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Geographical Differences Associated with Single-Nucleotide Polymorphisms (SNPs) in Nine Gene Targets among Resistant Clinical Isolates of *Mycobacterium tuberculosis*

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Alternative diagnostic methods, such as sequence-based techniques, are necessary for increasing the proportion of tuberculosis cases tested for drug resistance. Despite the abundance of data on drug resistance, isolates can display phenotypic resistance but lack any distinguishable markers. Furthermore, because resistance-conferring mutations develop under antibiotic pressure, different drug regimens could favor unique single-nucleotide polymorphisms (SNPs) in different geographical regions. A total of 407 isolates were collected from four geographical regions with a high prevalence of drug-resistant tuberculosis (India, Moldova, the Philippines, and South Africa). The "hot spot" or promoter sequences of nine genes (*rpoB*, *gyrA*, *gyrB*, *katG*, *inhA* promoter, *ahpC* promoter, *eis* promoter, *rrs*, and *tlyA*) associated with resistance to four types of antibiotics (rifampin, isoniazid, fluoroquinolones, and aminoglycosides) were analyzed for markers. Four genes contributed largely to resistance (*rpoB*, *gyrA*, *rrs*, and *katG*), two genes contributed moderately to resistance (the *eis* and *inhA* promoters), and three genes contributed little or no resistance (*gyrB*, *tlyA*, and the *ahpC* promoter) in clinical isolates. Several geographical differences were found, including a double mutation in *rpoB* found in 37.1% of isolates from South Africa, the C→T mutation at position −12 of the *eis* promoter found exclusively in 60.6% of isolates from Moldova, and the G→A mutation at position −46 of the *ahpC* promoter found only in India. These differences in polymorphism frequencies emphasize the uniqueness of isolates found in different geographical regions. The inclusion of several genes provided a moderate increase in sensitivity, and elimination of the examination of other genes might increase efficiency.

Determination of antibiotic resistance in clinical isolates of *Mycobacterium tuberculosis* is often performed through phenotypic test procedures in which the bacteria are inoculated onto growth media containing antibiotics. However, there are key weaknesses in using these methods despite being the gold standard for drug resistance testing. Due to the long generation time of *M. tuberculosis*, results of the tests often are not known until 28 to 40 days after the initial inoculation (1). In addition, testing for resistance to two first-line antibiotics used in tuberculosis treatment, i.e., rifampin and isoniazid, is rare. In 2011, the World Health Organization (WHO) estimated that only 1.8% of new global cases and 6.4% of existing global cases were tested for resistance to those two antibiotics (2). These flaws in the current system emphasize the need for rapid and efficient alternative diagnostic tests.

One alternative is sequence-based testing, in which genes that are associated with resistance are sequenced for known resistance-conferring mutations. The Hain GenoType MTBDRplus assay (3) and the GeneXpert test (4) are recommended tests for drug resistance genotyping and are used in some countries. Effective utilization of this method, however, requires that all mutations must be known before testing. Although resistance-conferring mutations have been studied extensively and compiled into databases (5), not all mutations have been documented, as shown by isolates that are phenotypically resistant but genotypically sensitive. These isolates are known as discordant isolates and are prime candidates for the study of novel resistance markers.

Because *M. tuberculosis* has been known to exchange DNA rarely, drug resistance mutations are confined to the bacterial chromosome (6), specifically within or near genes. Certain genes in the *M. tuberculosis* genome, such as *rpoB* and *gyrA*, have been noted to be strongly associated with resistance to antibiotics (7, 8). Furthermore, mutations that do occur within these genes tend to congregate within a specific region of the gene, known as a "hot spot." For example, mutations found in *rpoB* occur mostly within an 81-bp region known as the rifampin resistance-determining region. The *gyrA* gene contains a similar region, known as the quinolone resistance-determining region, that spans codon 88 to codon 95. Other genes, however, have been linked only recently to antibiotic resistance, such as the *tlyA* gene and capreomycin resistance (9) and the *eis* gene and kanamycin resistance (10). Inclusion of these genes within sequence-based diagnostic tests might lower the frequency of discordant isolates and thus increase test sensitivity.

The development of drug resistance mutations ultimately depends on selective pressure, i.e., exposure to the antibiotic. Although the WHO has made recommendations regarding some aspects of treatment regimens, such as the length, dosage, and

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types of antibiotic treatment (11), variations in regimens still exist between countries, especially within the private sector (12). Resistance-conferring mutations often cause a loss of fitness for the bacterium. One prime example is isoniazid and the catalase-peroxidase enzyme KatG, which is necessary for converting isoniazid into an active form. Mutations in the *katG* gene cause the enzyme to be less functional, preventing the activation of isoniazid but also hindering the bacterium from detoxifying organic peroxides. Because of the loss of fitness associated with drug resistance mutations, mutations that provide a balance between the benefits and costs of fitness are selected for. In the previous scenario, mutations in codon 315 of the *katG* gene are the most commonly observed in clinical isolates, because the resulting inhibition of the enzyme provides sufficient resistance and detoxification (8). Uneven selective pressure could occur among isolates from different countries, causing one isolate to favor mutations in one locus rather than others. These variations in mutations could impact the generalizability of sequence-based diagnostic tests and create differences in sensitivities for different geographical regions.

