Characterization of Mutations Conferring Extensive Drug Resistance to *Mycobacterium tuberculosis* Isolates in Pakistan[▽]

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The increasing incidence of extensively drug-resistant (XDR) *Mycobacterium tuberculosis* in high-tuberculosis-burden countries further highlights the need for improved rapid diagnostic assays. An increasing incidence of XDR *M. tuberculosis* strains in Pakistan has been reported, but drug resistance-associated mutations in these strains have not been evaluated previously. We sequenced the “hot-spot” regions of *rpoB*, *katG*, *inhA*, *ahpC*, *gyrA*, *gyrB*, and *rrs* genes in 50 XDR *M. tuberculosis* strains. It was observed that 2% of rifampin, 6% of isoniazid, 24% of fluoroquinolone, and 32% of aminoglycoside/capreomycin resistance in XDR *M. tuberculosis* strains would be undetected if only these common hot-spot regions were tested. The frequencies of resistance-conferring mutations were found to be comparable among all XDR *M. tuberculosis* strain families present, including the Central Asian Strain, Beijing, and East African Indian genogroups and the Unique isolates. Additional genetic loci need to be tested for detection of mutations conferring fluoroquinolone, aminoglycoside, and capreomycin resistance in order to improve molecular diagnosis of regional XDR *M. tuberculosis* strains.

Extensively drug-resistant tuberculosis (XDR-TB) has emerged as a major public health problem worldwide (22). Multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains are resistant to at least the first-line antituberculous agents, rifampin (RIF) and isoniazid (INH) (35). XDR *M. tuberculosis* strains are resistant to at least one fluoroquinolone (FQ) and at least one of the three second-line injectable agents amikacin (AMK), capreomycin (CAP), and kanamycin (KAN), in addition to MDR (15). According to World Health Organization (WHO) estimates of 2008, MDR-TB rates range between 1.6 and 22.3% in high-TB-burden countries, including Pakistan (52). Community-based MDR-TB cases are reported to be 4 to 5% of all TB cases in Pakistan (16). Global occurrence of XDR-TB among all MDR-TB cases is reportedly 6.6%, while in Asia, including Bangladesh and Indonesia, it has been reported as 1.5% (43). Recent data from Pakistan report that the number of XDR-TB cases has increased from 11 (or 1.5%) of 728 MDR isolates in 2006 to 53 (or 4.5%) of 1,181 MDR isolates in 2009 (27). Therefore, there is an urgent need for rapid and reliable tools for diagnosis of MDR- and XDR-TB to aid appropriate treatment and management of drug-resistant TB, particularly in this high-burden setting.

Molecular genotyping studies of mycobacterial strains have illustrated variations in the phylogeography of strains worldwide (19, 20). In addition, it has been shown that the drug resistance patterns of different *M. tuberculosis* genogroup families or clades differ worldwide. Beijing strains are the most predominant genogroup globally, but their resistance patterns differ regionally (25). Beijing strains are found to be associated with drug resistance in studies from Asia, Europe, and Africa

(7, 30, 41). T3 and Central Asian Strain (CAS) family strains have been shown to be associated with MDR (2). Studies from South Africa have reported higher rates of occurrence of F15/LAM4/KZN, known as KZN strains, among XDR *M. tuberculosis* isolates (21). In Pakistan, CAS (43) family isolates are most prevalent, followed by Beijing strains (49). However, Beijing strains were found to be associated with MDR (49). CAS strains were found to be the most prevalent (42.1%) among XDR *M. tuberculosis* isolates, followed by Beijing (8.8%) and T (7%) family strains (27).

Conventional drug susceptibility testing (DST) is highly sensitive and specific but takes around 6 to 9 weeks. Molecular assays for drug resistance detection may represent an alternative to conventional DST (28, 51). Molecular tests target common mutations in “variable” regions of drug resistance genes. Resistance to RIF is well characterized, and more than 95% of strains have mutations in an 81-bp “hot-spot” region of the *rpoB* gene (40, 50). INH resistance can occur due to mutations in several genes, such as *katG*, *inhA*, *kasA*, *oxyR*, and *ahpC* (9, 33, 56). However, 70 to 80% of INH resistance is associated with mutations in codon 315 of *katG* (9, 56). FQ resistance mutations in 320- and 375-bp hypervariable regions of *gyrA* and *gyrB* genes encoding DNA gyrase are thought to account for resistance in 50 to 90% of the phenotypic FQ-resistant isolates (14, 24, 48). Resistance to aminoglycosides (amikacin and kanamycin) and capreomycin is thought to be associated with mutations in the 16S rRNA gene (*rrs*), particularly at positions 1401, 1402, and 1484, leading to AMK, KAN, and CAP resistance, respectively (5, 32).

