Prevalences of levofloxacin resistance and *pncA* mutation in isoniazid-resistant *Mycobacterium tuberculosis* in Hong Kong

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A B S T R A C T

Introduction: In 2018, the World Health Organization recommended a 6-month treatment regimen that included levofloxacin and pyrazinamide for isoniazid-resistant *Mycobacterium tuberculosis* without rifampicin resistance (Hr-TB). Susceptibility testing for both drugs is not routinely performed for Hr-TB in Hong Kong. This study examined the prevalences of levofloxacin and pyrazinamide resistances in Hr-TB and explored associated risk factors.

Methods: All Hr-TB isolates archived during 2018 were retrieved. Isolates were de-duplicated to identify unique cases. Levofloxacin susceptibility testing was performed using the MGIT 960 System; *pncA* gene sequencing was used as a surrogate indicator of pyrazinamide susceptibility. Previous laboratory records for each case were analysed.

Results: In total, 160 phenotypic Hr-TB cases were identified from among 3411 patients with tuberculosis (4.7%). Among these, 157 were analysed, revealing 0.6% (n=1) levofloxacin resistance and 4.5% (n=7) pyrazinamide resistance, respectively. Independent risk factors associated with *pncA* mutations included history of tuberculosis in the affected patient and isoniazid poly-resistance (ie, double and triple resistances), but not mono-resistance.

Conclusion: For Hr-TB in Hong Kong, levofloxacin resistance is rare and pyrazinamide resistance-associated *pncA* mutations are uncommon. Routine susceptibility testing for these drugs is not indicated unless related risk factors are identified.

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New knowledge added by this study

- Levofloxacin (LVX) resistance is rare (0.6%) and pyrazinamide (PZA) resistance-associated *pncA* mutations are uncommon (4.5%) among isoniazid-resistant, rifampicin-susceptible *Mycobacterium tuberculosis* (Hr-TB) isolates in Hong Kong.
- Risk factors for *pncA* mutations in Hr-TB include history of tuberculosis in the affected patient and isoniazid poly-resistance.

Implications for clinical practice or policy

- Clinicians could initiate empirical treatment for patients with Hr-TB without routine susceptibility testing for LVX and PZA.
- Susceptibility testing for LVX and PZA could be considered in patients with Hr-TB and additional risk factors; or when clinical, radiological, and microbiological responses are suboptimal during early follow-up.

Introduction

There has been a gradual decline in the global tuberculosis (TB) incidence and mortality rates over time, by approximately 2% and 3% per year, respectively.[1] Despite this trend, TB remains a leading cause of death, and *Mycobacterium tuberculosis* drug resistance continues to be a major public health issue. Globally, isoniazid (INH)-resistant, rifampicin (RIF)-susceptible TB (Hr-TB) is the most common drug-resistant form of disease.[2] In 2017, the rates of Hr-TB were 7.1% among patients with new TB diagnoses and 7.9% in patients who had previously undergone treatment for TB.[1] In Hong Kong, the respective rates were 5.3% and 9.5%, with a combined rate of 5.7% in 2016.[3] The Hr-TB is associated with worse clinical outcome and development of multidrug resistance (MDR),[4,5] although the findings have been inconsistent.[6,7]

Isoniazid constitutes a key component in the treatment of drug-susceptible TB, through its inhibition of mycolic acid biosynthesis in the mycobacterial cell wall. Over 85% of INH resistance is conferred by mutations residing in the *katG* gene and *inhA* promoter region,[8] leading to high and low

香港異烟肼耐藥結核分枝桿菌的左氧氟沙星耐藥率和*pncA*突變率

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簡介：2018年，世界衛生組織推薦使用6個月包括左氧氟沙星和吡嗪酰胺治療異煙肼耐藥結核分枝桿菌（Hr-TB）。在香港，針對Hr-TB並不常規進行上述兩種藥物的敏感性測試。本研究檢視Hr-TB的左氧氟沙星和吡嗪酰胺耐藥率，並探討相關危險因素。

方法：檢索2018年存檔的所有Hr-TB分離株，並以病人識別代號對分離株進行重複數據刪除。使用MGIT 960進行左氧氟沙星藥敏測試；*pncA*基因排序則被用作吡嗪酰胺敏感度的替代指標。分析每宗病例過往的實驗室記錄。

結果：從3411名結核病患者中確定160名表型Hr-TB病例（4.7%），並對當中157例進行分析，左氧氟沙星耐藥率為0.6% (n=1)，吡嗪酰胺耐藥率為4.5% (n=7)。與*pncA*突變相關的獨立危險因素包括患者的結核病史和異煙肼多重耐藥（即雙重和三重耐藥），但不包括單耐藥。

結論：在香港，Hr-TB的左氧氟沙星耐藥很少見，與吡嗪酰胺耐藥相關的*pncA*突變也不常見。除非確定相關的危險因素，否則不需要對這些藥物進行常規藥敏試驗。

levels of resistance, respectively. These mutations can readily be detected by commercial molecular line-probe assays. The level of resistance can also vary according to the co-occurrences of less common mutations in other regions.