To evaluate the strength of the association between certain genetic markers and antibiotic resistance, as well as to identify regional differences in single-nucleotide polymorphisms (SNPs), genes from isolates of different geographical regions were sequenced and analyzed for known mutations associated with resistance. The presence or absence of mutations was then compared with drug susceptibility testing (DST) data to identify any isolates for which there were discordances between the two sets of data.

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MATERIALS AND METHODS

Gene selection. The hot spots or promoter regions of nine genes (with the exception of *tlyA*, for which the entire structural gene was sequenced) were analyzed to determine genotypic resistance to four types of antibiotics, i.e., rifampin (*rpoB*), isoniazid (*katG*, *ahpC*, and *inhA*), fluoroquinolones (*gyrA* and *gyrB*), and aminoglycosides (*rrs*, *eis*, and *tlyA*).

Isolate selection. A total of 407 isolates from four different study sites with high prevalence rates of drug-resistant tuberculosis were collected and provided by the University of California, San Diego. A total of 123 isolates were from the Tropical Disease Foundation in the Philippines (2000 to 2009), 100 isolates were from South Africa (30 isolates from the Glen Gray TrakCare laboratory, 51 isolates from the National Institute for Communicable Diseases in Sandringham, and 19 isolates from the Central Tuberculosis Laboratory in Braamfontein) (2006 to 2009), 96 isolates were from Hinduja Hospital in India (2007 to 2010), and 88 isolates were from the Center for Health Policies and Studies in Moldova (2008 to 2010). All isolates were collected by central and reference laboratories for each country (with the exception of the Glen Gray TrakCare laboratory in Eastern Cape, South Africa) and were chosen based on convenience sampling. Drug susceptibility testing data for a bank of clinical isolates were examined, and isolates for this study were selected to maximize the diversity of drug resistance patterns. The sample contained a small selection of isolates that were determined to be pansusceptible to rifampin, isoniazid, fluoroquinolones, and aminoglycosides through drug susceptibility testing, for the purposes of reference and control.

DNA extraction. *M. tuberculosis* DNA extraction was performed by a method developed by van Sooligen et al. with minor modifications at the University of Hawaii at Manoa (D. van Soolingen, P. E. W. de Haas, P. W. M. Hermans, and J. D. A. van Embden, unpublished data). Isolates were grown on Löwenstein-Jensen medium at the study sites. Several loopfuls of bacteria were collected on wooden applicator sticks and transferred to a microcentrifuge tube. The bacteria were then killed through exposure to ethanol and heat and were shipped to the University of Hawaii at Manoa. Samples were centrifuged for 5 min at 12,000 rpm using a 5415C centrifuge (Eppendorf), lysozyme (50 μl of a 10-mg/ml solution) was added to each sample, and the mixtures were incubated overnight at 37°C. The samples were collected, sodium dodecyl sulfate (75 μl of a 10% solution) and proteinase K (5 μl of a 10-mg/ml solution) were added, and the mixtures were incubated for 10 min at 65°C. After incubation, 5 M NaCl (100 μl) and cetrimonium bromide-NaCl solution (100 μl of 0.7 M NaCl, 274 mM cetrimonium bromide) were added, and the mixture was incubated for another 10 min at 65°C. A 750-μl volume of chloroform-isoamyl alcohol (24:1) was added to the samples, and the mixtures were centrifuged for 5 min at 12,000 rpm. The supernatant was collected, and isopropanol (450 μl) was used to precipitate the nucleic acids. Each sample was incubated for 30 min at −20°C and then centrifuged for 15 min at 12,000 rpm. The supernatant above the pellet of DNA was discarded, and 70% ethanol (750 μl) was added. The samples were then centrifuged for 5 min at 12,000 rpm, and the supernatant was again discarded, with a slight volume above the pellet remaining. After a final centrifugation step for 1 min at 12,000 rpm, the last of the supernatant was removed and the samples were allowed to air dry for 30 min. The DNA was then dissolved in sterile distilled water.

PCR. All genes were amplified using real-time PCR with the exception of *tlyA*, which was amplified using conventional PCR because of the large amplicon size. Real-time PCRs were performed with 2× LightCycler 480 SYBR Green I Master (Roche), 0.4 μM (each) forward and reverse primers (Table 1), and 10 ng of *M. tuberculosis* genomic DNA. The reactions began with initial denaturation at 95°C for 5 min, followed by 45 cycles of amplification consisting of denaturation at 95°C for 10 s, annealing at a temperature based on the primer set (Table 1) for 10 s, and extension at 72°C for 10 s. These reactions were conducted in a Roche LightCycler 480 II real-time PCR instrument. For each run, a positive control (H37Rv) and a negative control (reaction mixture lacking genomic DNA) were included. Positive reactions resulted in sigmoidal curves for fluorescence signals, and negative reactions showed no increase in fluorescence. Fluorescence curves were analyzed using the second derivative maximum method to determine the crossing point. Conventional PCR for amplification of the *tlyA* gene was performed using 2× TopTaq Master Mix (Qiagen), 4 μM (each) forward and reverse primers (Table 1), and 10 ng of *M. tuberculosis* genomic DNA. The reactions began with initial denaturation at 95°C for 5 min, followed by 35 cycles of amplification consisting of denaturation at 95°C for 60 s, annealing at 55° for 60 s, and extension at 72°C for 90 s. Reactions were conducted in a PerkinElmer Cetus DNA thermal cycler. For each run, a positive control (H37Rv) and a negative control (reaction mixture lacking genomic DNA) were included. Real-time PCR products were purified using the MinElute 96 UF PCR purification kit (Qiagen). Reactions amplified through conventional PCR were purified using silica membrane spin columns from the QIAquick PCR purification kit (Qiagen).

