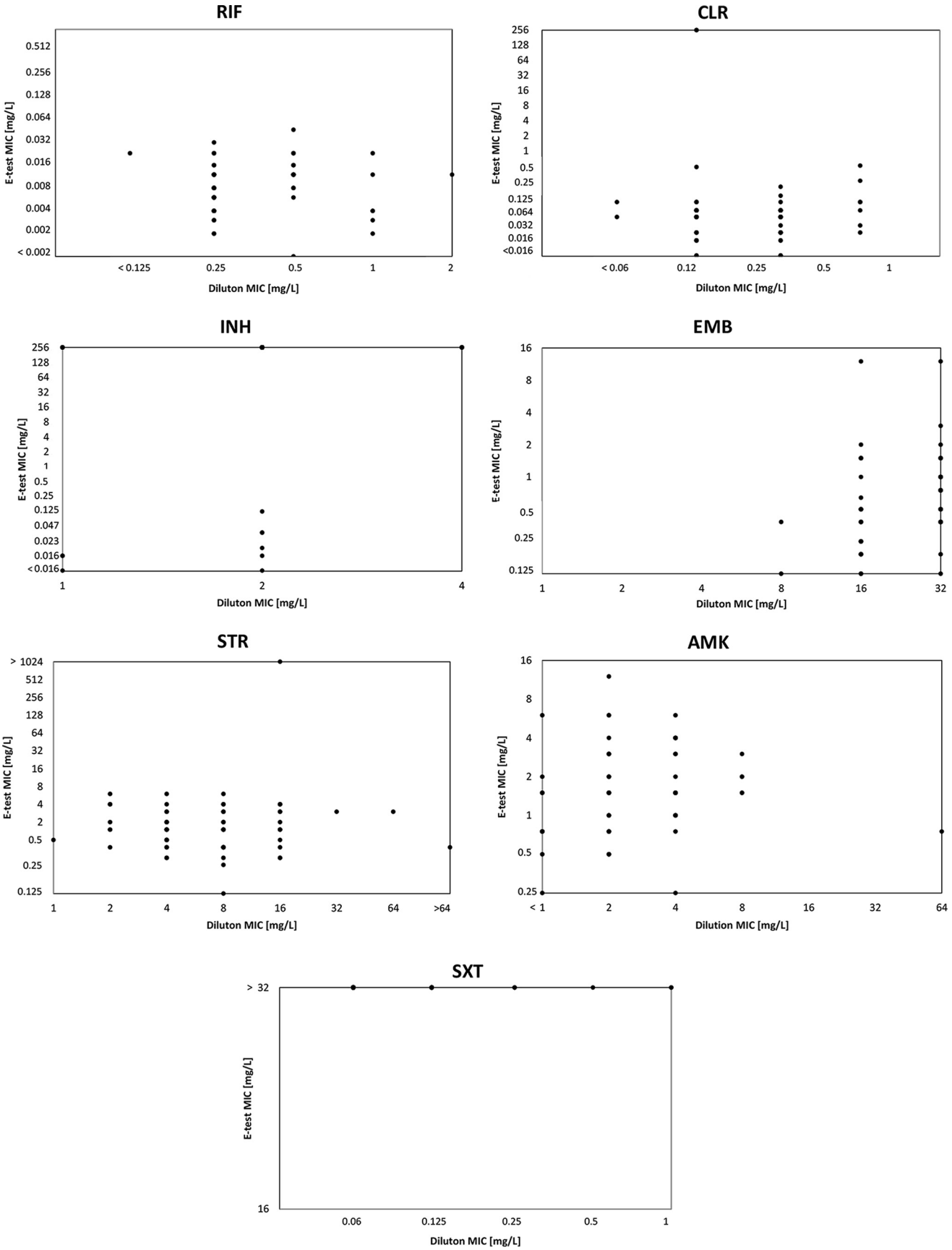

FIG 1 Scattergram results comparing MICs determined by Etest with those determined by the broth microdilution reference method (log_2 scale).