For the treatment of Hr-TB, current World Health Organization (WHO) guidelines recommend 6 months of RIF, pyrazinamide (PZA), ethambutol, and levofloxacin (LVX). The guidelines also recommend examination of resistances towards fluoroquinolones and PZA, prior to treatment.[9]

In our locality, routine testing of LVX and PZA susceptibility has not been implemented for the treatment of Hr-TB. To determine the most cost-effective testing strategy, this study reviewed an annual collection of Hr-TB isolates, with the aim of determining the prevalences of LVX resistance and *pncA* mutations (as a molecular marker of PZA resistance in Hr-TB) and identifying factors associated with these resistances.

Methods

Case identification and data collection

Data were reviewed from the TB Reference Laboratory of the Department of Health. This laboratory processes local *M tuberculosis* strains from specimens collected in out-patient clinics and in-patient hospitals in both public and private sectors. All phenotypic Hr-TB isolates in 2018 were identified and de-duplicated using unique patient identifiers. The following data were included in this analysis: basic patient demographics, phenotypic susceptibility results of first-line drugs (INH at critical concentrations 0.1 μg/mL and 0.4 μg/mL as recommended by the WHO, RIF, streptomycin, and ethambutol), and genotypic susceptibility results (*inhA* promoter region, *katG* codon 315, and *rpoB* hotspot region) from the GenoType MTBDRplus assay, version 2.0 (Hain Lifescience). Any discrepant or unsuccessful test results were resolved by the agar proportion method and/or DNA sequencing; when applicable, these additional data were reported as the final results. All available data from the Laboratory Information System were reviewed for each patient to determine any history of TB, fluorescence microscopy results (according to Global Laboratory Initiative grading[10]), site of isolation (pulmonary versus extrapulmonary), any documented sputum culture conversion and the duration (ie, time between the date of first positive culture for current infection and the date of consistently negative culture), and microbiological outcome.

Levofloxacin and pyrazinamide susceptibility testing and *pncA* gene sequencing

Selected *M tuberculosis* strains were retrieved from the laboratory archive and sub-cultured, then subjected to LVX susceptibility testing using the BD BACTEC MGIT 960 system (Becton, Dickson and Company), in accordance with the manufacturer's instructions. The LVX critical concentration at 1.0 μg/mL was used as recommended by the WHO. DNA extraction was performed using GenoLyse (Hain Lifescience). The *pncA* gene was amplified with the primers pncA-1F (5′-CGCTCAGCTGGTCATGTTC-3′) and pncA-1R (5′-CCCACCGGGTCTTCGAC-3′) to produce an amplicon of 798 bp, using the GeneAmp PCR System 9700 (Applied Biosystems). Each 25-μL reaction mixture contained 12.5 μL of GoTaq G2 Hot Start Colorless Master Mix (Promega) [1×], 0.25 μL of each primer (1.0 pM/μL), 9.5 μL PCR-grade water, and 2.5 μL of template DNA. Reaction mixtures were amplified using the following protocol: 2 minutes at 95°C; 35 cycles of 1 min at 95°C, 1 minute at 65°C, and 1 minute at 72°C; and 10 minutes at 72°C. Sequencing was performed using the 3730 × l DNA Analyzer (Applied Biosystems) with the same primers. Resulting sequences were compared with the sequence of wild-type *M tuberculosis* H37Rv for detection of *pncA* mutations. Strains with detected *pncA* mutations were subjected to further analysis of PZA susceptibility via the MGIT 960 system in accordance with the manufacturer's instructions, with a critical concentration of 100 μg/mL.

Statistical analysis

Univariate analysis comprised odds ratio calculations and Fisher's exact test. Firth logistic regression was

employed for multivariable analysis because of the low outcome frequency. IBM SPSS Statistics Subscription (Windows version, IBM Corp, Armonk [NY], United States) was used for data analysis. A P value of <0.05 was considered statistically significant. This article complies with the STROBE Statement reporting guidelines.