DNA sequencing. DNA sequencing of PCR products was performed at Advanced Studies of Genomics, Proteomics, and Bioinformatics, University of Hawaii at Manoa, using an Applied Biosystems 3730 XL analyzer in conjunction with a BigDye Terminator v3.1 cycle sequencing kit. Forward primers were used for sense sequencing of the products with the exception of *tlyA*, which was assembled via primer walking.

Drug susceptibility testing. Antibiotic resistance testing for each isolate was performed at the respective study site. Resistance was determined for isolates originating from the Philippines using either the proportion method (1) or a Bactec MGIT 960 system. Isolates originating from India, Moldova, and South Africa were tested only with the Bactec MGIT 960 system.

Statistical analyses. The data were analyzed using a chi-square test for differences. Chi-square and P values were calculated using Epi Info 3.5.1 (CDC, Atlanta, GA). All tests were two-tailed, and P values of ≤ 0.05 were considered statistically significant.

TABLE 1 Primers used for PCR amplification of nine genes

Gene	Primer sequence (5′ to 3′)	Annealing temperature (°C)	Amplicon size (bp)
rpoB	CGTGGAGGCGATCACACCGCAGTT AGTGCGACGGGTGCACGTCGCGGACCT	60	215
gyrA	GGTGCTCTATGAAATGTTCG GCTTCGGTGTACCTCATCG	60	234
gyrB	CGATGTTCCAGGCGATACTT ATCTTGTGGTAGCGCAGCTT	60	163
rrs	GTAATCGCAGATCAGCAACG TTTTCGTGGTGCTCCTTAGAA	60	216
tlyA	GTCTCTGGCCGAACTCGAAG ATTGTCGCCCAATACTTTTTCTAC	60	1,000
eis promoter	AAATTCGTCGCTGATTCTCG CGCGACGAAACTGAGACC	54	387
katG	CATGAACGACGTCGAAACAG CTCTTCGTCAGCTCCCACTC	55	270
inhA promoter	AGAAAGGGATCCGTCATGGT GTCACATTCGACGCCAAAC	55	340
ahpC promoter	CACTGCTGAACCACTGCTTT CAGTGGCATGACTCTCCTCA	55	196

RESULTS

Pansusceptible isolates. Of the 407 clinical isolates examined, 31 were determined to be pansusceptible to the four antibiotics by drug susceptibility testing. However, 5 of the 31 isolates contained a known mutation associated with drug resistance. The remaining 26 isolates lacked any known mutations, which is in agreement with the drug susceptibility testing data. These 26 pansusceptible isolates were excluded from further analysis, reducing the sample size to 381 isolates containing at least one known marker for resistance (India, 96 isolates; Moldova, 82 isolates; Philippines, 110 isolates; South Africa, 93 isolates). Table 2 displays the frequency of resistant markers found in the 381 isolates and further divides these isolates by origin, in four geographical regions.

Rifampin. The most common mutation observed within the rifampin resistance-determining region was the TCG→TTG mutation in codon 531. Based on the frequency of this marker, the four study sites were divided into two groups, i.e., a group with a high frequency of this mutation, including India (86.4%) and Moldova (81.0%), and a group with a lower frequency, including the Philippines (54.7%) and South Africa (44.9%) (Table 2). Study sites within the same grouping were not found to be significantly different. However, when study sites were compared with those in the other group, the difference in the proportions of this mutation was significant ($P < 0.001$). South Africa was noted to contain a larger proportion of codon 516 mutations as well (46.1%), which was significantly different from values for all other study sites. Double mutations were rarely observed in India, Moldova, and the Philippines. Only one isolate from India (ATG→ATA in codon 515 and CAC→AAC in codon 526) and one isolate from the Philippines (CAA→AAA in codon 513 and CAC→GAC in codon 526) contained double mutations, while one isolate from the Philippines contained a triple mutation (CTG→CCG in codon 511, AGC→ACC in codon 512, and GAC→TAC in codon 516). However, 33 (37.1%) of the 89 South African isolates that had a marker for rifampin resistance contained the double mutation of GAC→GGC in codon 516 and CTG→CCG in codon 533.