TABLE 3 Reported drug resistance of *M. kansasii* isolates

Drug and method	No. of tested isolates	Breakpoint value (mg/liter)	% of resistant isolates	Country	Study period	Reference or source
RIF						
NS[a]	71	1	18	USA	1980–1984	5
Agar proportion	464	1	3.7	USA	1989–1992	14
BACTEC[b]	20	NS	20	Czech Republic	1992[f]	6
Microdilution	169	1	12	Brazil	1993–1998	7
BACTEC	148	1	3.4	Spain	1997–2003	44
Resistance ratio[c]	56	NS	0	Israel	1994–2004	45
NS	314	NS	1%	Japan	2001–2005	16
Microdilution	37	1	21.6	Taiwan	2000–2004	9
Microdilution	104	1	1.9	Spain	2005[f]	8
Microdilution	262	1	2	The Netherlands	2000–2007	10
Etest	10	1	0	Greece	2000–2009	11
Agar proportion	7	NS	0	China	2008–2011	46
Microdilution	40	1	50	Iran	2010–2014	18
Microdilution	78	1	56.4	China	2008–2015	12
Microdilution	62	1	0	Poland	2000–2015	This study
CLR						
BACTEC	31	MIC_{50}, 0.25[d]		USA	1992	47
Agar proportion	31	MIC_{50}, 0.5		USA	1992	47
Microdilution	169	16	1	Brazil	1993–1998	7
BACTEC	148	MIC_{50}, 0.12		Spain	1997–2003	44
Resistance ratio	56	NS	0	Israel	1994–2004	45
Microdilution	104	16	0	Spain	2005[f]	8
Microdilution	262	16	1	The Netherlands	2000–2007	10
Etest	10	8	0	Greece	2000–2009	11
Microdilution	37	16	0	Taiwan	2009[f]	9
Microdilution	34	16	0	China	2014[f]	20
Microdilution	40	16	0	Iran	2010–2014	18
Microdilution	78	16	20.5	China	2008–2015	12
Microdilution	62	16	0	Poland	2000–2015	This study
INH						
NS	71	5	7	USA	1980–1984	5
Agar proportion	464	1	33	USA	1989–1992	14
BACTEC	20	NS	100	Czech Republic	1992[f]	6
Microdilution	169	5	8	Brazil	1993–1998	7
BACTEC	148	1	18.9	Spain	1997–2003	44
Microdilution	37	NS	27	Taiwan	2000–2004	9
Microdilution	104	5	2.9	Spain	2005[f]	8
Microdilution	262	1	100	The Netherlands	2000–2007	10
Etest	10	4	30	Greece	2000–2009	11
Agar proportion	7	NS	42.9	China	2008–2011	46
Microdilution	40	NS	0	Iran	2010–2014	18
Microdilution	62	MIC_{50}, 2[e]		Poland	2000–2015	This study
EMB						
NS	71	5	58	USA	1980–1984	5
Agar proportion	464	5	0.9	USA	1989–1992	14
BACTEC	20	NS	100	Czech Republic	1992[f]	6
Microdilution	169	5	94	Brazil	1993–1998	7
BACTEC	148	5	0	Spain	1997–2003	44
Resistance ratio	56	NS	0	Israel	1994–2004	45
Microdilution	37	4	73	Taiwan	2000–2004	9
Microdilution	104	5	2.9	Spain	2005[f]	8
Microdilution	262	5	8	The Netherlands	2000–2007	10
Etest	10	4	0	Greece	2000–2009	11
Agar proportion	7	NS	0	China	2008–2011	46
Microdilution	40	4	0	Iran	2010–2014	18
Microdilution	78	4	20.5	China	2008–2015	12
Microdilution	62	4	100	Poland	2000–2015	This study

(Continued on next page)

TABLE 3 (Continued)

