Genetic and phenotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates in Hong Kong

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Objectives: To characterize 250 drug-resistant *Mycobacterium tuberculosis* (MTB) isolates in Hong Kong with respect to their drug susceptibility phenotypes to five common anti-tuberculosis drugs (ofloxacin, rifampicin, ethambutol, isoniazid and pyrazinamide) and the relationship between such phenotypes and the patterns of genetic mutations in the corresponding resistance genes (*gyrA*, *rpoB*, *embB*, *katG*, *inhA*, *ahpC* and *pncA*).

Methods: The MIC values of the aforementioned anti-tuberculosis drugs were determined for each of the 250 drug-resistant MTB clinical isolates by the absolute concentration method. Genetic mutations in the corresponding resistance genes in these MTB isolates were identified by PCR-single-stranded conformation polymorphism/multiplex PCR amplimer conformation analysis (SSCP/MPAC), followed by DNA sequencing of the purified PCR products.

Results: Resistance to four or five drugs was commonly observed in these MTB isolates; such phenotypes accounted for over 34% of the 250 isolates. The most frequently observed phenotypes were those involving both rifampicin and isoniazid, with or without additional resistance to the other drugs. A total of 102 novel mutations, which accounted for 80% of all mutation types detected in the 7 resistance genes, were recovered. Correlation between phenotypic and mutational data showed that genetic changes in the *gyrA*, *rpoB* and *katG* genes were more consistently associated with a significant resistance phenotype. Despite this, however, a considerable proportion of resistant MTB isolates were found to harbour no detectable mutations in the corresponding gene loci.

Conclusions: These findings expand the spectrum of potential resistance-related mutations in MTB clinical isolates and help consolidate the framework for the development of molecular methods for delineating the drug susceptibility profiles of MTB isolates in clinical laboratories.

Keywords: MPAC, MDR-TB, tuberculosis

Introduction

*Mycobacterium tuberculosis* (MTB) infection remains a common infectious disease worldwide, with up to 10 million reported cases and 2 million deaths per year. Although the incidence of this disease seemed to have declined during the 1970s and early 1980s because of the success in chemotherapy, there has been a global resurgence of tuberculosis since the late
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1980s. In Hong Kong, a total of 6607 cases were recorded in the year 2002, at a rate of 97/100 000 people.

Treatment of MTB infection relies primarily on the use of two major first-line drugs, isoniazid and rifampicin, which are often included in a four-drug regimen that also includes ethambutol and pyrazinamide. The second-line fluoroquinolone drugs may be prescribed either when the two first-line drugs fail as a result of emergence of resistant organisms or in cases where their use is not appropriate due to hepatic problems in patients. Hence, the emergence of clinical isolates that are resistant to fluoroquinolones signals a significant compromise in the effectiveness of MTB treatment. When MTB strains exhibit resistance to both isoniazid and rifampicin, they are termed multidrug-resistant MTB (MDR-TB). These MDR-TB strains have been shown to be increasingly associated with infections in AIDS patients. Furthermore, treatment of MDR-TB infection has been complicated by the increased cost (up to 100 times higher than the treatment of diseases involving drug-susceptible organisms) and higher frequency of adverse reactions.

The genetic basis of antibiotic resistance in clinical MDR-TB isolates has been widely studied and is predominantly attributed to mutations in specific resistance genes. Despite the abundance of data regarding hot-spot mutations in MDR-TB isolates, however, we currently lack a systematic and comprehensive analysis on the prevalence of different mutation patterns and resistance phenotypes among clinical MDR-TB isolates, as well as the degree of correlation between various combinations of genotypes and phenotypes in Hong Kong. Owing to the advent of molecular detection techniques, it is envisaged that a more thorough understanding of the genetic basis of drug resistance shall facilitate the development of rapid methods for assessing the antibiotic susceptibility phenotypes of clinical MTB isolates, thereby allowing more effective drug usage and treatment of the disease, and hence reduction in resistance development.

We have recently completed an analysis on mutations in the *gyrA* gene of fluoroquinolone-resistant clinical isolates of MTB and showed that such mutations were strongly associated with a high-level fluoroquinolone resistance phenotype in Hong Kong. Hence, detection of such mutations using a rapid molecular technique such as multiplex PCR amplimer conformation analysis (MPAC), a mutation detection strategy we developed in the previous study, allows rapid prediction of the fluoroquinolone susceptibility phenotypes of clinical isolates. In the present study, we aimed to further perform a comprehensive survey of mutation patterns involving seven resistance genes in over 200 MTB clinical isolates, each exhibiting resistance to one or more of five commonly used drugs for MTB treatment in Hong Kong. The resistance genes studied included *rpoB*, *gyrA*, *katG*, *inhA*, *ahpC*, *embB* and *pncA*, the mutations of which were previously reported to be associated with resistance to the corresponding anti-tuberculosis drugs of rifampicin, fluoroquinolones, isoniazid, ethambutol and pyrazinamide, respectively. Through analysing the degree of correlation between the genetic profiles and their corresponding drug susceptibility phenotypes of a large collection of local MDR-TB isolates, we also hoped to provide an assessment of the predictive value of current molecular approaches in determining the antibiotic susceptibility profiles of MTB in a clinical setting.