Isoniazid. Resistance to isoniazid was largely due to the *katG* mutation of AGC→ACC in codon 315, with 305 of the 381 isolates containing this mutation (Table 2). Moldova had the largest proportion of this mutation, with all isolates containing it. This large proportion was significantly different from values for the three other study sites ($P \leq 0.05$). In the promoter region, T→A mutations at the −8 position of *inhA* were observed in a large proportion (38.9%) of South African isolates despite being rarely found in isolates from India (2.3%) and not found in isolates from the Philippines or Moldova. C→T mutations at the −15 position were observed in all study groups, with India having a significantly smaller proportion (19.5%) than the other sites ($P \leq 0.027$). Five isolates from India, 25 isolates from the Philippines, and six isolates from South Africa had mutations within the *inhA* promoter but wild-type *katG*. G→A mutations at position −46 of the *ahpC* promoter were found in only 18 isolates, all originating from India (20.7%). Nine of these isolates contained a *katG* mutation but wild-type *inhA*, one isolate had an *inhA* mutation but wild-type *katG*, one isolate had mutations in both *inhA* and *katG*, and seven isolates had wild-type *katG* and *inhA*.

Fluoroquinolones. Mutations that confer resistance to the fluoroquinolones were observed mostly within the quinolone resistance-determining region of *gyrA*, particularly codons 90 and 94. A larger proportion of GCG→GTG mutations in codon 90 were observed in isolates from South Africa (50.6%). However,

TABLE 2 Drug resistance mutations found in nine genes for 381 different isolates

Antibiotic[a]	Gene	Codon	Nucleotide	Polymorphism	No. (%) of isolates[b]			
					IN	MD	PH	SA
RIF	*rpoB*	511		CTG→CCG	0	0	1 (1.1)	0
		512		AGC→ACC	0	0	1 (1.1)	0
		513		CAA→AAA	2 (2.3)	0	2 (2.1)	1 (1.1)
				CAA→CCA	1 (1.1)	0	0	0
		515		ATG→ATA	1 (1.1)	0	0	0
		516		GAC→GGC	0	0	1 (1.1)	33 (37.1)
				GAC→GTC	4 (4.5)	11 (13.9)	2 (2.1)	8 (9.0)
				GAC→TAC	0	2 (2.5)	2 (2.1)	0
		522		TCG→TTG	0	0	2 (2.1)	0
		526		CAC→TAC	3 (3.4)	0	10 (10.5)	7 (7.9)
				CAC→TGC	1 (1.1)	0	3 (3.2)	0
				CAC→AAC	1 (1.1)	0	0	0
				CAC→CTC	0	1 (1.3)	3 (3.2)	0
				CAC→GAC	0	0	4 (4.2)	0
				CAC→GGC	0	0	1 (1.1)	0
				CAC→CGC	0	0	8 (8.4)	0
				CAC→CCC	0	0	1 (1.1)	0
		531		TCG→TTG	76 (86.4)	64 (81.0)	52 (54.7)	40 (44.9)
				TCG→TGG	0	1 (1.3)	5 (5.3)	0
		533		CTG→CCG	0	0	1 (1.1)	34 (38.2)
				Total with marker	88 (100.0)	79 (100.0)	95 (100.0)	89 (100.0)
INH	*katG*	315		AGC→ACC	75 (86.2)	82 (100.0)	64 (69.5)	84 (93.3)
	inhA		−8	T→A	2 (2.3)	0	0	35 (38.9)
			−8	T→C	5 (5.7)	2 (2.4)	0	0
			−15	C→T	17 (19.5)	40 (48.8)	34 (37.0)	32 (35.6)
	ahpC		−46	G→A	18 (20.7)	0	0	0
				Total with marker	87 (100.0)	82 (100.0)	92 (100.0)	90 (100.0)
FQL	*gyrA*	90		GCG→GTG	16 (19.5)	18 (32.1)	14 (43.8)	41 (50.6)
		91		TCG→CCG	1 (1.2)	3 (5.4)	0	0
		94		GAC→AAC	10 (12.2)	3 (5.4)	1 (3.1)	8 (9.9)
				GAC→CAC	2 (2.4)	2 (3.6)	0	0
				GAC→TAC	3 (3.7)	8 (14.3)	4 (12.5)	1 (1.2)
				GAC→GCC	13 (15.9)	7 (12.5)	1 (3.1)	3 (3.7)
				GAC→GGC	37 (45.1)	15 (26.8)	13 (40.6)	30 (37.0)
	gyrB	510		AAC→ACC	1 (1.2)	1 (1.8)	0	0
		515		GCG→GTG	2 (2.4)	0	0	0
				Total with marker	82 (100.0)	56 (100.0)	32 (100.0)	81 (100.0)
AMI	*rrs*		1401	A→G	66 (90.4)	21 (31.8)	18 (90.0)	84 (98.8)
			1484	G→T	2 (2.7)	1 (1.5)	0	0
	eis		−10	G→A	0	3 (4.5)	1 (5.0)	0
			−12	C→T	0	40 (60.6)	0	0
			−14	C→T	5 (6.8)	6 (9.1)	0	1 (1.2)
			−37	G→T	1 (1.4)	0	1 (5.0)	0
	tlyA			Any	0	0	0	0
				Total with marker	73 (100.0)	66 (100.0)	20 (100.0)	85 (100.0)

[a] RIF, rifampin; FQL, fluoroquinolones; AMI, aminoglycosides; INH, isoniazid.
[b] IN, India; MD, Moldova; PH, Philippines; SA, South Africa.

this difference was significant only when South Africa was compared with India only ($P < 0.001$). Codon 94 mutations were the most commonly found, with India having the largest proportion (79.3%) in comparison with the Philippines (59.4%), Moldova (52.2%), and South Africa (50.6%). Differences in the proportions of codon 94 mutations were found to be significant when India was compared with Moldova ($P = 0.049$) and South Africa ($P < 0.001$) but not the Philippines. Similar to the case with *rpoB*, all isolates contained only a single mutation within the quinolone resistance-determining region, with the exception of one isolate from the Philippines that had a double mutation (GCG→GTG in codon 90 and GAC→TAC in codon 94). Mutations in *gyrB* were rarely found, with only 4 isolates having mutations within this gene. Additionally, these mutations always occurred in conjunction with a mutation in *gyrA*.

