Prevalence and genetic basis of first-line drug resistance of *Mycobacterium tuberculosis* in Ca Mau, Vietnam

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Shareable abstract (@ERSpublications)
365 samples of *Mycobacterium tuberculosis* were collected in Ca Mau, Vietnam. 19.8% were phenotypically resistant to isoniazid. *katG* was the most common resistance mutation found in 12.8% of samples. *rpoB* mutations were found in 3.8% of samples. https://bit.ly/3axcaOE

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Abstract

Background and objective Data on the prevalence of anti-tuberculous drug resistance and its association with genetic mutations in *Mycobacterium tuberculosis* are limited. Our study explores the genomics of tuberculosis in Ca Mau, Vietnam.

Methods Patients ⩾15 years in Ca Mau Province, Vietnam, were screened annually for tuberculosis between 2014 and 2017. Isolates underwent drug susceptibility testing (DST) using the breakpoint method. DNA was extracted and whole genome sequencing (WGS) was performed.

Results We identified 365 positive sputum cultures for *M. tuberculosis* and processed 237 for DST and 265 for WGS. Resistance to isoniazid was present in 19.8% (95% CI 14.7 to 24.9%), rifampicin in 3.5% (1.1 to 5.7%) and ethambutol in 2.5% (0.9 to 5.4%) of isolates. Relevant mutations in *rpoB* gene were detected in 3.8% (1.8 to 6.8%). *katG*, *inhA* or *fabG1* mutations were found in 19.6% (15.0 to 24.9%) with *KatG* being most common at 12.8% (9.1–17.5%). We found 38.4% of isolates were of Beijing lineage, 49.4% East-African-Indian lineage and 8.4% European-American lineage. There were no associations between resistance profiles and clinical features.

Conclusion The high burden of isoniazid resistance and the *katG* mutation highlights the challenges facing Vietnam in its efforts to achieve its EndTB goals.

Introduction

Drug-resistance remains a significant barrier to managing tuberculosis (TB) globally. During 2020, 132 222 people were diagnosed with multi-drug resistant (MDR) or rifampicin resistant (RR) TB. Additionally, there were 25 681 new diagnoses of extended drug resistance (XDR) or pre-XDR-TB [1]. Only one in three of these patients with MDR/RR-TB accessed treatment [1]. Vietnam is one of ten countries that make up 70% of the estimated new cases MDR/RR-TB not enrolled in treatment [1]. A better understanding of the approaches to preventing and treating drug-resistant TB is key to reducing deaths and severe sequelae of TB.

One of the major impediments to a better understanding of the epidemiology of drug-resistant TB has been the lack of valid population data on the incidence and prevalence of the problem. In most high burden settings, mycobacterial culture and drug susceptibility testing (DST), is not routinely performed [1]. Hence, most people with drug resistant TB are not identified until treatment failure occurs.

Most of what is known about drug resistance in high burden settings comes from drug-resistance surveys, conducted among diagnosed TB cases [2]. However, these are often limited to specialised settings that are not representative of the general population. Furthermore, many people with TB lack typical symptoms or, if they do have symptoms, are never diagnosed with TB [3]. Hence, when drug-resistance prevalence surveys are limited to passively detected cases, that is, symptomatic patients who present for diagnosis and management, they may not reflect the population prevalence of drug resistance.

The identification of genetic mutations that are associated with phenotypic drug resistance has opened up new opportunities for early and simple diagnosis of drug resistance [4–6] and also insights into the mechanisms of drug resistance [7]. However, it has also raised questions about the interpretation of discordances between genotypic and phenotypic drug resistance.

The implementation of a four year population-wide active case finding survey for tuberculosis in Ca Mau Province of Vietnam [8], in which patients were screened for TB regardless of the presence of symptoms, provided the opportunity to define the population prevalence of and relationship between phenotypic and genotypic resistance to first-line anti-tuberculous drugs in *Mycobacterium tuberculosis* isolates co-circulating in the general community in this region of southern Vietnam. Secondary objectives were to assess the association of drug resistance with *M. tuberculosis* lineages and to examine whether any routinely available demographic or clinical features in the patients were associated with isoniazid resistance.