Drug and method	No. of tested isolates	Breakpoint value (mg/liter)	% of resistant isolates	Country	Study period	Reference or source
STR						
NS	71	10	23	USA	1980–1984	5
Microdilution	13	MIC_{50}, 2		USA	1986[f]	48
Agar proportion	464	2	11.6	USA	1989–1992	14
Microdilution	169	10	14	Brazil	1993–1998	7
BACTEC	148	2	0	Spain	1997–2003	44
Microdilution	37	NS	5.4	Taiwan	2000–2004	9
Microdilution	262	5	15	The Netherlands	2000–2007	10
Etest	10	10	40	Greece	2000–2009	11
Agar proportion	7	NS	85.7	China	2008–2011	46
Microdilution	40	NS	35	Iran	2010–2014	18
Microdilution	62	MIC_{50}, 8		Poland	2000–2015	This study
AMK						
Microdilution	13	MIC_{50}, 8		USA	1986[f]	48
Microdilution	169	32	3	Brazil	1993–1998	7
Microdilution	37	32	2.7	Taiwan	2000–2004	9
Microdilution	262	5	54	The Netherlands	2000–2007	10
Etest	10	10	0	Greece	2000–2009	11
Agar proportion	7	NS	14.3	China	2008–2011	46
Microdilution	40	32	5	Iran	2010–2014	18
Microdilution	78	32	5.1	China	2008–2015	12
Microdilution	85	32	0	Poland	2000–2015	This study
SXT						
Microdilution	13	$MIC_{50,}$ $\leq$1.0		USA	1986[f]	48
Microdilution	37	2/38	18.9	Taiwan	2000–2004	9
Etest	10	32	0	Greece	2000–2009	11
Microdilution	40	32	8	Iran	2010–2014	18
Microdilution	78	32	16.7	China	2008–2015	12
Microdilution	62	2/38	0	Poland	2000–2015	This study
RFB						
BACTEC	20	NS	0	Czech Republic	1992[f]	6
Microdilution	169	2	7	Brazil	1993–1998	7
Microdilution	37	2	2.7	Taiwan	2000–2004	9
Microdilution	262	2	0	The Netherlands	2000–2007	10
Microdilution	40	2	50	Iran	2010–2014	18
Microdilution	78	2	34.6	China	2008–2015	12
Microdilution	62	2	0	Poland	2000–2015	This study
MXF						
BACTEC	148	MIC_{50}, 0.06		Spain	1997–2003	44
Microdilution	37	2	40.5	Taiwan	2000–2004	9
Microdilution	104	2	0	Spain	2005[f]	8
Etest	10	1	0	Greece	2000–2009	11
Microdilution	40	2	0	Iran	2010–2014	18
Microdilution	78	2	16.7	China	2008–2015	12
Microdilution	62	2	0	Poland	2000–2015	This study
LZD						
BACTEC	148	MIC_{50}, 1		Spain	1997–2003	44
Microdilution	19	8	0	USA	2003[f]	21
Microdilution	104	32	0	Spain	2005[f]	8
Etest	10	8	0	Greece	2000–2009	11
Microdilution	40	16	0	Iran	2010–2014	18
Microdilution	78	16	32.1	China	2008–2015	12
Microdilution	62	16	0	Poland	2000–2015	This study
CIP						
BACTEC	20	NS	40	Czech Republic	1992[f]	6
Microdilution	169	2	66	Brazil	1993–1998	7
Resistance ratio	56	NS	29	Israel	1994–2004	45
Microdilution	37	2	29.7	Taiwan	2000–2004	9

(Continued on next page)

TABLE 3 (Continued)

Drug and method	No. of tested isolates	Breakpoint value (mg/liter)	% of resistant isolates	Country	Study period	Reference or source
Microdilution	262	2	15	The Netherlands	2000–2007	10
Etest	10	2	20	Greece	2000–2009	11
Microdilution	40	2	50	Iran	2010–2014	18
Microdilution	62	2	25.8	Poland	2000–2015	This study
DOX						
Microdilution	169	4	99	Brazil	1993–1998	7
Microdilution	40	NS	100	Iran	2010–2014	18
Microdilution	62	MIC_{50}, 8		Poland	2000–2015	This study
ETO						
Agar proportion	464	5	3.4	USA	1989–1992	14
Resistance ratio	56	NS	1	Israel	1994–2004	45
Microdilution	169	4	5	Brazil	1993–1998	7
Etest	10	10	0	Greece	2000–2009	11
Microdilution	62	MIC_{50}, <0.3		Poland	2000–2015	This study
KAN						
NS	71	5	86	USA	1980–1984	5
Microdilution	13	MIC_{50}, 8		USA	1986[f]	48
Agar proportion	464	5	99.8	USA	1989–1992	14
Etest	62	MIC_{50}, 2		Poland	2000–2015	This study

[a]NS, not specified in the literature.
[b]Susceptibility testing was performed with the BACTEC radiometric system (Becton, Dickinson, New Jersey, USA).
[c]Resistance ratio method (49).
[d]Percentage of resistant strains not indicated in the literature.
[e]For the drugs with no established critical concentrations, only MIC values are reported (as recommended by the CLSI).
[f]Study period not specified in the literature. Year of publication is indicated.

microdilution methods, the others found the Etest MICs of those drugs differed up to 16-fold from those calculated using the proportion method, with overall concordance rates of 72% to 87% for AMK, 63 to 77% for CLR, and 36 to 78% for CIP (25, 26).

