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Mutations Prevalent among Rifampin- and Isoniazid-Resistant *Mycobacterium tuberculosis* Isolates from a Hospital in Vietnam

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Vietnam is ranked 13th among the WHO list of 22 high-burden countries, based upon estimated total number of tuberculosis cases. Despite having a model national tuberculosis program, consistently achieving and exceeding WHO targets for detection and cure, drug-resistant and multidrug-resistant tuberculosis cases continue to rise. Rapid multidrug-resistant tests applicable in this setting, coupled with effective treatment regimens, would be a useful tool in reversing this trend, allowing early identification of patients with multidrug-resistant tuberculosis and avoiding resistance-amplifying regimens. Sequencing of consecutive isolates identified by the National Tuberculosis Program showed 89% of isoniazid-resistant isolates could be detected by targeting just 2 codons, *katG* 315 and −15C→T in the *inhA* promoter, while rifampin resistance will be more complex to detect, with many different mutation and insertion events in *rpoB*. The most prevalent rifampin resistance-conferring mutations, as in other countries, were in *rpoB* codons 531 (43%), 526 (31%), and 516 (15%). However, a hybridization-based resistance test with probes targeting the 5 most common mutations would only detect 78% of rifampin-resistant isolates. Overall, these data suggest that rifampin resistance may be used as a surrogate marker for multidrug-resistant tuberculosis and that a sensitivity of between 70 to 80% may be possible for rapid molecular detection of multidrug-resistant tuberculosis in this setting.

Few data are available on resistance among *Mycobacterium tuberculosis* isolates in Vietnam. The latest figures from the WHO are derived from the 1997 report on global drug resistance, which estimates resistance to any drug from a 1996 survey at 32.5% and multidrug-resistant (MDR) tuberculosis (TB) at 2.3% (35). The second national drug resistance survey was carried out between 2001 and 2002, but there were problems with data collection. The third survey is under way.

There are indications that the incidence of MDR TB has risen significantly since the first survey and, in the face of a rapidly increasing human immunodeficiency virus prevalence in Vietnam, is likely to continue to increase in the coming years. The International Organization for Migration (IOM) reported from a study of potential immigrants to Canada between 1989 and 2000 that the estimated MDR TB rate was approximately 4.5% (32), and data from our own studies show the rate of MDR TB in 2002 to 2004 to be approximately 6% (unpublished data).

The National Tuberculosis Program (NTP), while apparently achieving high rates of detection and cure (12), is not able to offer sensitivity testing on all isolates. Patients currently receive a standard regimen upon diagnosis of 2 months of streptomycin (S), isoniazid (H), rifampin (R), and pyrazinamide (Z), followed by 6 months of isoniazid and ethambutol (E) (2SHRZ/6HE). Following microbiological failure or relapse, there is a retreatment regimen of 2 months of streptomycin, isoniazid, rifampin, pyrazinamide, and ethambutol, followed by 1 month of isoniazid, rifampin, pyrazinamide, and ethambutol, and finally, 5 months of isoniazid, rifampin, and ethambutol ($2SHRZE/1HRZE/5H_3R_3E_3$). A 2003 study showed that 47% of patients who failed treatment within the NTP had primary MDR TB (24), and in a separate study, only 33% of MDR TB patients were sputum smear negative upon completion of 8 months of retreatment (17).

Effective control and treatment of MDR TB will require earlier diagnosis and a change in treatment patterns to avoid potential resistance-amplifying regimens (7).

Studies have shown that the mutations responsible for drug resistance in *M. tuberculosis* vary geographically (22). It is not clear if this is secondary to prescribing practices, *M. tuberculosis* strain subtypes, or other factors.

The development of a sensitive, rapid, and economical genotypic test for MDR TB will require knowledge of the prevalent mutations among MDR TB isolates in Vietnam. This study aimed to characterize the mutations conferring resistance in 100 consecutive rifampin (RIF)-resistant isolates and 100 consecutive isoniazid (INH)-resistant isolates identified at Pham Ngoc Thach Hospital, the WHO coordinating center for the NTP in southern Vietnam.