Methods

Study population and setting

Ca Mau is the southern-most province of Vietnam with a population of 1 194 476 on the 2019 census [9]. Sitting on the peninsula of the Mekong Delta Region it has a tropical climate distinguished by a wet monsoon season from May to August, and a dry season from September to April [10]. The incidence of TB in Vietnam is 170 000 per year with 5500 occurring in HIV positive patients among a total of ~250 000 HIV positive Vietnamese patients [11, 12].

Study design

We conducted a cluster randomised trial of active case finding for TB (Australian New Zealand Clinical Trials Registry number ACTRN12614000372684) between March 2014 and February 2018. It has been described in detail elsewhere [8].

The cluster sampling unit was the sub-commune, which had an average population of 1000 persons 15 years of age or older. In 60 randomly selected clusters active case finding for TB was conducted annually for three years (intervention group). In another 60 randomly selected clusters (control group) no active case finding was conducted. In the fourth year of the trial a prevalence survey for TB was performed in both the intervention and control groups. The intervention included a household census of the cluster, followed by active screening of all consenting household members aged 15 years and over. The screening consisted of a questionnaire about symptoms (cough, production of sputum and presence of haemoptysis), smoking status and basic demographic data and a request to provide a single, spontaneously expectorated sputum sample. Sputum samples were transferred to a central (provincial) laboratory and tested using the Xpert MTB/RIF platform (Cepheid). Those who were Xpert MTB positive were asked to provide two further sputum specimens for mycobacterial culture and undergo a chest x-ray. In addition, in the fourth year, all participants were offered a chest x-ray and those with abnormalities suggestive of TB were requested to provide a single, spontaneously expectorated sputum sample for mycobacterial culture.

Participants who were Xpert MTB positive were also asked to complete a detailed clinical questionnaire collecting information on: symptoms, history of previous TB and treatment for TB and their self-reported history of diabetes.

Mycobacterial culture and identification of M. tuberculosis

Sputum specimens for mycobacterial culture were transferred, by cold chain, to a regional reference laboratory approximately four hours by car from field sites.

Specimens were processed with N-acetylcysteine and sodium hydroxide, followed by centrifugation. All processing sputum specimens were cultured in the liquid medium (BACTEC mycobacteria growth indicator tube (MGIT) 960 culture) and solid medium (BD BBL TM Prepared Lowenstein-Jensen Media). Cultures were incubated for up to 8 weeks. If the liquid cultures were positive, MPT64 antigen testing were performed to confirm the presence of *Mycobacterium tuberculosis* complex. In cases with multiple positive cultures only the first culture was included in the analysis.

Phenotypic drug resistance testing

M. tuberculosis MGIT or LJ cultures were sent from the regional reference laboratory to the TB laboratory at the National Institute for Hygiene and Epidemiology for phenotypic DST. MGIT cultures were decontaminated by 1% NaOH (2 mL culture+2 mL 2% NaOH) then subcultured on LJ medium for 3–4 weeks. For LJ cultures, 1–2 colonies of *M. tuberculosis* were picked up and subcultured on LJ medium for 3–4 weeks. In case of contamination, repeat subcultures were performed from original cultures after decontamination with 2% NaOH according to standardised procedures.

The phenotypic DST for first-line agents was performed on purified and confirmed *M. tuberculosis* colonies using proportional agar microplate assay as described previously. This was the standard method in the reference laboratory [13]. Critical concentrations (breakpoints) were isoniazid 0.2 $\mu g \cdot mL^{-1}$, rifampicin 1 $\mu g \cdot mL^{-1}$ and ethambutol 5 $\mu g \cdot mL^{-1}$.