The frequencies of specific resistance-conferring gene mutations have been found to differ between *M. tuberculosis* genogroups. In MDR strains of the Beijing genotype, the most common mutation for RIF resistance is at codon 531 of the *rpoB* gene (23, 37, 46). Data from Pakistan shows the mutation at codon 526 of *rpoB* gene to be the second most common

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mutation among CAS MDR *M. tuberculosis* isolates, after mutation at codon 531 (6).

Currently, the World Health Organization has endorsed the use of line probe assays (INNO-LiPA Rif and MTBDR_{plus}), in addition to the conventional culture susceptibility testing method, for rapid detection of first-line-drug resistance (36, 53). The MTBDR_{sl} line probe assay is for rapid detection of second-line-drug resistance (11). Together, these tests target *rpoB*, *katG*, *inhA*, and *gyrA* hot-spot-region mutations. As prevalent *M. tuberculosis* strain types differ geographically, as do their frequencies and patterns of drug resistance mutation (36, 37), local data are essential to the development of appropriate molecular assays. No data on mutations of XDR *M. tuberculosis* strains are currently available from Pakistan, and this is the first study where first- and second-line-drug-resistance-conferring mutations in XDR *M. tuberculosis* strains have been characterized.

MATERIALS AND METHODS

Mycobacterium tuberculosis isolates. *M. tuberculosis* strains were obtained from the strain bank at Aga Khan University Clinical Microbiology laboratory, Pakistan. Fifty XDR *M. tuberculosis* isolates which had previously been spoligotyped were selected randomly for the study (27). These strains were from the period of 2004 to 2009 and included 26 CAS isolates, 4 Beijing and 4 T family strains, 2 East African Indian (EAI) and 2 U family strains, 1 Pak3 and 1 X family strain, and 10 Unique strains.

Culture and antibiotic susceptibility testing. Susceptibility testing was performed using the agar proportion method on enriched Middlebrook 7H10 medium (BBL) at the following cutoff concentrations: for RIF, 1 µg/ml; for isoniazid, 0.2 µg/ml; for streptomycin (S), 10 µg/ml; and for ethambutol (EMB), 5 µg/ml. Pyrazinamide (PZA) sensitivity was carried out using Bactec 7H12 medium, pH 6.0, at 100 µg/ml (Bactec PZA test medium; Becton Dickinson). MDR-TB strains were further tested with ciprofloxacin (CIP) at 2 µg/ml, ethionamide (ETH) at 25 µg/ml, CAP at 10 µg/ml, AMK at 6 µg/ml, and KAN at 6 µg/ml on Middlebrook 7H11 agar. The sensitivity data in this study report breakpoints, and MICs were not determined. The susceptibility of XDR strains included in this study was confirmed by repeating the susceptibility testing by the same methodology. Reference strain *M. tuberculosis* H37Rv (ATCC 27294) was used as a control with each batch of susceptibility testing (54).

DNA extraction and amplification. DNA was extracted by the cetyltrimethylammoniumbromide (CTAB) method (29). XDR strains were further subjected to PCR amplification and DNA sequencing in specific hot-spot target regions of the following genes: for *rpoB*, the RIF region comprising bp 1694 to 2189 (38); for INH, the *katG* region comprising bp 872 to 1568 (3), the *inhA* region comprising bp -168 and 79, and the *ahpC* region comprising bp -182 to 100 (37); and for FQ, the *gyrA* region comprising bp 78 to 397 and the *gyrB* region comprising bp 1330 to 1742 (18). For AMK, CAP, and KAN, the *rs* region comprising bp 1202 to 1565 was sequenced using specific primers (18). PCR products were purified using a QIAquick Qiagen PCR purification kit and subjected to DNA sequencing in order to determine the genetic sequence in the hot-spot regions of the genes under investigation.

DNA sequence analysis. DNA sequences were analyzed after DNA sequencing by BLAST using multiple sequence alignments (<http://www.ncbi.nlm.nih.gov/BLAST>). Mutations were detected in the respective gene by comparison with *Mycobacterium tuberculosis* H37Rv (wild-type reference laboratory strain).