Aminoglycosides. Mutations in the *rrs* gene, particularly at nucleotide position 1401, contributed most commonly to aminoglycoside resistance. This mutation was highly prevalent in isolates from India (90.4%), the Philippines (90.0%), and South Africa (98.8%) but less so in isolates from Moldova (31.8%). The largest proportion of position 1401 mutations, seen in the isolates from South Africa, was significantly different from values for In-

dia ($P = 0.041$) and Moldova ($P < 0.001$) but not the Philippines. Additionally, the small proportion of position 1401 mutations found in Moldova was significantly different from values for all other study sites ($P < 0.001$). Mutations in the *eis* promoter were rare with the exception of Moldova, where a large proportion of isolates (60.6%) contained a C→T mutation at position −12 that was unique to that region. Of the 58 isolates with mutations in the *eis* promoter, 5 isolates from India, 44 isolates from Moldova, 2 isolates from the Philippines, and 1 isolate from South Africa had wild-type *rrs*. Lastly, no resistance-conferring mutations were observed in the entire *tlyA* structural gene.

Discrepancies between DST and sequence data (discordance and reverse discordance). Drug susceptibility testing data and sequence data were compared and each isolate was classified based on the agreement between the two sets of data. Discordant isolates were identified as isolates that were determined to be resistant through DST but lacked any known resistance-conferring mutation. Similarly, reverse discordant isolates were identified as isolates that were determined to be sensitive through DST but contained a known resistance-conferring mutation.

Discordance for rifampin was the lowest of the four antibiotic groups. No significant differences in rifampin discordance were found across the four study sites. Isoniazid discordance was not observed in Moldova (0.0%) and was low in South Africa (3.2%) but was higher in India (9.8%) and the Philippines (16.7%). The absence of discordance at the Moldova study site was significantly different from findings for India ($P = 0.011$) and the Philippines ($P < 0.001$) but not South Africa. Additionally, the high rate of discordance in the Philippines was significantly different from findings for South Africa ($P = 0.004$) but not India. Overall, fluoroquinolone discordance was the highest of the four antibiotics, particularly in Moldova (28.4%) and the Philippines (42.2%). Both of these study sites were significantly different from India and South Africa, which had lower discordance ($P \leq 0.01$). No significant differences between the study groups in discordance for the aminoglycosides were found.

A total of 45 isolates were determined to be reverse discordant by containing a marker for resistance despite being phenotypically sensitive. The aminoglycosides had the highest frequency of reverse discordance with 25 isolates (52.1%). This high frequency of reverse discordance was largely due to the C→T mutation at the −12 position of the *eis* promoter found in 24 reverse discordant isolates from Moldova. Geographical differences also were observed. Most of the reverse discordant isolates (71.1%) were from the Moldova group. The Philippines had 12 reverse discordant isolates (25.0%), while India had only 8 (8.3%). South Africa had a high level of agreement, with no reverse discordant isolates being observed in that group.

Isolates with multidrug discordance were identified and defined as isolates that had more than a single discordance for the four antibiotics. A total of 19 isolates were determined to be multidrug discordant, which may be indicative of alternative resistance mechanisms that could provide a broader spectrum of resistance. Isolates with multidrug reverse discordance also were examined, and 3 isolates were determined to have more than one reverse discordance. Interestingly, one isolate was reverse discordant for all four antibiotic groups.

DISCUSSION

Alternative diagnostic tests such as sequence-based methods are necessary for increasing the number of tuberculosis cases analyzed for drug resistance. These tests require an established set of known mutations that are linked to resistance. Not all mutations have been recorded, however, as shown by isolates that lack mutations but still display resistance to antibiotics. These discordant isolates are drug-resistant strains that are able to evade detection with sequence-based methods through the absence of discernible mutations. Although certain mutations have been highly associated with drug resistance, other mutations, such as those discovered recently, can be utilized in sequence-based testing to lower the discordance frequency and thus increase the sensitivity. In this study, significant geographical differences in both the frequencies of different types of polymorphisms and the discordances have been found and could impact the generalizability of sequence-based tests and possibly necessitate a more-regional approach for the detection of drug resistance.