Extraction of mycobacterial DNA

M. tuberculosis colonies no more than 4 weeks old in subcultures were harvested in TE buffer (in cryovials) and inactivated at 80°C for 30 mins then kept at −80°C until they were sent for DNA extraction and whole genome sequencing (WGS).

All procedures of subcultures, DST and bacterial cell harvest/inactivation were conducted in BSL-3 laboratories.

Whole genome sequencing

Genomic DNA from 50–100 µL of subcultures were extracted as described before [14]. Libraries were constructed using Nextera XT DNA extraction kit (Illumina, San Diego, CA). Genome sequencing was performed on the NextSeq500 (Illumina) at the Center for Infectious Diseases and Microbiology, New South Wales Health Pathology in Sydney, Australia. Fastq were processed through an established pipeline whilst contamination screening and organism identification were performed using Centrifuge v1.0.4 with trimming of reads by Trimmomatic v0.38.

Mykrobe predictor was used to assign phylogenetic lineages using lineage-informative single nucleotide polymorphisms (SNPs) (https://github.com/Mykrobe-tools/mykrobe) [15–17].

Identification of resistance-associated SNPs

Sequencing reads were mapped to the reference genome H37Rv (Genbank NC_000962) using the RedDog pipeline (https://github.com/katholt/RedDog) [18]. An initial phylogenetic tree was inferred using FastTree v2.1.8. Variant calling was performed using Snippy version 3.1 and in-house scripts use to filter mutations associated with drug resistance as per CRyPTIC database (https://github.com/tseemann/snippy) [19, 20]. Indels were detected from RedDog output and in-house scripts.

Statistical analysis

First, we report the prevalence of phenotypic drug resistance and of drug resistance conferring mutations, with 95% binomial confidence intervals. Then, we report on the prevalence of the major *M. tuberculosis* lineages and their association with drug resistance. The observed associations and significance were tested using a logistic regression model. Finally, we examined the predictive value of demographic and clinical characteristics for the most common form of drug resistance, isoniazid resistance, using logistic regression models.

Analysis was conducted using STATA 16.1.

Ethics

The protocol was approved by the Human Research Ethics Committee of the University of Sydney (Ref: 2013/73) on 9 April 2013. The protocol was also approved by the Institutional Review Board of the National Lung Hospital, Vietnam Ministry of Health (Ref: 407/QD-BVPTU) on 29 August 2013. The authors have no conflicts of interest to disclose.

Results

There were 365 participants with one or more positive cultures for *M. tuberculosis* over the four years of the study. There were 12 participants who had more than one occurrence of a positive culture. Mycobacterial DNA was extracted for WGS from the cultures for 265 participants (73%). Of these, 237 participants (89%) had phenotypic DST performed (figure 1).