MATERIALS AND METHODS

Pham Ngoc Thach Hospital is a 500-bed hospital for tuberculosis and lung disease serving Ho Chi Minh City and is a tertiary referral hospital for southern Vietnam. The microbiology laboratory is a WHO international reference laboratory. Between January and March 2005, consecutive *M. tuberculosis* isolates identified at Pham Ngoc Thach Hospital and shown to be resistant to either RIF or INH had their relevant genes sequenced at the Hospital for Tropical Diseases, Ho Chi Minh City. Isolates are tested in the NTP following failure of directly observed therapy-short course (DOTS) or at the request of the treating physician.

Susceptibility testing was routinely performed by the standard 1% proportion method on Lowenstein-Jensen media for INH (0.2 μg/ml), RIF (40 μg/ml),

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TABLE 1. Primers for sequencing of rifampin and isoniazid resistance-determining regions

Primer (reference)	Sequence (5′–3′)	T_a (°C)	Concn (nM)	Target	Product length (bp)
katGwgF1	GTC CTC TAT ACC GGA CTA CGC	61	120	*katG* whole gene	2,899
katGwgR1	TCG CAC ATC CAG CAC ATT TC				
katGP5	GGT CGA CAT TCG CGA GAC GTT	68		*katG* codon 315, sequencing primers	518
katGP6 (21)	CGG TGG ATC AGC TTG TAC CAG				
TB92	CCT CGC TGC CCA GAA AGG GA	56	150	*inhA* promoter region	248
TB93 (29)	ATC CCC CGG TTT CCT CCG GT				
AhpC1	GCC TGG GTG TTC GTC ACT GGT	56	150	*oxyR-ahpC* intergenic region	359
AhpC2 (28)	CGC AAC GTC GAC TGG CTC ATA				
RPOBF	GGG AGC GGA TGA CCA CCC A	65	125	*rpoB* RRDR	350
RPOBR (14)	GCG GTA CGG CGT TTC GAT GAA C				
TB146-F	CTT CTC CGG GTC GAT GTC GTT G	64	300	*rpoB* N-terminal region	365
TB146-R (8)	CGC GCT TGT CGA CGT CAA ACT C				
RpoB-C3F	GAG TAC GTG CCC TCG TCT GA	56	300	*rpoB* cluster 3 region	319
RpoB-C3R	ACT TGC GCA TCC GGT AGG TA				

streptomycin (4 μg/ml), and ethambutol (2 μg/ml). All isolates were identified as *M. tuberculosis* by standard phenotypic identification tests. Data on the residential district were collected to ensure that isolates were not part of a single outbreak or from one residential district of the city (data not shown).

PCR and DNA sequencing. For isolates resistant to rifampin, the *rpoB* rifampin resistance-determining region (RRDR, codons 409 to 493) was sequenced. If no mutations were found, further sequencing was done at the *rpoB* N-terminal (codons 111 to 200) and cluster 3 (codons 575 to 656) regions. For isoniazid-resistant isolates, a 381-bp (codons 266 to 392) region of *katG* was sequenced. If no mutations were seen in *katG*, two further regions involved in INH resistance were sequenced: the *oxyR-ahpC* intergenic region (codons *oxyR* 56 to *ahpC* 97) and *inhA* promoter region (codons −93 to 27). Primers (Proligo, Singapore) are given in Table 1.

PCRs for *rpoB* N-terminal, cluster III, *oxyR-ahpC* intergenic, and *inhA* promoter regions were performed with 0.75 U of Bioline *Taq* (Bioline, United Kingdom) polymerase, RRDR PCR used 0.75 U of Amersham *Taq* polymerase (Amersham, United Kingdom), and *katG* whole-gene PCR used 1.3 U of the Expand high-fidelity polymerase system (Roche, United Kingdom). Amplifications were carried out in an Eppendorf Mastercycler as follows: an initial step of 95°C for 3 min, 30 cycles of 95°C for 15 s, appropriate annealing temperature (T_a) for 15 s, 72°C for 15 s, and a final step of 72°C for 2 min. *katG* whole-gene PCR used 2.5 min at 72°C for extension and a final step of 72°C for 7 min.