Mutations within codon 531 of the *rpoB* gene were the most frequently found across the four study groups (43.0% to 79.2%) (Table 1), suggesting that overall global resistance to rifampin is largely due to mutations in this codon. Furthermore, other studies that have sequenced the *rpoB* gene of clinical isolates also have found this mutation to be the most prevalent (13, 14). Despite other study groups having only a few isolates with double mutations in *rpoB*, 33 (37.1%) of the isolates from South Africa contained the double mutation of GAC→GGC in codon 516 and CTG→CCG in codon 533. Because resistance mutations ultimately develop from selective pressure, such as exposure to the antibiotic, the South African isolates could be under unique pressure to develop double mutations. However, the effects of mutations in codon 533 have been relatively unclear, with some studies proposing that they are not related to rifampin resistance (15, 16). These isolates could also be from a rapidly disseminated clonal expansion event that occurred in this South African population, because they share the same sequences for the eight other genes, the same spoligotype, and the same 12-locus mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) pattern with the exception of one isolate (data not shown). Finally, selection bias might have played a role, with these identical isolates being overrepresented in the South African sample. Twenty-eight of the 33 isolates originated from the Glen Gray TrakCare Laboratory and the other 5 isolates originated from the Central Tuberculosis Laboratory in Braamfontein, despite having the same MIRU-VNTR pattern. Although only a single target was analyzed for the determination of resistance to rifampin, discordance frequencies were the lowest for this antibiotic, indicating that resistance is highly associated with the 81-bp sequence of *rpoB* known as the rifampin resistance-determining region. Differences in discordant frequencies were found not to be significant across study groups ($P > 0.05$). Therefore, the sensitivity of sequence-based tests for rifampin resistance should be stable globally.

Although isoniazid resistance was largely associated with the *katG* gene, inclusion of the *inhA* promoter has been shown to allow detection of resistant isolates that lack *katG* mutations. If resistance to isoniazid were based solely on *katG*, then 67 (18.2%) of the 368 isoniazid-resistant isolates would have been discordant. However, 36 isolates had wild-type *katG* but also had mutations in the *inhA* promoter. Of these 36 isolates, 35 isolates were resistant

TABLE 3 Discordant and reverse discordant isolates by antibiotic and study site

Antibiotic and discordance[a]	No. (%) of isolates[b]			
	IN	MD	PH	SA
RIF				
Discordant isolates	4 (4.3)	3 (3.7)	6 (6.2)	3 (3.3)
Total resistant isolates	92 (100.0)	81 (100.0)	97 (100.0)	92 (100.0)
Reverse discordant isolates	0	1 (14.3)	4 (15.4)	0
Total sensitive isolates	4 (100.0)	7 (100.0)	26 (100.0)	8 (100.0)
INH				
Discordant isolates	9 (9.8)	0	17 (16.7)	3 (3.2)
Total resistant isolates	92 (100.0)	81 (100.0)	102 (100.0)	93 (100.0)
Reverse discordant isolates	4 (100.0)	1 (14.3)	7 (33.3)	0
Total sensitive isolates	4 (100.0)	7 (100.0)	21 (100.0)	7 (100.0)
FQL				
Discordant isolates	10 (10.9)	19 (28.4)	19 (42.2)	7 (8.0)
Total resistant isolates	92 (100.0)	67 (100.0)	45 (100.0)	88 (100.0)
Reverse discordant isolates	0	8 (38.1)	2 (4.0)	0
Total sensitive isolates	4 (100.0)	21 (100.0)	50 (100.0)	12 (100.0)
AMI				
Discordant isolates	19 (20.7)	5 (10.9)	5 (20.8)	8 (8.6)
Total resistant isolates	92 (100.0)	46 (100.0)	24 (100.0)	93 (100.0)
Reverse discordant isolates	0	25 (59.5)	1 (1.0)	0
Total sensitive isolates	4 (100.0)	42 (100.0)	99 (100.0)	7 (100.0)

[a] Resistance and sensitivity were determined by DST. RIF, rifampin; INH, isoniazid; FQL, fluoroquinolones; AMI, aminoglycosides.
[b] IN, India; MD, Moldova; PH, Philippines; SA, South Africa.

to isoniazid and one isolate was sensitive. Thus, analysis of the *inhA* promoter lowered the frequency of discordance to 32 (8.7%) of 368 isolates, for a percentage difference of 9.5%. Although T−8A mutations were found in a large proportion of South African isolates (38.9%), this mutation might be misrepresented because it belonged to the aforementioned clonal strain. Mutations in the *ahpC* promoter were observed only in the Indian study group. These mutations had little impact on lowering overall discordance for isoniazid. Inclusion of the *ahpC* promoter in a sequence-based test utilizing both *katG* and *inhA* would have lowered the discordance frequency by 3 isolates to 29 (7.9%) of 368 isolates, for a percentage difference of 0.8%. In regard to the Indian group only, discordance was lowered from 13.0% to 9.8%, for a percentage difference of 3.2%. Significant differences in discordance for isoniazid were found, with Moldova and South Africa having lower discordance frequencies than India and the Philippines (Table 3). Thus, the overall sensitivity for isoniazid would be higher at the sites with low discordance (Moldova, 100%; South Africa, 96.8%) than at those with higher discordance (India, 90.2%; Philippines, 83.3%).