Materials and methods

Strain selection

Clinical isolates of MTB collected from the Grantham Hospital and the Public Health Laboratory of Hong Kong were routinely screened for their susceptibility to commonly used anti-tuberculosis drugs. On the basis of the drug susceptibility data in a 10 year period (1994–2004), all isolates (a total of 250) that exhibited resistance to one or more of the following five anti-tuberculosis drugs in routine antimicrobial susceptibility testing were included in this study: rifampicin, isoniazid, ethambutol, pyrazinamide and ofloxacin. Preliminary epidemiological data and patient information, such as the dates of collection of clinical specimens, suggested that there were no apparent clonal features among the 250 isolates. Hence we consider each of the test strains an independent isolate. Two MTB reference strains, ATCC 27294 H37Rv and ATCC 25117 H37Ra, and a collection of 50 randomly selected clinical isolates that were susceptible to all the five test drugs were also included as control strains in the study.

Antibiotic susceptibility tests

The drug susceptibility profiles of the selected strains were evaluated by the absolute concentration method. The MICs at which the isolates were considered resistant were as follows: 64 mg/L for rifampicin, 1.0 mg/L for isoniazid, 4 mg/L for ethambutol and 4.8 mg/L for ofloxacin. For assessment of pyrazinamide susceptibility, the BACTEC™ MGIT™ 960 PZA Kit (Becton Dickinson Microbiology, Franklin Lakes, NJ, USA) was used, and 100 mg/L was taken as the breakpoint.

DNA extraction procedures

All isolates were cultured on Löwenstein–Jensen slants. Chromosomal DNA was extracted by a recommended method. The purified DNA pellet was allowed to air-dry overnight and finally dissolved in 20 µL of sterile double-distilled water and stored at 4°C until use.

PCR-SSCP/MPAC and nucleotide sequencing

The SSCP/MPAC analysis was performed as described to elucidate the genetic profiles of the five resistance genes. These included the quinolone resistance-determining region of the *gyrA* gene, the region containing hot-spot mutations in the *rpoB* gene and those in the *embB*, *katG*, *inhA*, *ahpC* and *pncA* genes, respectively. Information regarding primer sequences is listed in Table S1 [available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. The drug susceptibility phenotypes of MTB isolates in the specimens were predicted on the basis of the SSCP/MPAC patterns generated. Wild-type and known mutation controls were included in the analysis to facilitate the identification of SSCP/MPAC or SSCP types of specimens. All PCR products exhibiting mutation profiles and randomly selected PCR products that exhibited the wild-type pattern were analysed by nucleotide sequencing (Macrogen, Seoul, Korea).

Results

Resistance profiles

This study comprised a total of 250 MTB isolates, each of which exhibited resistance to one or more of the following
anti-tuberculosis agents: ofloxacin, rifampicin, isoniazid, ethambutol and pyrazinamide. Detailed analysis of the resistance phenotypes of these isolates revealed that only 33 (13%) isolates were resistant to one antibiotic, whereas 43 isolates (17%) were found to be resistant to all the five test drugs. Prevalence of resistance to two, three and four drugs was 29%, 23% and 18%, respectively. Resistance to the first-line drugs of rifampicin and isoniazid was the most common, with 88% and 97% of isolates exhibiting resistance to these two drugs, respectively (see Table 1, which provides a summary of the correlation analysis of the genetic and phenotypic characteristics of the 250 MTB isolates). A wide variety of resistance phenotypes were observed among the 250 strains. These phenotypes are listed in Table 2, which also shows the relative frequency of each of the resistance patterns exhibited by the 250 isolates. Table 3 carries a list of isolates that exhibit rare phenotypic characteristics and/or mutational profiles.