RESULTS

Demographic information for XDR *M. tuberculosis* strains. All strains were from pulmonary specimens. Forty-six percent of isolates were from the province of Sindh, 40% from Punjab, 12% from the Khyber Pakhtunkhwa province, and 2% from Baluchistan. Twenty-eight isolates were from male patients and 22 from female patients. The average ages of the male and female patients from which isolates were obtained were 37.28

TABLE 1. Antimicrobial susceptibility profile of antituberculous drugs against XDR *M. tuberculosis* isolates

Drug category	Antituberculous agents to which isolates are resistant (cutoff concn [µg/ml]) ^a	No. of isolates
First line	RMP (1), INH (0.2), PZA (100)	50
	RMP (1), INH (0.2), PZA (100), EMB (5)	49
	RMP (1), INH (0.2), PZA (100), EMB (10), S (10)	30
Second line	CIP (2), AMK (6), KAN (6)	50
	CIP (2), AMK (6), KAN (6), CAP (10)	28
	CIP (2), AMK (6), KAN (6), CAP (10), ETH (25)	8

^a Drug resistance of isolates was determined as described in Materials and Methods. The values in parentheses represent cutoffs for each drug tested. RIF, rifampin; INH, isoniazid; PZA, pyrazinamide; EMB, ethambutol; S, streptomycin; CIP, ciprofloxacin; AMK, amikacin; KAN, kanamycin; CAP, capreomycin; and ETH, ethionamide.

years and 33.09 years, respectively, with median ages of 33 years for males and 34.5 years for females.

Antimicrobial susceptibility pattern of XDR *M. tuberculosis* strains. The drug susceptibility patterns of XDR *M. tuberculosis* strains and the cutoff values for first- and second-line antituberculous agents are summarized in Table 1. All isolates were resistant to RIF, INH, and PZA among first-line drugs and CIP, AMK, and KAN among second-line drugs. Twenty isolates were susceptible to S, and only 1 isolate was susceptible to EMB, while 30 isolates were resistant to all first-line drugs, including EMB and PZA. Twenty-two isolates were sensitive to CAP, and 28 were sensitive to ETH, while 8 XDR isolates were resistant to all the second-line antituberculous agents tested.

Detection of mutations in *rpoB* gene for rifampin resistance. To investigate the mutations associated with rifampin resistance, we examined the 495-bp region including the 81-bp hypervariable RIF resistance detection region (RRDR) of the *rpoB* gene in each XDR isolate. Mutations affecting four amino acid codons of *rpoB*, codons 531 (68%), 516 (24%), 526 (4%), and 513 (2%), were detected in 49 of 50 isolates (Table 2). Forty-four isolates (88%) had a single mutation, and five (10%) had two mutations each. Two strains had a mutation outside the RRDR at codon 575 along with one at codon 516.

Detection of mutations in *katG*, *inhA*, and *ahpC* genes leading to INH resistance. Mutations leading to INH resistance were first investigated in the hot-spot region of *katG*. Forty-one (82%) isolates had mutations at codon 315 (Table 2). The corresponding amino acid mutations were either S315T (76%) or S315N (6%). One strain had a change at codon 328. No mutations were detected in eight (16%) isolates.

The 8 strains without mutations in *katG* were further subjected to sequencing in the *inhA* and *ahpC* promoter regions. Two strains had a transition at the *inhA* position -15 promoter site. Three of the six strains without either *katG* or *inhA* mutations had a transition at *ahpC* position -88. None had dual mutations in *inhA* and *ahpC* genes. We could not detect any mutations responsible for INH for three strains based on *katG*, *inhA*, and *ahpC*.

Detection of mutations in *gyrA* and *gyrB* genes for fluoroquinolone resistance. Sequencing of the *gyrA* gene revealed

TABLE 2. Mutations conferring resistance to first-line TB drugs in 50 XDR isolates