For *M. kansasii*, only one study has looked at the MIC values measured by the agar proportion and Etest methods (on Löwenstein-Jensen medium). The results were in agreement in 95 to 100% of cases (27).

Our study is the first to compare the reference broth microdilution method with the Etest for *M. kansasii*. In general, the results from these two methods were widely discrepant for EMB, INH, SXT, RIF, CLR, and STR, with calculated MIC level concordance below 5%. It was somewhat higher, but still low (22.6%), for AMK.

DST methods, including broth microdilution, agar proportion, and Etest, vary by the medium used, supplements and inoculum added, incubation conditions, and endpoint interpretations (4). All of those features presumably influence the test outcomes.

Genetic determinants of drug resistance. The genetic background of drug resistance in NTM, including *M. kansasii*, has only anecdotally been addressed and largely explained through extrapolation of findings from studies on *M. tuberculosis*. The only two mutations possibly associated with drug resistance were A2266C in the *rrl* gene and A128G (K42R) in the *rpsL* gene, found in single *M. kansasii* isolates resistant to CLR, and with the highest STR MICs established with broth microdilution ($>$64 mg/liter). Mutations in the *rrl* gene have been associated with high levels of macrolide resistance among *M. kansasii* and *M. avium* isolates, with mutations at *rrl* codon 2058 or 2059 found with frequencies of 56.2% (9/16) and 92.3% (24/26), respectively (12, 28). In contrast, only 1.4% (2/140) of CLR-resistant *M. abscessus* isolates had mutations at this locus (29). The A128G (K42R) substitution is the leading alteration among STR-resistant *M. tuberculosis* isolates, with a frequency of detection of 24 to 64% (30–32). However, it has yet to be described in NTM (33).

In *M. tuberculosis*, most of the EMB resistance-associated mutations are located in the *embB* gene (34). In our study, all *M. kansasii* types harbored M306I, G406P, and M423I amino acid substitutions, frequently described in EMB-resistant *M. tuberculosis*

strains but unreported in NTM, including *M. kansasii*, *M. abscessus*, *M. fortuitum*, and *M. avium* (35, 36). As these alterations were found in both EMB-resistant (types I to IV and VI) and EMB-susceptible (type V) isolates, they cannot be considered specific for EMB resistance in *M. kansasii*. However, they might be responsible for reduced susceptibility of *M. kansasii* to EMB, since EMB-susceptible isolates presented MICs around the breakpoint level (4 mg/liter), as opposed to EMB-resistant isolates with MICs of 8 to >16 mg/liter.

The role of mutations in *gyrA* and/or *gyrB* in FQ resistance determination in NTM is uncertain. A study on *M. abscessus* showed that resistance to FQ may, to some extent, relate to mutations in the same codons as those responsible for FQ resistance in *M. tuberculosis* (A83 in *gyrA* and R447 and D464 in *gyrB*) (37). However, only 3.7% (2/54) of FQ-resistant *M. abscessus* isolates were mutated at these loci compared to sequences of susceptible isolates (30). Here, 17 *M. kansasii* isolates resistant to CIP did not harbor any changes in the *gyrA* and *gyrB* genes.

The key role in developing resistance to INH in *M. tuberculosis* is played by mutations in the *katG* gene, with reported frequencies of 67.7% to 95% among INH-resistant strains (38). To our knowledge, literature devoted to the role of *katG* or *inhA* in the formation of the INH-resistant phenotype among NTM is absent.

Drug resistance in NTM might be acquired (drug induced) through specific mutations in a number of different genetic loci or could be preexistent (intrinsic), mediated by the cell envelope and active efflux. For instance, low permeability of the mycobacterial cell wall shields the bacilli from environmental stress and contributes to their drug-resistant phenotype. Therefore, enzymes involved in cell wall integrity (e.g., MurA, MurB, Ldt, PonA1, and PonA2) might play an important role in the development of drug resistance (39). NTM also have a broad repertoire of multidrug efflux pumps, such as P55, Tap, LfrA, and EfpA, involved in the extrusion of STR (P55, Tap), INH (P55, EfpA), RMP (P55), β-lactams, and FQs (LfrA) (4). Among other plausible mechanisms of intrinsic drug resistance in NTM are porin channels, enzymatic modification or degradation of antibiotics, and dormancy of mycobacterial cells (39).

To get better insight into the molecular mechanisms of drug resistance in *M. kansasii*, further investigations, involving whole-genome sequencing, are needed.