Results

In total, 8865 *M tuberculosis* isolates from 3411 patients were processed in 2018. Susceptibility profiles were available for all except repeated strains collected from the same patient within 3 months. De-duplication of 393 isolates yielded 160 patients with phenotypic Hr-TB, amounting to a case rate of 4.7%. *rpoB* hotspot mutations were identified in five cases by GenoType MDRTBplus, three of which were confirmed to confer RIF resistance according to *rpoB* sequencing results. These three cases were considered to be RIF-resistant and excluded from further analysis. The mean age of the patients in the remaining 157 cases was 61.3 years (range, 15-95 years); the male to female ratio was 1.8. Pulmonary TB was present in 90.4% of affected patients (n=142), while 9.6% of affected patients (n=15) had extrapulmonary involvement. There was a documented history of TB by positive culture in 4.5% of affected patients (n=7). Microscopy results were available for cases in which direct specimens were received by our laboratory at the time of initial diagnosis (n=45). The majority of specimens was acid-fast bacillus smear-negative (n=33), followed by 1-4 acid-fast bacilli per length (n=4), scanty (n=4), 1+ (n=2), and 2+ and 3+ (n=1 each). In terms of microbiological outcome, 76.4% of affected patients (n=120) had documented sputum culture conversion without recurrence after follow-up of at least 9 months, among which 106 attained conversion within 5 months after diagnosis. The median interval required for culture conversion was 72.5 days (range, 9-622 days). No clearance was documented for patients in the remaining 37 cases, for whom no follow-up specimens were received for >1 year.

Table 1 shows the patterns of resistance among first-line drugs and distributions of cases with LVX resistance and *pncA* mutations. Table 2 shows the patterns of mutations detected. With respect to INH, 45.2% (n=71) of the isolates exhibited low-level resistance (minimum inhibitory concentration 0.1-0.4 μg/mL) and 54.8% (n=86) of the isolates exhibited high-level resistance (minimum inhibitory concentration >0.4 μg/mL).

For LVX, only one strain was resistant, while 155 were sensitive (one isolate failed to be recovered). The number of resistant cases was insufficient for correlation analysis. *pncA* mutations were detected in 4.5% (7/157 cases), and all detected mutations were previously identified as PZA resistance–related. All isolates with *pncA* mutations were phenotypically resistant to PZA. Univariate analysis showed that *pncA* mutations were associated with documented history of TB in the affected patient (odds ratio [OR]=11.60, 95% confidence interval [CI]=1.79-75.00; P=0.03) and INH poly-resistance (OR=19.06, 95% CI=1.07-339.78; P=0.04) but not mono-resistance, as shown in Table 3. In multivariable logistic regression, *pncA* mutations were associated with documented history of TB in the affected patient (OR=18.03, 95% CI=2.25-153.85; P=0.008), INH triple resistance (OR=409.11, 95% CI=6.52 to >1000; P<0.001), and INH double resistance (OR=13.50, 95% CI=1.43 to >1000; P=0.019).

TABLE 1. Resistance patterns among first-line drugs

Resistance pattern	No. (%)	Levofloxacin resistance	*pncA* mutation
Mono (INH only)	84 (53.5)	0	0
Double (INH + SM)	67 (42.7)	1	5
Double (INH + EMB)	3 (1.9)	0	0
Triple (INH + SM + EMB)	3 (1.9)	0	2

Abbreviations: EMB = ethambutol; INH = isoniazid; SM = streptomycin

TABLE 2. Mutation patterns among isolates with high and low levels of isoniazid resistance*

Mutation	Low resistance	High resistance
inhA + *katG* –	44 (62.0)	5 (5.8)
inhA – *katG* +	1 (1.4)	67 (77.9)
inhA + *katG* +	0	1 (1.2)
inhA – *katG* –	25 (35.2)	10 (11.6)
Could not be determined†	1 (1.4)	3 (3.5)
Total	71 (100)	86 (100)

Abbreviations: + detected; – not detected; PCR = polymerase chain reaction

* Data are shown as No. (%)

† No evaluable resistance pattern at either *inhA* or *katG* by GenoType MTBDRplus with subsequent conventional PCR/sequencing failure

Discussion

Levofloxacin resistance

In this study, the frequency of LVX resistance was very low in Hr-TB. Levofloxacin is an important drug in the management of drug-resistant TB. In a 2009 study in Shanghai, Xu et al[11] estimated the rates of LVX resistance to be 1.9% in strains pan-susceptible to first-line drugs, 6.7% in INH mono-resistant strains, and ≤25% in MDR-TB. Independent risk factors