Fluoroquinolone resistance was caused mostly by *gyrA* mutations, particularly in codon 94 of the gene (Table 2). Mutations in this codon were the most commonly found at all four study sites, which suggests that mutations in this codon are favored globally. Additionally, the clonal strain with double mutations in *rpoB* that was found at the South African study site had codon 90 mutations, which may have been responsible for the large proportion of mutations found in this codon, compared with other study sites. Although it was not related to resistance, 371 (97.4%) of the total of 381 isolates had the AGC→ACC mutation in codon 95 of *gyrA* (data not shown), indicating that a majority of the isolates belonged to principal genetic group 1 or 2 of the *M. tuberculosis* complex, whereas *M. tuberculosis* reference strain H37Rv belongs to principal genetic group 3 (17). Only four isolates contained a mutation in *gyrB*; three of these isolates were from India and one of the isolates was from Moldova. The few *gyrB* mutations detected always coincided with *gyrA* mutations, which might indicate that sequencing of the *gyrB* gene might not be necessary for determination of fluoroquinolone resistance, because of its rarity and lack of impact in increasing the sensitivity of the test. Very high rates of discordance for the fluoroquinolones were observed for the sites in the Philippines (42.2%) and, to a lesser extent, Moldova (28.4%). However, it should be noted that the sampling for fluoroquinolone resistance in the Philippines (45 isolates) and Moldova (67 isolates) was lower than in India (92 isolates) and South Africa (88 isolates). Further analyses of the isolates from these two study sites are necessary to determine the exact cause of the high discordance, which might be a result of alternative resistance mechanisms or mutations in other genes. Because of this high discordance, the sensitivity of fluoroquinolone resistance detection through sequence-based methods would be lower in Moldova (71.6%) and the Philippines (57.8%) than in India (89.1%) and South Africa (92.0%).

The A→G transition at nucleotide position 1401 in the *rrs* gene was responsible for the majority of aminoglycoside resistance for all study sites (Table 2) except Moldova. Mutations at position 1484 were found to be rare, with only three isolates (two from India and one from Moldova) having such mutations. Mutations were rarely found in the *eis* promoter with the exception of Moldova, where 60.6% of the isolates had mutations at the −12 position, which was exclusive to that study site. Compared to other study sites, Moldova had a significantly smaller proportion of mutations at position 1401 in the *rrs* gene; therefore, selective pressure could cause isolates from this site to favor this particular mutation in the *eis* promoter instead. However, whether the C→T mutation at position −12 provides resistance to aminoglycosides is questionable, due to the fact that this mutation alone contributed to 50.0% of all reverse discordant isolates found in this study. Zaunbrecher et al. found that clinical isolates that contained this mutation were not resistant to kanamycin, and they hypothesized that this mutation provides only minor resistance, with a MIC that is near the critical level used for drug susceptibility testing (10). Similar to the *inhA* promoter, inclusion of the *eis* promoter could lower the frequency of discordance. If resistance to aminoglycosides were based solely on *rrs*, then 64 (25.1%) of the 255 isolates that were resistant to aminoglycosides would have been discordant. Sequence data revealed that 52 isolates had wild-type *rrs* but contained a mutation in the *eis* promoter. Of the 52 isolates, 27

TABLE 4 Reverse discordance patterns found in isolates by antibiotic and study site

Antibiotic(s) with reverse discordance[a]	No. (%) of isolates[b]				
	IN	MD	PH	SA	Total (by drug)
RIF	0	0	2 (4.2)	0	2 (4.2)
FQL	0	7 (14.6)	1 (2.1)	0	8 (16.7)
AMI	0	24 (50.0)	1 (2.1)	0	25 (52.1)
INH	4 (8.3)	0	6 (12.5)	0	10 (20.8)
RIF + INH	0	0	1 (2.1)	0	1 (2.1)
RIF + FQL	0	0	1 (2.1)	0	1 (2.1)
RIF + FQL + AMI + INH	0	1 (2.1)	0	0	1 (2.1)
Total (by study site)	4 (8.3)	32 (66.7)	12 (25.0)	0	48 (100.0)

[a] RIF, rifampin; FQL, fluoroquinolones; AMI, aminoglycosides; INH, isoniazid.
[b] IN, India; MD, Moldova; PH, Philippines; SA, South Africa.

TABLE 5 Multidrug discordant isolates found at the four study sites

No. of discordances	No. (%) of isolates[a]			
	IN	MD	PH	SA
0	64 (66.7)	58 (70.7)	76 (67.3)	81 (87.1)
1	25 (26.0)	22 (26.8)	32 (28.3)	7 (7.5)
2	5 (5.2)	1 (1.2)	2 (1.8)	2 (2.2)
3	1 (1.0)	1 (1.2)	2 (1.8)	2 (2.2)
4	1 (1.0)	0	1 (0.9)	1 (1.1)

[a] IN, India; MD, Moldova; PH, Philippines; SA, South Africa.

isolates were resistant to the aminoglycosides and 25 isolates were sensitive. Additionally, 35 isolates contained a C−12T mutation and wild-type *rrs*, of which 13 isolates were resistant and 22 isolates were sensitive. Thus, inclusion of the *eis* promoter lowered the discordance frequency to 37 (14.5%) of 255 isolates, for a percentage difference of 10.6%. Although it was the only gene where the entire structure was analyzed, no resistance-conferring mutations in *tlyA* were found in any of the isolates. These data are supported by another study that found that *tlyA* mutations were more likely to occur in mutants selected *in vitro*, rather than clinical isolates (18). Thus, inclusion of the *tlyA* gene in a sequence-based diagnostic test may not be necessary and should be studied further to determine its impact on capreomycin resistance in clinical isolates. Similar to findings for rifampin, rates of discordance for the aminoglycosides were not significantly different across study sites, indicating that the sensitivity of aminoglycoside resistance detection would be consistent geographically.