Concluding remarks. In conclusion, this study brings two important advantages to the study of *M. kansasii*. First, it provides DST results for a wide panel of antimycobacterials by means of two different methodologies, i.e., reference broth microdilution and Etest. Second, it gives a snapshot of the genetic background of drug-resistant and drug-susceptible phenotypes in *M. kansasii*.

Overall, the results presented here show high activity of RIF, CLR, AMK, SXT, RFB, MXF, LZD, and ETO against *M. kansasii* isolates. Discrepancies of the results between the reference microdilution method and Etest precludes the use of the latter for drug susceptibility determination in *M. kansasii*. Finally, drug resistance in *M. kansasii* may have genetic determinants somewhat similar to those of resistance to the same drugs in *M. tuberculosis* for CLR, STR, and EMB and others for INH and CIP. Further studies are required to pinpoint the specific mutations or mechanisms conferring drug resistance in *M. kansasii*.

MATERIALS AND METHODS

Isolates. A total of 85 *M. kansasii* isolates were included in the study. Within this number were 82 strains representing six subtypes (type I, $n = 67$; type II, $n = 7$; type III, $n = 3$; type IV, $n = 2$; type V, $n = 2$; type VI, $n = 1$) and three representing intermediate (type I/II, $n = 1$) and atypical (type IIb, $n = 2$) types. The isolates were originally collected from Poland ($n = 62$), the Netherlands ($n = 10$), the Czech Republic ($n = 4$), Germany ($n = 3$), South Korea ($n = 3$), Spain ($n = 2$), Belgium ($n = 1$), and Italy ($n = 1$). Also, the *M. kansasii* ATCC 12478 type strain was used. The isolates were categorized as representing (or not) *M. kansasii* disease according to the American Thoracic Society (ATS) diagnostic criteria (15). Since no data on patient management and long-term treatment outcomes were available, they were excluded from the analysis.

DNA extraction and genotyping. Genomic DNA was extracted using the cetyl-trimethyl-ammonium bromide (CTAB) method. For PCR-restriction enzyme analysis (PCR-REA) genotyping, protocols described by Telenti et al. and Bakuła et al. were used (40, 41).

Broth microdilution. Drug susceptibility testing using the broth microdilution method was performed on all 85 isolates. A panel of 13 drugs, including RIF, CLR, INH, EMB, STR, AMK, SXT, RFB, MXF, LZD,

CIP, doxycycline (DOX), and ethionamide (ETO), using Sensititre SLOMYCO plates (TREK Diagnostic Systems, Cleveland, USA) was tested. Inocula were prepared according to the CLSI protocol (13) and the manufacturer's instructions, and the SLOMYCO plates were incubated at 36°C until the controls showed sufficient growth. The MICs were determined visually using an inverted mirror and read as the lowest concentration of the antibiotic yielding no visible growth. The CLSI-recommended breakpoints were applied: RIF, >1 mg/liter; CLR, >16 mg/liter; EMB, >4 mg/liter; AMK, >32 mg/liter; SXT, >2/38 mg/liter; RFB, >2 mg/liter; MXF, >2 mg/liter; LZD, >16 mg/liter; CIP, >2 mg/liter. In the absence of breakpoints for INH, STR, DOX, and ETO, only MIC values were reported for these drugs, as recommended by the CLSI (13). *M. kansasii* ATCC 12478, *M. avium* ATCC 700898, and *M. peregrinum* ATCC 700686 reference strains were used as controls.

Etest. For 62 *M. kansasii* clinical isolates from Poland, MIC values were also determined using the Etest method according to the manufacturer's instructions (bioMérieux, Durham, NC). Eight antimycobacterial drugs, available under the Etest strip formula, were employed, i.e., RIF, CLR, INH, EMB, STR, AMK, SXT, and kanamycin (KAN). The *M. kansasii* ATCC 12478 reference strain was used as a control.

DNA sequencing and data analysis. The search for mutations possibly associated with drug resistance was performed by PCR amplification and sequencing of nine genetic loci, i.e., *rrl* (CLR), *katG* and *inhA* (INH), *embB* and *embCA* (EMB), *rpsL* and *rrs* (STR), and *gyrA* and *gyrB* (CIP). All primers used for the amplification and sequencing were newly designed (see Table S1 in the supplemental material). The PCRs were performed with a TopTaq master mix kit as recommended by the manufacturer (Qiagen, Hilden, Germany).