Gene	Codon/nucleotide change(s)	Amino acid/nucleotide change(s)	Total no. of isolates	Genotype(s) (no. of isolates per genotype) ^a
<i>rpoB</i>	TCG → TTG	S531L	33	CAS (16), EAI (1), Beijing (3), T (4), U (2), X (1), Pak3 (1), Unique (5)
	A inserted at nt 1942 and G deleted from nt 1944	A replaced G at codon 531	1	CAS (1)
	CAA → AAA	Q513K	1	Unique (1)
	ATG → ATC, GAC → TAC	M515I, D516Y	3	CAS (1), Unique (2)
	GAC → TAC	D516Y	2	CAS1 (2)
	GAC → GTC	D516V	5	CAS (3), Beijing (1), Unique (1)
	GAC → TAC, CTG → CGG	D516Y, L575R	1	CAS (1)
	GAC → GTC, CTG → CGG	D516V, L575R	1	CAS1
	CAC → AAC	H526N	1	CAS
	CAC → CGC	H526R	1	EAI
		No mutation	1	Unique (1)
<i>katG</i>	AGC → ACC	S315T	37	CAS (18), EAI (1), Beijing (3), T (4), U (2), X (1), Pak3 (1), Unique (7)
	AGC → AAC	S315N	3	CAS (2), Unique (1)
	AGC → ACC, GCG → GGG	S315T, A242G	1	CAS (1)
	TGG → TAG	W328Stop	1	CAS (1)
		No mutation	8 ^b	CAS (4), Beijing (1), EAI (1), Unique (2)
<i>inhA</i>	C → T	At position –15	2	EAI (1), Unique (1)
		No mutation	6	CAS (4), Beijing (1), Unique (1)
<i>ahpC</i>	G → A	At position –88	3	CAS (1), Beijing (1), Unique (1)
		No mutation	5	CAS (3), EAI (1), Unique (1)

^a CAS, Central Asian strain; EAI, East African Indian; T, T clade; U, U clade; Unique isolates, shared types which did not match any in the SpolDB 4.0 database (Institut Pasteur de Guadeloupe).

^b Isolates sequenced for *inhA* and *ahpC* gene promoter regions.

that 11/50 (22%) isolates had a mutation at codon 90, while 26/50 (52%) had variable mutations at codon 94 (Table 3). Forty-seven strains had an AGC-to-ACC polymorphism at codon 95, and 11 of these had no other mutation. No *gyrA* mutation was detected in one isolate. As the codon 95 mutation is reportedly not associated with drug resistance, all 11 isolates and the one without a mutation were further tested for *gyrB* gene mutations. Only 1 of the 12 tested displayed a mutation at codon 471 of the *gyrB* gene.

Detection of mutations in *rrs* genes for AMK resistance. We next analyzed the nt 1400 region of the *rrs* gene to investigate resistance-conferring mutations for AMK, KAN, and CAP. Mutations were detected in 35/50 (70%) strains (Table 3). Thirty-three of the strains had an A-to-G transition at nucleotide (nt) 1401, while 1 had a mutation at nt 1344 and another at nt 1484.

Relationship between *M. tuberculosis* genogroups and the resistance-conferring mutations in XDR *M. tuberculosis* isolates. The distribution of mutations according to genogroups of the *M. tuberculosis* clinical isolates showed the most common single *rpoB* mutation to be S531L (Table 4). Most strains had a single mutation, but 3 CAS and 2 Unique genotype strains revealed 2 mutations each.

Of the *katG* gene mutations, that at codon 315 was the most common mutation (Table 4). Of the 8 strains without *katG* mutations, 4 were CAS family strains, 2 were Unique strains, and 1 was an EAI and another a Beijing family isolate. Of these, only the EAI and Unique group isolates showed an *inhA*

position –15 region mutation. The 3 strains with mutations in the *ahpC* region belonged to the CAS, Beijing, and Unique genogroups, while the 3 strains for which INH resistance could not be detected belonged to the CAS family.

gyrA gene mutations at codons 90 and 94 were present among all XDR genogroups tested. The 12 strains where *gyrA* gene resistance was not detected belonged to the CAS, EAI, T, and Unique genogroups (Table 4). Analysis of *gyrB* gene mutations identified a codon 271 mutation in one Unique isolate but none in the CAS, EAI, and T genogroups.

All XDR isolates showed a predominant *rrs* gene mutation at nt 1401. One CAS isolate had additional mutations at nt 1344/nt 1358, while one Unique isolate had a mutation at nt 1484. The 15 XDR strains where no *rrs* mutation were detected comprised the CAS, EAI, Beijing, T, U, and Unique genogroups (Table 4).