TABLE 3. Univariate analysis of risk factors for *pncA* mutation

Characteristic	*pncA* mutation		Odds ratio (95% CI)	P value
	Detected	Not detected		
Age, y				
<65	6	75	6.00 (0.71-51.05)	0.118
≥65	1	75		
Sex				
Female	4	52	2.51 (0.54-11.65)	0.248
Male	3	98		
History of TB				
Yes	2	5	11.60 (1.79-75.00)	0.032
No	5	145		
Site of involvement				
Extrapulmonary	1	14	1.62 (0.18-14.43)	0.512
Pulmonary	6	136		
Sputum conversion within 5 months				
No	3	48	1.59 (0.34-7.40)	0.683
Yes	4	102		
INH resistance level				
High	4	82	1.11 (0.24-5.11)	1.000
Low	3	68		
INH resistance pattern				
Poly	7	66	19.06 (1.07-339.78)	0.04
Double	5	65	3.27 (0.62-17.40)	0.243
Triple	2	1	59.60 (4.61-771.38)	0.005

Abbreviations: 95% CI = 95% confidence interval; INH = isoniazid; TB = tuberculosis

associated with LVX resistance included MDR, RIF mono-resistance, poly-resistance (resistance to ≥2 first-line drugs, but not MDR), age ≥46 years, and TB re-treatment, but not INH mono-resistance. These findings were consistent with the results of a 2017 study in Ningbo (a city near Shanghai), whereby Che et al[12] revealed no LVX resistance in strains pan-susceptible to first-line drugs, 3% in strains with any resistance to first-line drugs except MDR, and ≤30% in MDR-TB. Most fluoroquinolone resistance was present in the MDR group. Che et al[12] also found that prevalence of LVX resistance in MDR-TB increased with duration of inappropriate treatment before LVX; this relationship was stronger than any relationships with previous fluoroquinolone exposures. Further studies are needed to determine whether this finding also applies to Hr-TB. Levofloxacin resistance is uncommon in non-MDR strains, especially in the present study. Since 2015, our laboratory has performed LVX susceptibility testing on 640 Hr-TB strains, identifying only two resistant cases (0.31%; unpublished data), including the one in this study. Clinicians could treat affected patients empirically, in accordance with WHO recommendations; further testing could be arranged based on clinical, radiological, and microbiological responses during early follow-up. In patients with Hr-TB that exhibits LVX resistance or poly-resistance to other primary agents, individualised adjustment of the treatment regimen is recommended in accordance with WHO guidelines[9] (eg, exclusion of LVX or inclusion of second-line TB medicines). In our single case with LVX resistance, the patient had no history of TB. His sputum acid-fast bacillus smear result was 2+. The Hr-TB strain isolated was initially LVX-sensitive without mutations in *rpoB*, *gyrA*, or *gyrB* on diagnosis and follow-up at 8 months. The patient continued to exhibit sputum culture-positive results after 15 months of treatment; further analysis revealed that the isolate had acquired *gyrA* mutation (detected by line-probe assays) and LVX phenotypic resistance. It then developed into an MDR strain; the patient eventually achieved culture conversion at approximately 18 months after diagnosis. Underlying hetero-resistance was a possible contributing factor.

Pyrazinamide resistance

Phenotypic PZA susceptibility testing has often been problematic. *pncA* mutations constitute the most common and primary determinant of PZA resistance, with reported sensitivity of approximately 80% to 95% and specificity of approximately 85% to 100%.[13,14] Resistance-conferring mutations are known to be diverse and scattered over the gene's full length. Our seven isolates all had unique mutations (Gln10Arg, His51Tyr, Trp68Stop, Thr47Pro, Ala102Thr, Asp63Gly, and Val139Ala), which were associated with PZA resistance at a high probability of 0.985 according to available literature[15] (except Val139Ala, probability of resistance=0.783). These seven isolates were also confirmed to be phenotypically resistant to PZA in our study.

Thus far, investigations of phenotypic and genotypic PZA resistance have generally focused on MDR-TB. Concerning non-MDR-resistant strains, there is considerable geographical variation in the reported rates, from 2.92% to 4.8% in the United States,[16,17] to 24% in the Western Pacific and 75% in South East Asia.[18] For Hr-TB in particular, phenotypic PZA resistance was reported in 2% of INH mono-resistant strains in South Africa,[19] no PZA resistance was detected (phenotypic or *pncA* mutation) in Georgia (Eastern Europe) among Hr-TB strains,[20] and ≤14.9% of Hr-TB strains exhibited *pncA* mutations in Vietnam.[21] In our study, we observed that 4.5% of Hr-TB isolates had *pncA* mutations. Such variation may be related to multiple factors, including differences in inclusion criteria, testing method, treatment, infection control practice, and disease burden. In terms of risk factors, we found that history of TB in the affected patient and poly-resistance (but not mono-resistance) were significantly associated with *pncA* mutations. These results are consistent with findings from previous studies, which also showed an association with MDR.[21-23] Clinicians are advised to consider the possibility of PZA resistance in patients with Hr-TB and these risk factors. Routine testing for LVX or PZA susceptibility in patients with INH mono-resistant TB alone (ie, no other risk factors) is not considered cost-effective, based on our findings.