PCR amplicons, after purification (Clean-Up; A&A Biotechnology, Gdynia, Poland), were sequenced in both directions using the same primers as that for PCR amplification and, when needed, with additional sequencing primers (Table S1). Sequence analysis was performed with MEGA8 tool using ClustalW and visualized with ESPript (42, 43).

Screening for mutations associated with resistance to all drugs but EMB was performed on *M. kansasii* type I isolates which, upon using the microdilution method, were declared resistant based on the published breakpoints or showed, for a given drug, the five highest MIC values if no breakpoints were available.

Sequencing of the EMB resistance-associated loci (*embB* and *embCA*) was carried out for 8 isolates, each representing a different subtype, declared EMB resistant ($n = 7$) or EMB susceptible ($n = 1$) upon using the dilution method.

The sequences obtained for *M. kansasii* isolates all were compared to sequences of the respective genetic loci of the *M. kansasii* ATCC 12478 and *M. tuberculosis* H37Rv reference strains and to isolates with the five lowest MICs.

Statistical analysis. The MIC_{50}, MIC_{90}, and geometric means (GMs) of the MICs were calculated. Nonparametric statistical methods were used. Mann-Whitney U and chi-square tests were applied for correlations between MICs and interpretation with disease status according to the ATS diagnostic criteria. The differences in the MICs between the two drug susceptibility methods employed (microdilution versus Etest method) were assessed by the Wilcoxon test. Statistical significance was set at a P value of <0.05. All calculations were performed using IBM SPSS v.23 software.

Accession number(s). All nucleotide sequences were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/) under the accession numbers indicated in Fig. S1 to S8 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01788-17.

SUPPLEMENTAL FILE 1, PDF file, 25.2 MB.

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REFERENCES

1. 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, Gómez-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, Mannsåker T, Marras TK, Maugein J, Milburn HJ, Mlinkó T, Morcillo N, Morimoto K, Papaventsis D, Palenque E, Paez-Peña M, Piersimoni C, Polanová M, Rastogi N, Richter E, Ruiz-Serrano MJ, Silva A, da Silva MP, Simsek H, Szabó N, Thomson R, Tórtola Fernandez T, Tortoli E, Totten SE,