DISCUSSION

Culture and DST remain the most sensitive and reliable methods for detection of *M. tuberculosis* drug resistance. However, phenotypic testing is time-consuming and requires specialized laboratory facilities. Therefore, there is a need for rapid molecular methods for detection of drug resistance. This study illustrates that molecular methods for antimicrobial susceptibility testing of XDR *M. tuberculosis* isolates test only primary mutational hot-spot regions of the *rpoB*, *katG*, *gyrA*, and *rrs* genes, a proportion of phenotypically resistant XDR

TABLE 3. Mutations conferring resistance to second-line TB drugs in 50 XDR isolates

Gene	Sequence change(s)	Amino acid change(s)	Total no. of isolates	Genotype(s) (no. of isolates per genotype)
<i>gyrA</i>	GCG → GTG, AGC → ACC	A90V, S95T	7	CAS (5), Unique (2)
	GCG → GTG	A90V	2	Unique (1), T (1)
	TCG → CCG, AGC → ACC	S91P, S95T	1	CAS (1)
	GCG → GTG, TCG → CCG, AGC → ACC	A90V, S91P, S95T	1	CAS (1)
	GCG → GTG, AGC → ACC, CTG → GGC	A90V, S95T, L96P	1	Unique (1)
	GAC → GGC, AGC → ACC	D94G, S95T	14	CAS (7), Beijing (2), U (1), Pak3 (1), Unique (3)
	GAC → TAC, AGC → ACC	D94Y, S95T	5	CAS (3), X (1), Beijing (1)
	GAC → GCC, AGC → ACC	D94A, S95T	2	CAS (1), Beijing (1)
	GAC → AAC	D94N	1	T (1)
	GAC → AAC, AGC → ACC	D94N, S95T	1	T (1)
	GAC → GGC, AGC → ACC, GAC → AAC	D94G, S95T, D111N	2	CAS (2)
	AGC → ACC	S95T	11 ^a	CAS (6), EAI (2), Unique (3)
		No mutation	1 ^a	T (1)
<i>gyrB</i>	GCC → GTC	A471V	1	Unique (1)
		No mutation	11	T (1), CAS (6), EAI (2), Unique (2)
<i>rrs</i>	A → G	At position 1401	29	CAS (16), EAI (1), Beijing (2), T (2), U (1), X (1), Pak3 (1), Unique (5)
	A → G, A → T	At positions 1401 and 1252	2	CAS (1), Unique (1)
	A → G, A → T	At positions 1401 and 1462	1	CAS (1)
	A → G, G → T	At positions 1401 and 1472	1	Unique (1)
	C → G, C → T	At positions 1344 and 1358	1	CAS (1)
	G → T	At position 1484	1	Unique (1)
		No mutation	15	CAS (7), EAI (1), Beijing (2), T (2), U (1), Unique (2)

^a Isolates subjected to *gyrB* gene sequencing.

strains, particularly those with fluoroquinolone (24%), amikacin/kanamycin, and capreomycin (32%) resistance, would remain undetected.

The difficulty in treatment of XDR isolates is further highlighted by the observation that 8 of 50 strains resistant to all first- and second-line antituberculous agents tested.

Rifampin resistance due to common mutation at codons 531, 516, and 526 of the *rpoB* gene is consistent with data from Pakistan and other countries (4, 6, 12, 47). We observed a high frequency of mutations at codon 516 of the *rpoB* gene in XDR strains, especially in CAS spoligotypes. This differs from re-

ports of low frequencies of codon 516 mutations among XDR *M. tuberculosis* isolates from India and China (4, 47). Also, it has previously been reported that the codon 526 mutation of *rpoB* is associated with MDR among CAS isolates (6).

The most common mutation for INH resistance in the XDR strains was at codon 315 of *katG* gene, correlating with the data reported in other studies (4, 12). We also observed one isolate with a mutation at codon 328 of *katG*, which has been identified previously among XDR isolates (12, 47). Testing of *katG* and *inhA* hot-spot regions identified mutations in 44 of 50 isolates, while another *ahpC* mutation was found in 3 other

TABLE 4. Drug resistance-associated gene mutations in XDR *M. tuberculosis* isolates versus genotypes^a

Genotype (no. of isolates)	No. of strains with mutation at:																						
	<i>rpoB</i> codon							<i>katG</i> codon					<i>gyrA</i> codon						<i>rrs</i> nucleotide				
	513	515	516	526	531	575	ND	315	328	242	379	ND	90	91	94	96	111	ND	1344	1401	1484	Others	ND
CAS (26)		1*	7	2	16	2*	1	21	1	1*		4	6	1, 1*	13		1*	6	1	18		2*	7
EAI (2)				1	1			1				1						2		1			1
Beijing (4)			1		3			3				1			4					2			2
Unique (10)	1	2*	4		5	1*		8			2*	2	4		3	1*		3		7	1	2*	2
T (4)					4			4					1		2			1		2			2
X (1)					1			1							1					1			
U (2)					2			2							2					1			1
Pak3 (1)					1			1							1					1			
Total	1	3*	12	3	33	3*	1	41	1		3*	8	11	1, 1*	26	1*	1*	12	1	33	1	4*	15

^a An asterisk denotes concurrent mutations. ND, none detected.

strains. INH resistance can also be due to changes in the *kasA* and *oxyR* genes (13), and it may be necessary to study these genes to investigate the molecular basis for isoniazid resistance in these XDR *M. tuberculosis* isolates.