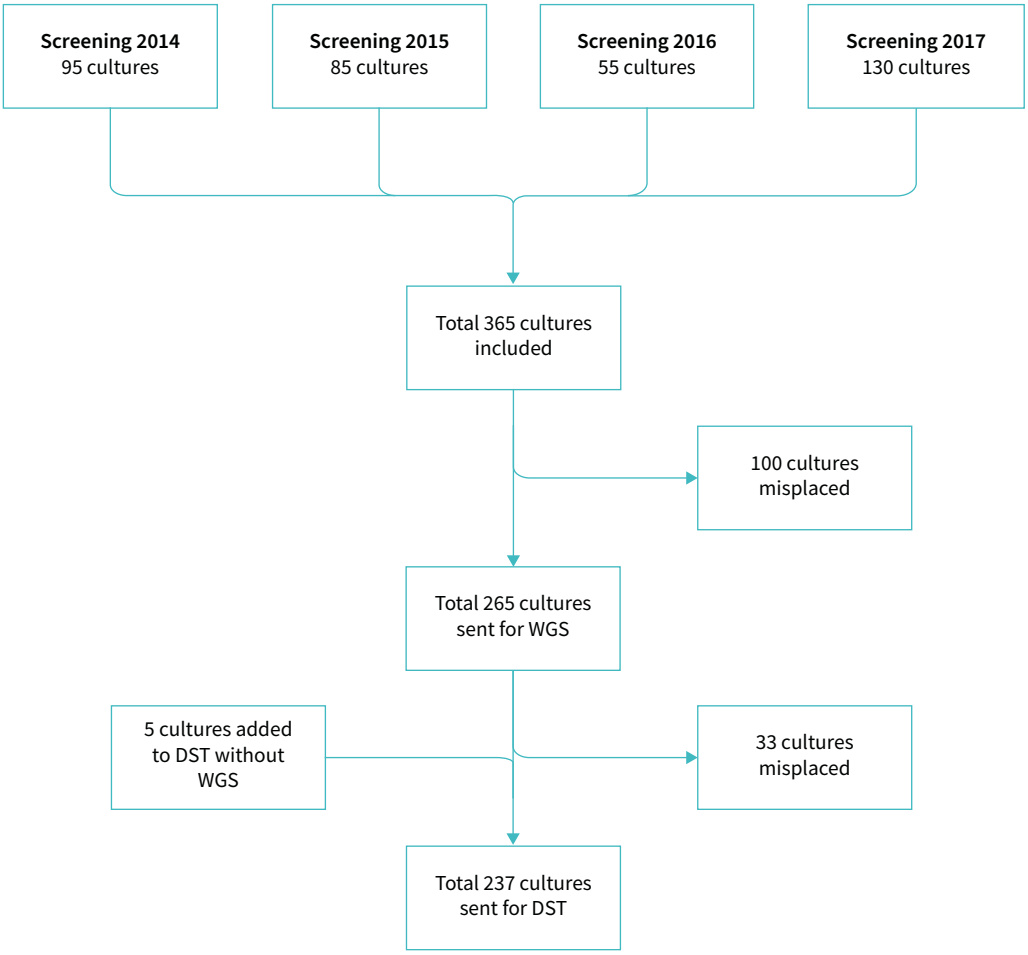

FIGURE 1 Study flowchart. The first positive culture for a participant was included in the study if a positive culture was obtained on more than one occurrence. WGS: whole genome sequencing; DST: drug susceptibility testing.

Most *M. tuberculosis* positive cultures were from male participants (83.8%) and the mean age was 52.9 years (SD=15.4). Over half (53.9%) were current smokers, 8.8% had diabetes, and 12.2% of participants had a history of previous TB. Fever and night sweats within the last two weeks were reported in 27.2% and 24.4% of participants respectively (table 1).

Phenotypic drug resistance shown higher levels of resistance to isoniazid compared with the other first line drugs (table 2), with a total of 19.8% (95%CI: 14.7, 24.9%). There were seven participants (3.0%, 95%CI: 1.2, 6.0%) with multi-drug resistant tuberculosis and one with rifampicin mono-resistance. Testing numbers were lower for DST because of misplaced samples.

From a total of 265 isolates sequenced, we found 88 isolates (33.2%, 95% CI 27.5, 38.9%) had an identifiable drug resistance mutation (table 2). There were 10 cases with a *rpoB* mutation. Two of these did not have phenotypic DST performed. Seven of remaining eight cases with rifampicin-resistance mutations in the *rpoB* gene demonstrated phenotypic resistance. One case was reported as susceptible by phenotypic DST. In this case, the *rpoB* mutation was L430P, which has been reported to be associated with lower MIC values [21].

There were 40 cases of isoniazid monoresistance. Several patients with *fabG1* (5 of 18), *inhA* (2 of 2) and *katG* (2 of 31) mutations were phenotypically susceptible to isoniazid (using a breakpoint of 0.2 $\mu g \cdot mL^{-1}$). One isolate expressed a mutation associated with fluoroquinolone resistance (A90V mutation in *gyrA* gene) with no other resistance conferring mutations were identified. The complete list of mutations/substitutions and their corresponding phenotypic resistance patterns can be seen in table 3. No other resistance-associated mutations were detected beyond those listed in table 3. Phenotypic resistance with no