Tyrrell G, Vasankari T, Villar M, Walkiewicz R, Winthrop KL, Wagner D. 2013. The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples: a NTM-NET collaborative study. Eur Respir J 42:1604–1613. https://doi.org/10.1183/09031936.00149212.
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 41:1240–1144. https://doi.org/10.1128/JCM.41.3.1240-1244.2003.
3. Iwamoto T, Saito H. 2005. 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. van Ingen J, Kuijper EJ. 2014. Drug susceptibility testing of nontuberculous mycobacteria. Future Microbiol 9:1095–1100. https://doi.org/10.2217/fmb.14.60.
5. Good RC, Silcox VA, Kilburn JO, Plikaytis BD. 1985. Identification and drug susceptibility test results for *Mycobacterium* spp. Clin Microbiol News 7:18. https://doi.org/10.1016/S0196-4399(85)80012-X.
6. Hjelm U, Kaustová J, Kubín M, Hoffner SE. 1992. Susceptibility of *Mycobacterium kansasii* to ethambutol and its combination with rifamycins, ciprofloxacin and isoniazid. Eur J Clin Microbiol Infect Dis 11:51–54. https://doi.org/10.1007/BF01971272.
7. 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 54:957–959. https://doi.org/10.1099/jmm.0.45965-0.
8. Guna R, Muñoz C, Domínguez V, García-García A, Gálvez J, de Julián-Ortiz JV, Borrás 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.
9. 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.
10. 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.
11. 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.
12. 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.
13. Clinical and Laboratory Standards Institute. 2011. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes; approved standard, 2nd ed. CLSI document M24-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
14. Wallace RJ, Jr, Dunbar D, Brown BA, Onyi G, Dunlap R, Ahn CH, Murphy DT. 1994. Rifampin-resistant *Mycobacterium kansasii*. Clin Infect Dis 18:736–743. https://doi.org/10.1093/clinids/18.5.736.
15. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, Holland SM, Horsburgh R, Huitt G, Iademarco MF, Iseman N, Olivier K, Ruoss S, von Reyn CF, Wallace RJ, 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.
16. Yoshida S, Suzuki K, Tsuyuguchi K, Iwamoto T, Tomita M, Okada M, Sakatani M. 2006. Detection of *rpoB* mutations in rifampicin-resistant *Mycobacterium kansasii*. Kekkaku 81:475–479.
17. Klein JL, Brown TJ, French G. 2001. Rifampin resistance in *Mycobacterium kansasii* is associated with *rpoB* mutations. Antimicrob Agents Chemother 45:3056–3058. https://doi.org/10.1128/AAC.45.11.3056-3058.2001.
18. 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.
19. Ahn CH, Wallace RJ, Jr, Steele LC, Murphy DT. 1987. Sulfonamide-containing regimens for disease caused by rifampin-resistant *Mycobacterium kansasii*. Am Rev Respir Dis 135:10–16.
20. Nie W, Duan H, Huang H, Lu Y, Chu N. 2015. Species identification and clarithromycin susceptibility testing of 278 clinical nontuberculosis mycobacteria isolates. BioMed Res Int 1:506598.
21. Brown-Elliott BA, Crist CJ, Mann LB, Wilson RW, Wallace RJ. 2003. In vitro activity of linezolid against slowly growing nontuberculous mycobacteria. Antimicrob Agents Chemother 47:1736–1738. https://doi.org/10.1128/AAC.47.5.1736-1738.2003.
22. Higgins PG, Fluit AC, Hafner D, Verhoef J, Schmitz FJ. 2002. Evidence of cross-resistance between ciprofloxacin and non-fluoroquinolones in European Gram-negative clinical isolates. J Antimicrob Chemother 50:438–440. https://doi.org/10.1093/jac/dkf150.
23. Schmalstieg AM, Srivastava S, Belkaya S, Deshpandea D, Meekc C, Leffc R, van Oersb NSC, Gumboa T. 2012. The antibiotic-resistance arrow of time: efflux pump induction is a general first step in the evolution of mycobacterial drug-resistance. Antimicrob Agents Chemother 56:4806–4815. https://doi.org/10.1128/AAC.05546-11.
24. Woods GL, Bergmann JS, Witebsky FG, Fahle GA, Boulet B, Plaunt M, Brown BA, Wallace RJ, Jr, Wanger A. 2000. Multisite reproducibility of Etest for susceptibility testing of *Mycobacterium abscessus*, *Mycobacterium chelonae*, and *Mycobacterium fortuitum*. J Clin Microbiol 38:656–661.
25. Hoffner SE, Klintz L, Olsson-Lijequist B, Bolmström A. 1994. Evaluation of Etest for rapidly growing susceptibility testing of *Mycobacterium chelonae* and *M. fortuitum*. J Clin Microbiol 32:1846–1849.
26. Biehle JR, Cavalieri SJ, Saubolle MA, Getsinger LJ. 1995. Evaluation of Etest for susceptibility testing of rapidly growing mycobacteria. J Clin Microbiol 33:1760–1764.
27. Fabry W, Schmid EN, Ansorg R. 1995. Comparison of the E-test and a proportion dilution method for susceptibility testing of *Mycobacterium kansasii*. Chemotherapy 41:247–252. https://doi.org/10.1159/000239352.
28. Inagaki T, Yagi T, Ichikawa K, Nakagawa T, Moriyama M, Uchiya K, Nikai T, Ogawa K. 2011. Evaluation of a rapid detection method of clarithromycin resistance genes in *Mycobacterium avium* complex isolates. J Antimicrob Chemother 66:722–729. https://doi.org/10.1093/jac/dkq536.
29. Lee SH, Yoo HK, Kim SH, Won-Jung Koh BS, Kim CK, Park YK, Kim HJ. 2014. The drug resistance profile of *Mycobacterium abscessus* group strains from Korea. Ann Lab Med 34:31–37. https://doi.org/10.3343/alm.2014.34.1.31.
30. Jnawali HN, Yoo H, Ryoo S, Lee KJ, Kim BJ, Koh WJ, Kim CK, Kim HJ, Park YK. 2013. Molecular genetics of *Mycobacterium tuberculosis* resistant to aminoglycosides and cyclic peptide capreomycin antibiotics in Korea. World J Microbiol Biotech 29:975–982. https://doi.org/10.1007/s11274-013-1256-x.
31. Spies FS, von Groll A, Ribeiro AW, Ramos DF, Ribeiro MO, Dalla Costa ER, Martin A, Palomino JC, Rossetti ML, Zaha A, da Silva PE. 2013. Biological cost in *Mycobacterium tuberculosis* with mutations in the *rpsL*, *rrs*, *rpoB*, and *katG* genes. Tuberculosis 93:150–154. https://doi.org/10.1016/j.tube.2012.11.004.
32. Jagielski T, Ignatowska H, Bakuła Z, Dziewit L, Napiórkowska A, Augustynowicz-Kopeć E, Zwolska Z, Bielecki J. 2014. Screening for streptomycin resistance-conferring mutations in *Mycobacterium tuberculosis* clinical isolates from Poland. PLoS One 9:e100078. https://doi.org/10.1371/journal.pone.0100078.
33. Portillo-Gomez L, Mair J, Rouse D, Morris S. 1995. The absence of genetic markers for streptomycin and rifampicin resistance in *Mycobacterium avium* complex strains. J Antimicrob Chemother 36:1049–1053. https://doi.org/10.1093/jac/36.6.1049.
34. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. 2009. Tuberculosis drug resistance mutation database. PLoS Med 6:e1000002. https://doi.org/10.1371/journal.pmed.1000002.
35. Alcaide F, Pfyffer GE, Telenti A. 1997. Role of *embB* in natural and acquired resistance to ethambutol in mycobacteria. Antimicrob Agents Chemother 41:2270–2273.
36. Cohen KA, Bishai WR, Pym AS. 13 June 2014. Molecular basis of drug resistance in *Mycobacterium tuberculosis*. Microbiol Spectr https://doi.org/10.1128/microbiolspec.MGM2-0036-2013.
37. de Moura VC, da Silva MG, Gomes KM, Coelho FS, Sampaio JL, Mello FC, Lourenço MC, Amorim Ede L, Duarte RS. 2012. Phenotypic and molecular characterization of quinolone resistance in *Mycobacterium abscessus subsp. bolletii* recovered from postsurgical infections. J Med Microbiol 61:115–125. https://doi.org/10.1099/jmm.0.034942-0.
38. Jagielski T, Bakuła Z, Roeske K, Kamiński M, Napiórkowska A, Augusty-