Outcomes and treatment of isoniazid-resistant *Mycobacterium tuberculosis* without rifampicin resistance

In our study, most patients with Hr-TB had a satisfactory microbiological outcome without recurrence. However, the outcome was unknown in 23.6% of cases. Most of these involved patients were diagnosed and managed in hospitals, where only positive isolates were subjected to reference testing at our laboratory. Based on the recommended management regimen for TB in Hong Kong, these patients would have been followed up until sputum culture conversion. Considering the time elapsed since the latest positive laboratory result, the patients in most cases with unknown outcomes likely already had culture conversion.

In general, Hr-TB is presumably associated with higher rates of treatment failure and MDR acquisition, compared with drug-susceptible strains.[4,5] Important considerations for disease control include recognition of risk factors associated with Hr-TB (eg, history of TB treatment, incarceration in prisons, and homelessness[7,24]), early detection of INH resistance, exclusion of RIF resistance by appropriate molecular methods (eg, line-probe assays),[2] and compliance with current treatment guidelines. With the maturation of whole-genome sequencing, the technology is becoming integrated into the local routine drug susceptibility testing protocol for all *M tuberculosis* isolates; multiple and uncommon molecular markers of drug resistance might thus be identified earlier in a single set of assays for proper treatment guidance.

Strengths and limitations

Because our laboratory serves as a local TB reference laboratory, this study was able to use a representative *M tuberculosis* collection that reflected the status of Hr-TB in Hong Kong. Furthermore, because our laboratory serves as a supranational reference laboratory, we were able to perform an array of confirmatory tests in accordance with WHO recommendations, thus ensuring reliable results. However, this study had some limitations. First, it lacked data regarding microscopy results, drug treatment, and clinical outcome, thus preventing a more comprehensive assessment. Second, the small sample size and low outcome frequencies of LVX and PZA non-susceptibilities led to limited correlation analysis. Third, less common mutations mediating INH resistance were not examined to determine their prevalences; Hr-TB cases with these mutations would only be identified on the basis of phenotypic results. A substantial proportion of cases (22.3%; n=35) had negative GenoType MDRTBplus results. Because this test only detects the most common mutations at the *inhA* promoter region and *katG* codon 315, other relevant mutations might have been missed. Furthermore, in 3.2% (n=5) of cases with high-level INH resistance, mutations were detected in the *inhA* promoter region alone. The availability of preliminary information before receiving the INH phenotypic susceptibility results might lead to mismanagement of patient treatment (eg, INH inclusion in the therapeutic regimen). Fourth, low-level RIF resistance mediated by mutations outside the *rpoB* hotspot region and undetected by phenotypic testing was not ruled out

in this study. Inclusion of such isolates might have led to overestimation of true resistance in Hr-TB. Fifth, PZA resistance could be mediated by other less common mutations, which were not examined by performing phenotypic PZA susceptibility testing on all Hr-TB isolates; thus, we may have underestimated its prevalence. Our laboratory has found that all PZA-resistant *M tuberculosis* isolates thus far could be confirmed through *pncA* mutation analysis. Nevertheless, we presume that these limitations do not greatly affect the reliability and overall interpretation of our findings.

Conclusion

In Hong Kong, the rate of Hr-TB was 4.7% among active TB cases in 2018. Levofloxacin and PZA resistances among Hr-TB were uncommon (0.6% and 4.5%, respectively). History of TB in the affected patient and INH poly-resistance, including both double and triple resistances, were independent risk factors for *pncA* mutations. The findings from this study do not support routine susceptibility testing for these two drugs in patients with INH mono-resistant TB who lack additional risk factors.

Author contributions

Concept or design: KKM Ng.
Acquisition of data: All authors.
Analysis or interpretation of data: All authors.
Drafting of the manuscript: KKM Ng.
Critical revision of the manuscript for important intellectual content: KKM Ng.

All authors had full access to the data, contributed to the study, approved the final version for publication, and take responsibility for its accuracy and integrity.

Conflicts of interest

All authors have disclosed no conflicts of interest.

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Ethics approval

Ethics approval for this study was obtained from the Ethics Committee of the Department of Health, Hong Kong SAR Government (Ref: LM 425/2019). All included patients consented to testing for tuberculosis.

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