TABLE 1 Demographic and clinical characteristics of the study participants

	Participants with positive mycobacterial cultures for *M. tuberculosis.*	Participants with a culture sample analysed by WGS	Participants with a culture sample analysed for DST
Subjects N	365	265	237
Male	306/365 (83.8)	221/265 (83.4)	198/237 (83.5)
Age, years	52.9±15.4	52.0±15.8	51.9±15.9
Cough in the last 2 weeks	208/279 (74.6)	149/196 (76.0)	128/169 (75.7)
Current smoker	193/358 (53.9)	137/262 (52.3)	123/236 (52.1)
Self-reported diabetes	26/295 (8.8)	16/212 (7.5)	14/190 (7.4)
Previous TB treatment	41/336 (12.2)	24/244 (9.8)	18/217 (8.3)
Fever in the last 2 weeks	76/279 (27.2)	51/196 (26.0)	48/169 (28.4)
Night sweats in the last 2 weeks	68/279 (24.4)	45/196 (23.0)	37/169 (21.9)
Weight loss in the last month	73/279 (26.2)	51/196 (26.0)	46/169 (27.2)

Data are presented as n/N (%) and mean±SD. *M. tuberculosis: Mycobacterium tuberculosis*; WGS: whole genome sequencing; DST: drug susceptibility testing; TB: tuberculosis.

detectable mutation was found in 2/46 INH resistant cultures, 0/8 RIF resistant cultures, and 1/6 ethambutol resistant cultures. One INH resistant sample did not have WGS performed.

There were 106 isolates identified as Beijing lineage, 130 as EAI lineage and 23 European-American lineage. Six did not have lineage identified. The prevalence of isoniazid resistance was significantly related to lineage (table 4) and was higher among those with Beijing lineage isolates. All seven cases of MDR-TB were in Beijing strains.

Finally, there was no statistically significant relationship between isoniazid resistance and gender, smoking status, cough, haemoptysis, fever, night sweats, weight loss or smear positivity (table 5).

Discussion

This study has demonstrated a high prevalence of isoniazid resistance and a low prevalence of other drug resistance among screen-detected cases of TB in the rural province of Ca Mau, Vietnam. The dominant genes in which mutations are associated with isoniazid resistance in this population are *katG* and *fabG1*,

TABLE 2 Prevalence of drug resistance and resistance mutations in Ca Mau, Vietnam

Drug resistance	Prevalence, n/N	Prevalence, % (95% CI)
Phenotypic testing (DST)		
Isoniazid	47/237	19.8 (14.7 to 24.9)
Rifampicin	8/237	3.4 (1.1 to 5.7)
Ethambutol	6/237	2.5 (0.9 to 5.4)
MDR-TB	7/237	3.0 (1.2 to 6.0)
Resistance mutations (WGS)		
Rifampicin		
rpoB	10/265	3.8 (1.8 to 6.8)
Isoniazid		
inhA	2/265	0.8 (0.1 to 2.7)
katG	34/265	12.8 (9.1 to 17.5)
fabG1	20/265	7.5 (4.7 to 11.4)
Any isoniazid mutation	52/265	19.6 (15.0 to 24.9)
Ethambutol		
embA	5/265	1.9 (0.6 to 4.3)
Pyrazinamide		
pncA	6/265	2.3 (0.8 to 4.9)
Ethionamide		
ethA	18/265	6.8 (4.1 to 10.5)

DST: drug susceptibility testing; MDR-TB: multi-drug resistant tuberculosis; WGS: whole genome sequencing.