Isolates that were determined to contain a resistance-conferring mutation but were still susceptible to the antibiotic, i.e., reverse discordant isolates, could provide an estimate of the specificity of the test and indicate which mutations are more likely to result in false-positive results. Reverse discordance was found to occur mostly with respect to the aminoglycosides, more specifically at the site in Moldova (Table 4), due to the C→T mutation found at position −12 in the *eis* promoter. However, this mutation may not be a specific marker for aminoglycoside resistance and should be further evaluated regarding whether the level of resistance it confers is sufficient for clinical resistance. Other mutations that are highly associated with resistance, such as the codon 315 mutations in *katG*, have been observed in some reverse discordant isolates. These types of reverse discordances are a reflection of possible laboratory or technical errors, and repeat DST of these isolates could resolve these discordances and increase specificity.

Discordance with respect to more than one antibiotic, or multidrug discordance, was found in 19 (5.0%) of 381 isolates (Table 5). Of the 19 isolates, 10 isolates (2.6%) were discordant for two antibiotics, 6 isolates (1.6%) were discordant for three antibiotics, and 3 isolates (0.8%) were discordant for all four antibiotics. Because of the low percentage of multidrug-discordant isolates, it is unlikely that *M. tuberculosis* possesses a generalized resistance mechanism (such as permeability barriers or efflux pumps) that provides resistance to a series of antibiotics. Additional research is necessary to determine the cause of the multiple discordances, although it is likely that stepwise acquisition of resistance to antibiotics, rather than generalized mechanisms that provide a broader spectrum of resistance, played a role in these discordances. Previous studies identified resistance mechanisms, such as efflux pumps, that provide resistance to more than one antibiotic. For example, overexpression of the drug efflux gene *jefA* led to resistance to both isoniazid and ethambutol (19). Furthermore, when rifampin-resistant isolates were exposed to rifampin, resistance to the fluoroquinolone ofloxacin also occurred. When efflux pump inhibitors were added, however, no fluoroquinolone resistance was observed (20). Despite the evidence for alternative resistance mechanisms, it is believed that the development of multidrug-resistant tuberculosis (MDR-TB) is due not to a single mechanism providing multiple resistances but instead to the accumulation of single resistances (8). Thus, it is highly likely that these isolates contain various unknown genetic markers in other genes. Unidentified markers are a weakness of sequence-based diagnostic methods, and analysis of the isolates for the exact cause of discordance could provide further insight into drug resistance through the determination of novel markers. The presence of these isolates emphasizes that sequence-based methods are not yet complete and that improvements such as the addition and evaluation of other markers are necessary to increase sensitivity.

Utilizing nine genetic markers for resistance, significant differences in the proportions of discordant isolates and the sensitivities to fluoroquinolones and isoniazid were found for the different geographical regions. The specificities of the antibiotics were calculated in a similar fashion using reverse discordant isolate proportions, although, due to the low rate of sampling of drug-sensitive isolates, results may be misleading. Overall, rifampin would have the highest sensitivity (95.6%). Isoniazid would have the next highest sensitivity (92.1%), followed by the aminoglycosides (85.5%) and then the fluoroquinolones (81.2%). These different sensitivities demonstrate the strength of the association between the combination of genetic markers and drug resistance, establishing that resistance mutations rarely occur outside known genes for rifampin and isoniazid and that mutations for the aminoglycosides and fluoroquinolones remain elusive. Discrepancies within drug susceptibility testing data for each study site also might have accounted for low sensitivity values, providing a potential source of error for the identification of discordant isolates.

Due to the weaknesses of standard resistance testing for *M. tuberculosis*, the proportion of clinical cases tested remains low, with only 1.8% of new cases and 6.4% of existing cases being tested (2). Sequence-based methods are viable alternative methods that analyze specific genes for discernible mutations associated with resistance. Analysis of a total of 3,663 sequences has shown that

certain genes (*rpoB*, *gyrA*, *katG*, and *rrs*) are highly associated with resistance on a geographical scale. Conversely, other genes are only moderately associated with resistance (the *eis* and *inhA* promoters) or are rarely associated (*gyrB*, *tlyA*, and the *ahpC* promoter). Furthermore, certain polymorphisms in these genes were found to yield a high degree of resistance, while other polymorphisms, such as the C→T mutation at position −12 in the *eis* promoter, remain relatively unclear. Because significant geographical differences in the types of polymorphisms as well as the discordances were found, sequence-based tests can be tailored to specific regions, rather than a single general test being used worldwide. Additional studies are necessary to strengthen sequence-based testing, through either identification of resistance markers or evaluation of the strength of the associations between markers and resistance. With increased data and insight into resistance mechanisms in *M. tuberculosis*, sequence-based testing can be improved to provide accurate rapid assessment of resistance to various antibiotics in clinical isolates.

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