PCR products were purified with QIAgen PCR purification kits (QIAGEN, United Kingdom) and then served as templates for cycle sequencing reactions. Both strands of each product were sequenced with CEQ dye terminator cycle sequencing quick start kits (Beckman Coulter, Singapore) in a half-volume reaction using PCR primers, except for *katG*, which was sequenced with katGP5 and katGP6 (21). The thermal cycling program was 96°C for 20 s, appropriate T_a for 20 s, and 60°C for 4 min for 30 cycles, followed by holding at 4°C. The cycle sequencing products were subjected to ethanol precipitation steps according to the manufacturer's instructions and sequenced on the CEQ8000 system (Beckman Coulter, Singapore).

RESULTS

One hundred thirty-one consecutive isolates resistant to either RIF or INH were collected. Resistance patterns to first-line drugs are shown in Table 2. One hundred four isolates (79%) were RIF resistant; 129 isolates (98%) were INH resistant. Only 2 (1.9%) RIF-resistant isolates were not MDR: one of these was RIF monoresistant and the other was resistant to RIF and streptomycin.

One hundred four RIF-resistant isolates had the *rpoB* RRDR sequenced. The most prevalent mutations were at codons 531 (43%), 526 (31%), and 516 (15%). In total, mutations were found in 11 different codons, 10 isolates had mutations at multiple codons, and two insertions in codons 514 and 521 were also identified. Mutations identified in *rpoB* in 104 rifampin-resistant isolates are shown in Table 3.

Five isolates showing no RRDR mutations had their N-terminal regions sequenced. One previously reported mutation, V146F, was found in the N-terminal region, and three isolates carried a mutation at codon 561, just outside cluster 2. These five isolates also had the cluster 3 region sequenced, but no further mutations were identified.

The first 100 isolates resistant to INH had *katG* sequenced. The majority of isolates, 71%, carried the G944C (S315T) mutation, while two isolates carried G944A (S315N) mutations, two carried G944T (S315I) mutations, and one carried a A943G (S315G) mutation. Two isolates carried double mutations: one G944C/C924T (S315T/T308T) and one a dual mutation in codon 315, G944C/C945G (S315T). One isolate appeared to have a deleted *katG* gene.

Twenty-one isolates with wild-type *katG* had their *inhA* and *oxyR-ahpC* region sequences determined. Ten isolates carried a −15C→T mutation in the *inhA* promoter, 1 isolate had a silent mutation in *ahpC* (C21A), 11 isolates carried a silent mutation in the *oxyR* pseudogene (G37A), and 6 isolates carried different mutations in the *oxyR-ahpC* intergenic region (−81C-T, −51G-A, −48G-A, −74G-A, −72C-T, and −51G-A).

TABLE 2. Resistance patterns in 131 consecutive isolates resistant to either isoniazid or rifampin identified at Pham Ngoc Thach Hospital for Tuberculosis and Lung Diseases in southern Vietnam

Resistance pattern[a]	No. (%) of isolates
H	5 (3.8)
R	1 (0.8)
HE	2 (1.5)
HR	4 (3.0)
HS	19 (14.5)
RS	1 (0.8)
HRE	3 (2.3)
HRS	58 (44.3)
HSE	1 (0.8)
HRSE	37 (28.2)
Total	131 (100)

[a] H, isoniazid; R, rifampin; S, streptomycin; E, ethambutol.

TABLE 3. Mutations in the *rpoB* gene of 104 consecutive rifampin-resistant isolates identified in southern Vietnam