TABLE 3 Full list of genetic mutations/substitutions and their corresponding phenotypic resistance profiles

Gene	Mutation/ substitution	Frequency	Drug resistant to INH	Drug resistant to rifampicin	Drug resistant to ethambutol	MDR-TB
gyrA	A_A90V	2	0	0	0	0
embA	C-16G	1	1	1	1	1
embB	G406C	1	0	0	0	0
embB	M306I	1	1	1	1	1
embB	M306V	2	2	2	2	2
ethA	S266R	18	3	1	0	1
fabG1	C-15T	15	12	0	0	0
fabG1	G-17T	2	0	0	0	0
fabG1	T-8A	3	1	0	0	0
inhA	I21V	2	0	0	0	0
katG	S315N	4	2	1	0	1
katG	S315T	30	27	6	5	6
pncA	D12A	1	1	0	0	0
pncA	E111	1	0	0	0	0
pncA	T135P	1	1	1	1	1
pncA	T2289252C	1	1	1	0	1
pncA	V139A	1	1	0	0	0
pncA	W68G	1	1	0	0	0
rpoB	D435V	1	1	1	0	1
rpoB	H445D	1	1	1	0	1
rpoB	H445N	1	0	0	0	0
rpoB	H445S	1	1	1	1	1
rpoB	H445Y	1	1	1	0	1
rpoB	L430P	1	0	0	0	0
rpoB	S450L	3	3	3	3	3
rpoB	V170F	1	0	0	0	0
rpsL	K43R	29	15	7	5	6
rpsL	K88R	14	7	1	0	1

INH: isonicotinic acid hydrazide: MDR-TB: multi-drug resistant tuberculosis.

with only a small number of isoniazid resistant cases associated with *inhA* mutations. While the EAI strain is the dominant lineage in Ca Mau, the Beijing strain is common and is strongly associated with anti-tuberculous drug resistance. We did not find any clinical or demographic predictors of drug resistance in this study population though this may reflect limitations of the questionnaire style of data collection.

The high prevalence of isoniazid resistance in this study population is consistent with other reports from Vietnam. Previous studies in southern Vietnam have demonstrated a similar prevalence of isoniazid resistance of 16.6% and rifampicin resistance of 2.0% [22]. While much higher rates of resistance have been reported, this has been in selected populations. For example, one study conducted on isolates drawn from central reference hospitals reported that 58% of patients had isoniazid resistance and 39% had rifampicin resistance [23]. The reported prevalence of drug resistance across all of Vietnam ranges from 22.4% to 39.6% for isoniazid and 4.9% to 24.5% for rifampicin [23–25]. The prevalence of drug resistance is influenced by the breakpoint used for defining resistance. In this study we defined rifampicin

TABLE 4 Phylogenetic lineage as a predictor of resistance

Lineage	Total WGS	Isoniazid resistance/lineage, n/N (%)	Isoniazid resistance/lineage, OR (95% CI)
Subjects n	265		
Beijing (lineage 2)	106	25/91 (27.5%)	Reference
East-Africa-India (lineage 3)	130	16/117 (13.7%)	0.42 (0.21 to 0.84)
European American (lineage 4)	23	3/20 (15.0%)	0.47 (0.13 to 1.73)
Other	6	N/A	N/A

WGS: whole genome sequencing; N/A: not available.

TABLE 5 The relationship between clinical features and phenotypic isoniazid resistance

Clinical features	Isoniazid resistance, OR (95% CI)	p-value[#]
Age (>40 years *versus* ⩽40 years)	0.77 (0.38 to 1.57)	0.48
Gender (male *versus* female)	1.16 (0.48 to 2.81)	0.75
Current smoker (yes *versus* no)	1.82 (0.94 to 3.52)	0.07
Cough in the last 2 weeks (yes *versus* no)	0.86 (0.37 to 2.04)	0.74
Haemoptysis in the last month (yes *versus* no)	1.14 (0.23 to 5.77)	0.87
Fever in the last 2 weeks (yes *versus* no)	0.47 (0.18 to 1.23)	0.13
Night sweats in the last 2 weeks (yes *versus* no)	0.91 (0.36 to 2.29)	0.84
Weight loss in the last month (yes *versus* no)	0.95 (0.41 to 2.23)	0.91
Smear positive (yes *versus* no)	1.02 (0.53 to 1.95)	0.96

[#]: p-value from separate logistic regression models with isoniazid resistance as the outcome and each clinical feature as an exposure variable.

resistance using a breakpoint of 1 μg·mL^{-1}. However, the latest WHO guidelines have recommended lowering the 7H10 and MGIT breakpoints (critical concentrations) to 0.5 μg·mL^{-1}. This may have led to an under-estimation of rifampicin resistance (and MDR-TB) in the study population [26].