The most common mutations for FQ resistance were at *gyrA* gene codons 94 and 90, with a low frequency of mutation at codon 91. This is consistent with reports of XDR strains from India, China, the United States, and Germany (4, 12, 18, 47). In contrast, a study from Portugal reported higher rates of mutations at codon 91 of *gyrA* (42). We found that the sequence change at codon 94 exhibited the highest variability. Ninety-four percent of the XDR strains revealed a mutation at codon 95 of the *gyrA* gene; however, this mutation does not contribute to FQ resistance and instead serves as an evolutionary marker for classification of *M. tuberculosis* strains into three principal genetic group (PGGs) (44). The ACC polymorphism at codon 95 confirms the PGG1 grouping of CAS and Beijing strains (26). However, we could detect only 76% of FQ-resistant isolates via mutations at *gyrA* codons 91 and 94, compared with 100% detection of FQ resistance in XDR *M. tuberculosis* isolates from a recent hospital based study in Mumbai, India (4).

One XDR isolate had a novel A471V mutation in the *gyrB* gene protein, which may be responsible for FQ resistance. Eleven (22%) phenotypically resistant FQ isolates did not appear to have mutations in either *gyrA* or *gyrB* genes. Alternative mechanisms for FQ resistance have been attributed to drug efflux pumps, and it may be possible that FQ resistance in these isolates may be attributable to those (1, 45).

Screening of the *rrs* gene for detection of resistance to AMK, KAN, and CAP revealed the A1401G mutation to be the most common and present mutation in 66% of XDR isolates, consistent with reports from India and China (4, 34, 47). All isolates with an *rrs* nt 1401 mutation were KAN and AMK resistant, but 51.5% were CAP susceptible. Mutations at nt 1484 have been reported to be associated with AMK, KAN, and CAP resistance (4, 42). Additional point mutations in *rrs* may contribute to resistance, perhaps also the concomitant mutations at nt 1344 and 1358. The impact of *rrs* mutations at nt 1252, 1462, and 1472 could not be determined, as these were concomitant with the mutation at nt 1401. No *rrs* gene mutations were detected in 15 (30%) XDR isolates, which included both amikacin/kanamycin- and capreomycin-resistant strains. We report more XDR *M. tuberculosis* isolates with mutations other than those in *rrs* at nt 1401 and 1484 than those reported for XDR *M. tuberculosis* isolates from India, where a detection rate of 98% for KAN, AMK, and CAP resistance is stated (4). CAP and KAN resistance can also be due to mutations in the *thyA* and *eis* genes, respectively (39, 55). Therefore, additional genetic loci may be responsible for resistance to aminoglycosides and capreomycin in *M. tuberculosis*, and additional sites need to be included for molecular testing for resistance to second-line drugs in order to avoid misidentification of XDR isolates.

It is possible that the discordance between the phenotypic and genotypic resistance to FQ and AMK/KAN/CAP observed may be due to a lower sensitivity of currently available genotypic methods than of phenotypic methods. These differences could be due to alternate mutations in regions other than the currently known target regions for these drugs (17). In addition,

mycobacterial cultures may also comprise a mixed population of resistant and sensitive bacteria (8), and therefore, DNA extracted from sample specimens may be a lower-level representation of the resistant genotype, leading to a falsely low detection rate in the specimen. However, given the increasing levels of drug resistance in *M. tuberculosis* strains worldwide and the imperative to rapidly detect and treat these strains, the molecular testing needs to be emphasized. A comparative analysis of the genogroups of the XDR strains indicated that the mutations were common to all genogroups and that there was no association between a specific mutation and the strain type.

Overall, our data highlight the need to include testing of additional genes for surveillance of drug resistance and indicate that molecular testing based on common mutational hotspot regions of *rrs* and *gyrA* genes is not sufficient to detect resistance in XDR strains globally. Insufficient detection of XDR *M. tuberculosis* strains would lead to a further increase in drug-resistant TB in an environment already overburdened by the disease.

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