Mutated *E. coli* codon(s)	*M. tuberculosis* mutation(s)	Amino acid change(s)	No. (%) of isolates
146	G508T	V170F	1 (0.96)
511, 526	T1288C, C1333A	L430P, H445N	1 (0.96)
511, 512	T1288C, A1290G	L430P, S431G	1 (0.96)
511, 516	T1288C, A1304G	L430P, D435G	1 (0.96)
513	C1293A	Q432K	2 (1.92)
513	A1294T	Q432L	1 (0.96)
513, 526	C1293A, C1333G	Q432L, H445D	1 (0.96)
514	Ins[a]: 1299TTC1300	Ins: F, QFM→QFFM	1 (0.96)
516	A1304T	D435V	9 (8.65)
516	A1304C	D435A	1 (0.96)
516, 526	A1304C, C1333A	D435A, H445N	1 (0.96)
516, 526	A1304T, C1335G	D435V, H445Q	1 (0.96)
516, 533	A1304G, T1355C	D435G, L452P	2 (1.92)
521, 516	Ins: 1320TCGGGGTTG1333, A1304G	Ins: SGL, PLSGL→PLSGLSGL, D435G	1 (0.96)
526	C1333T	H445Y	14 (13.46)
526	C1333G	H445D	6 (5.76)
526	A1334G	H445R	5 (4.81)
526	C1333A, A1334G	H445S	1 (0.96)
526	C1335G	H445Q	1 (0.96)
526	A1334T	H445L	1 (0.96)
526	C1333T, A1334T	H445F	1 (0.96)
531	C1349T	S450L	42 (40.38)
531, 561	C1349T, A1387G	S450L, I480V	3 (2.88)
533	T1355C	L452P	2 (1.92)
No mutations			4 (3.85)

[a] Ins, insertion.

DISCUSSION

These data indicate that rifampin resistance would be a good surrogate marker for MDR TB in this setting, with 98% of rifampin-resistant isolates also MDR. However, the molecular detection of rifampin resistance in Vietnam will be complex, with many single point mutations scattered in the RRDR, multiple mutations, and insertion events. The general pattern of *rpoB* mutations reflects those found worldwide, with codons 531 (43%), 526 (31%), and 516 (15%) being the most commonly mutated. Four isolates (4%) had no mutations in any region sequenced for rifampin resistance, as has been previously reported (11, 22). While it is possible that these isolates are due to laboratory error in the assignment of resistance, consistent reports from many laboratories suggest that another mechanism is responsible for resistance in these isolates. Alternatively, these isolates may contain a mixed population of resistant and susceptible organisms (8).

Two rare insertions were identified, 514F, reported elsewhere (10) and 521SGL. The V146F mutation, in the N-terminal (cluster 2) region, has also been reported elsewhere and associated with low-level resistance, though not exclusively so (9).

Seventy-one percent of INH-resistant isolates carried an S315T mutation in *katG*. This mutation has been associated with high-level (>5 μg/ml) INH resistance (31) but, again, not exclusively so (25, 31), with MICs ranging from 0.38 to 12 μg/ml. A further 7 (7%) isolates had different mutations in codon 315. In total, 78% of INH-resistant isolates carried a mutated *KatG* codon 315. One of these isolates carried a double mutation in codon 315 and one carried a second mutation at base 924. However, this mutation is synonymous, T308T, and therefore does not contribute to the resistant phenotype. Although S315T is the most common mutation worldwide, the precise functional effect of the mutation remains unclear. The threonine residue may block access to the active site and reduce the INH affinity of the enzyme, or it may alter redox potentials and local hydrogen bonds (2). The catalase peroxidase remains functional but with reduced activity (26, 33).

One isolate appears to have a deleted *katG* gene, a rare but previously reported event. Loss of *katG* confers high-level (>256 μg/ml) INH resistance but probably confers a biological cost for the bacteria in the form of a decreased ability to fight oxidative stress (34). Twenty-one isolates carried a wild-type *katG* gene. Ten of these (50%) had a −15C→T mutation in the *inhA* promoter. This mutation generally confers intermediate-level (0.1 to 0.4 μg/ml) resistance (18). Mutations in this promoter region lead to increased *inhA* expression, overwhelming the effect of INH at low levels. The INH reactive form, isonicotinic acyl radical, reacts with NAD(H) to form a covalent adduct which blocks the active site of the NADH-dependent enoyl ACP reductase product of *inhA* (27).

No mutations were found in *ahpC* which conferred resistance to INH. One isolate carried a C21A synonymous mutation. Ten isolates also carried the G37A polymorphism in *oxyR*, which is nonfunctional in *M. tuberculosis*, carrying multiple deletions and mutations (4); therefore, this mutation is unlikely to confer INH resistance.