Our findings in relation to the prevalence of mutations conferring drug resistance were also broadly consistent with previous reports which have shown that the *katG* mutation is predominant among isolates with isoniazid resistance [27–29]. Compared to *katG* mutations there was a low prevalence of *inhA* mutations among the isoniazid resistant isolates in this study population [30]. However, we did find a relatively high prevalence of mutations in *fabG1* which, similar to *inhA*, encodes an enzyme in the FAS II process inhibited by isoniazid [31]. The clinical importance of *katG* mutation predominance among the isoniazid resistant isolates is that, unlike *inhA & fabG1*, it often confers a high level of resistance which cannot be overcome with high-dose Isoniazid [32, 33].

In contrast to our finding in this southern, rural province of Ca Mau, a study in Hanoi, a large urban centre in the north, reported that Beijing strain is the dominant lineage with a prevalence of 56.6% compared to EAI of 24.7% [34]. Previous studies in Vietnam have found the Beijing strain to be more common in young females and patients previously treated for TB [35] Our finding that Beijing strain was strongly associated with drug resistance is consistent with several previous observations that have reported a link with fluoroquinolone resistance, increased multidrug-resistance and increased relapse rates [36–38].

We found that many isolates with mutations in the genes associated with isoniazid resistance (*inhA and fabG1*) did not demonstrate phenotypic resistance to isoniazid. It is possible that the critical concentration (breakpoint) for determining isoniazid resistance in the phenotypic DST was too high to detect the low-level resistance associated with these mutations [39, 40]. It is worth noting that two of the samples that were phenotypically susceptible to isoniazid but had *fabG1* mutations where the SNP was in the −8 position. Our experience has been that phenotypic isoniazid resistance associated with this mutation is only observed when the broth microdilution method is used [41]. In contrast, to these findings with *inhA* and *fabG1*, most isolates that demonstrated *katG* mutations did demonstrate phenotypic isoniazid resistance consistent with its association with high level isoniazid resistance [33]. The high specificity of the *katG* gene mutation for isoniazid resistance along with the high prevalence of isoniazid resistance in the general population supports its inclusion in rapid molecular tests that are used for screening for resistance [42]. This also supports the current Vietnam national TB protocol regimen for the inclusion of three drug therapy in the continuation phase to avoid acquired MDR-TB [43].

We did not find any correlations between clinical features and isoniazid resistance. This is consistent with the findings of other investigators who have found a relationship with previous history of TB, but not with presenting clinical symptoms [44]. It remains possible that other clinical characteristics, not measured here, might be predictive of isoniazid resistance. However, in general, our findings reinforce the importance of molecular strategies for rapid identification of drug resistance in people with TB.

This study has several strengths. The setting within a population-wide active case finding program, in which participants provided sputum for testing regardless of symptoms, means that the data can be considered representative of the general population. The low proportion of women among the cases, reflects the low

prevalence of TB among women in Vietnam [45, 46]. The major limitation was the incompleteness of WGS and DST testing of the available cases. We assume that missing samples were missing a random. Another weakness was the lack of DST for pyrazinamide so that phenotypic resistance to this drug could not be assessed.

In conclusion, this population-based study has shown results consistent with previous, less representative studies in Vietnam: a high prevalence of isoniazid resistance that is strongly associated with *katG* mutations and with Beijing lineage. In the context of the rapid progress in development of molecular tests for selecting anti-tuberculous drug regimens, data on the association between phenotypic resistance and genetic mutations, particularly in unselected, community-based study populations such as this, should be helpful in designing future testing strategies.

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