nowicz-Kopeć E, Zwolska Z, Bielecki J. 2014. Detection of mutations associated with isoniazid resistance in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates. J Antimicrob Chemother 69:2369–2375. https://doi.org/10.1093/jac/dku161.
39. Nasiri MJ, Haeili M, Ghazi M, Goudarzi H, Pormohammad A, Imani Fooladi AA, Feizabadi MM. 2017. New Insights in to the intrinsic and acquired drug resistance mechanisms in *Mycobacteria*. Front Microbiol 8:681. https://doi.org/10.3389/fmicb.2017.00681.
40. Telenti A, Marchesi F, Balz M, Bally F, Böttger EC, Bodmer T. 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 31:175–178.
41. Bakuła 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.
42. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948. https://doi.org/10.1093/bioinformatics/btm404.
43. Robert X, Gouet P. 2014. Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res 42:320–324. https://doi.org/10.1093/nar/gku316.
44. Alcaide F, Calatayud L, Santín M, Martin R. 2004. Comparative in vitro activities of linezolid, telithromycin, clarithromycin, levofloxacin, moxifloxacin, and four conventional antimycobacterial drugs against *Mycobacterium kansasii*. Antimicrob Agents Chemother 48:4562–4565. https://doi.org/10.1128/AAC.48.12.4562-4565.2004.
45. Shitrit D, Baum GL, Priess R, Lavy A, Shitrit AB, Raz M, Shlomi D, Daniele B, Kramer MR. 2006. Pulmonary *Mycobacterium kansasii* infection in Israel, 1999-2004: clinical features, drug susceptibility, and outcome. Chest 129:771–776. https://doi.org/10.1378/chest.129.3.771.
46. Wang X, Li H, Jiang G, Zhao L, Ma Y, Javid B, Huang H. 2014. Prevalence and drug resistance of nontuberculous mycobacteria, Northern China, 2008-2011. Emerg Infect Dis 20:252–253. https://doi.org/10.3201/eid2007.131801.
47. Biehle J, Cavalieri SJ. 1992. In vitro susceptibility of *Mycobacterium kansasii* to clarithromycin. Antimicrob Agents Chemother 36:2039–2041.
48. Wallace RJ, Jr, Dunbar D, Brown BA, Onyi G, Dunlap R, Ahn CH, Murphy DT. 1986. Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 broth. J Clin Microbiol 24:976–981.
49. Collins CH, Grange JM, Yates MD. 1985. Organization and practice in tuberculous bacteriology. Butterworth, London, United Kingdom.