Six isolates carried mutations in the *oxyR-ahpC* intergenic region, but the significance of this is unclear. Some mutations in this region, such as C−30T, have been shown to upregulate alkylhydroperoxidase (15), thought to be a compensatory mechanism for loss of catalase peroxidase activity; other mutations, such as −46G→A (1), have been found in both resistant and susceptible isolates and are not related to INH resis-

tance but have phylogenetic significance. The functional effect, if any, of the mutations seen here is not yet known.

Recent reports (5) and theoretical models (6) suggest that MDR TB can be controlled and reduced through effective first-line DOTS programs such as the one functioning in Vietnam. The reasons for increasing drug resistance, particularly MDR, despite an effective NTP network remain unclear. However, both DOTS studies were based on lower underlying levels of primary drug resistance, and the use of a primary ethambutol-based continuation phase, as in Vietnam, has been shown to be less effective than rifampin-based regimens and may be a contributing factor (13). High rates of primary INH (20%) and streptomycin (31%) resistance and resistance to other first-line drugs (40% resistant to one or more) may well be a contributing factor, with many patients effectively receiving ethambutol monotherapy in the continuation phase.

A previous report suggests that 9% of smear-positive TB patients detected in the Vietnamese NTP in 2000 were not registered for treatment, suggesting that while detection rates were high, many of these patients did not progress through NTP-controlled DOTS (3). Efforts have been made to address this issue by changes in data management to record all patients at time of diagnosis, whereas prior to 2000, patients were only registered at commencement of therapy (12). Private treatment in Vietnam is associated with significantly lower treatment success rates than the NTP (48.9% versus 85%, $P < 0.001$) (20), probably due to incorrect prescriptions and poor adherence to therapy for financial and other reasons. It has been estimated that 40% of all antituberculosis drug dispensing in Vietnam occurs outside the NTP and that a quarter of these sales are made without a prescription (19).

It is likely that the prevalence figures on which WHO estimates of NTP performance are based (incidence of smear-positive TB, 85/100,000; prevalence, 96/100,000) (36) are also low. Wide regional variation in NTP performance, particularly in rural areas, may be hindering TB control in Vietnam. A prevalence survey in a rural district of Northwest Vietnam (30) estimated smear-positive prevalence at 90 to 110/100,000, but detection in the NTP is only 39% for females and 12% for males, significantly below the national detection rates. Efforts are being made by the government to improve NTP performance in rural areas.

Screening of applicants for Australian visas in Ho Chi Minh City by the IOM estimates rates of 157/100,000 for new smear-positive TB and 489/100,000 of new bacteriologically confirmed (smear and culture) TB, a rate 2.5 times that estimated by the WHO (23). The true incidence rates are unknown. TB notification rates vary widely across Vietnam, and it is also possible that the incentive system in the NTP leads to an overestimation of the cure rate (12). More accurate tuberculosis incidence and drug resistance prevalence data will be useful in addressing MDR TB in Vietnam.

It is likely that the sensitivity of hybridization tests for RIF resistance will be low in this setting. The commercial INNO-LiPA Rif.TB test (Innogenetics, Belgium) will directly detect only 73% through the resistant probes, though sensitivity may be higher when prediction of resistance through nonhybridization to probes for wild-type sequence is included. Probes directed to the 5 most prevalent mutations would achieve a sensitivity of only 78%. Molecular INH resistance detection may be simpler, though still incomplete, with combined detection of mutated codon 315 and −15C→T mutation in the *inhA* promoter, allowing a sensitivity of up to 89%. Early detection of INH resistance may be particularly important in Vietnam, with high underlying INH resistance (>20%) (16; also unpublished data) in conjunction with the use of an ethambutol-based continuation phase possibly fuelling the development of MDR TB. Ideally, such a test should be cheap, rapid, and applicable in low-technology environments to have an impact in Vietnam.

However, while the molecular detection of neither RIF nor INH resistance is 100% accurate and the importance of phenotypic tests for the exclusion of drug resistance remains, the earlier detection of resistant cases, particularly MDR TB, will be an important tool in controlling MDR TB and achieving the millennium development goals for reductions in TB mortality.

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