More than 85% of the isolates were resistant to both rifampicin and isoniazid, often with additional resistance to other drugs. Nevertheless, there were 6 strains that exhibited resistance to rifampicin but not to isoniazid, whereas 26 strains were found to be resistant to isoniazid but not to rifampicin (Table 2). Except for strain M45 (Table 3), no isolates were found to display resistance towards one or more of the other three drugs (ofloxacin, ethambutol and pyrazinamide) without involving either rifampicin or isoniazid, or both. The clustering of resistance towards rifampicin and isoniazid was also signified by the observation that phenotypes involving these two drugs are much more prevalent than those involving other antibiotic combinations, although some discrepancy in the frequency of recovery was observed for those involving additional drugs (Table 2). For instance, 9 strains were found to be resistant to rifampicin, isoniazid and ofloxacin, yet 32 strains displayed co-resistance to rifampicin, isoniazid and ethambutol; another 14 were found to be resistant to rifampicin, isoniazid and pyrazinamide. Co-resistance to four drugs, such as those resistant to rifampicin, isoniazid, ethambutol and pyrazinamide, was also a common phenomenon. A total of 28 isolates were found to display such a phenotype. Another 13 isolates exhibited resistance to rifampicin, isoniazid, ethambutol and ofloxacin. Interestingly, the aforementioned strain M45 was resistant to ethambutol but not to other drugs including rifampicin and isoniazid (Table 3). Another strain (M47) was found to be resistant to ofloxacin, rifampicin and pyrazinamide, but not to isoniazid. Strain M82, however, was resistant to ethambutol, isoniazid and pyrazinamide but not to rifampicin and ofloxacin. Contrary to the high rates of resistance towards rifampicin and isoniazid, >50% of the isolates remained susceptible to the other three drugs: ofloxacin, ethambutol and pyrazinamide. In fact, 72% of the isolates remained susceptible to ofloxacin, 53% were susceptible to ethambutol and 64% of the 250 MDR-TB isolates were still susceptible to pyrazinamide (Table 1).

### Mutation patterns

The corresponding resistance genes in which mutations are known to be associated with resistance to the five test drugs were analysed by SSCP/MPAC, as well as nucleotide sequencing to depict all possible genetic alterations in the known mutation hot-spot regions. Table S2 [available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)] tabulates the full spectrum of mutations recovered in the 250 isolates. Mutations were not found in the 50 control strains that are susceptible to all five drugs. Among the seven genes studied, pncA and katG were found to comprise the largest range of mutation genotypes, with a total of 45 and 38 mutation profiles identified, respectively. It should be noted that deletions and double mutations are common in the katG and pncA genes of these isolates. For the other genes, the number of mutation profiles recovered was 25, 10, 7, 3 and 2 for rpoB, gyrA, embB, ahpC and inhA, respectively. Except for the inhA and ahpC genes where mutations were recovered in only three and four isolates, respectively, hot-spot mutations in each of the other resistance genes were identified (Table S2). For gyrA, 19 isolates were found to harbour the mutation that gave rise to the 94 Asp → Gly alteration, which accounted for 34% of all gyrA mutants. For rpoB, the most frequently found changes were the 531 Ser → Leu and 526 His → Tyr amino acid alterations, which were recovered in 134 and 26 isolates, respectively. These two mutation types together accounted for 77% of all rpoB mutants. For embB, 67 and 32 isolates were found to harbour the 306 Met → Val and 306 Met → Ile changes, respectively. These 99 isolates represented 94% of all embB mutants. For katG, the most frequently found mutation type, recovered in 100 isolates or 63% of all katG mutants, was the one that gave rise to the 315 Ser → Thr amino acid alteration. For pncA, 22 isolates

### Table 1. Summary and correlation of genetic and phenotypic characteristics of 250 clinical MTB isolates

| Drug/resistance gene | Resistance to specific drug (%) | Resistant isolates that harbour mutations (%) | Mutants exhibiting resistance (%) | Drug-susceptible strains that harboured mutations (%) |
|----------------------|-------------------------------|---------------------------------------------|----------------------------------|-----------------------------------------------|
| OFX/gyrA             | 71/250 (28)                   | 55/71 (78)                                  | 55/56 (98)                       | 1/179 (0.6)                                  |
| RIF/rpoB             | 221/250 (88)                  | 206/221 (93)                                | 206/208 (99)                     | 2/29 (7)                                     |
| EMT/embB             | 118/250 (47)                  | 98/118 (83)                                 | 98/106 (93)                      | 8/132 (6)                                    |
| INH/katG             | 241/250 (96)                  | 159/241 (66)                                | 159/159 (100)                    | 0/9 (0)                                      |
| INH/inhA             | 241/250 (96)                  | 3/241 (1.2)                                 | 3/3 (100)                       | 0/9 (0)                                      |
| INH/ahpC             | 241/250 (96)                  | 4/241 (1.6)                                 | 4/4 (100)                       | 0/9 (0)                                      |
| PNZ/pncA             | 89/250 (36)                   | 71/89 (80)                                  | 71/89 (80)                       | 18/161 (11)                                  |

OFX, ofloxacin; RIF, rifampicin; EMT, ethambutol; INH, isoniazid; PNZ, pyrazinamide.
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Table 2. Prevalence of each of the possible combinations of resistance phenotypes among 250 clinical MTB isolates

| Resistance phenotype | Frequency of recovery |
|----------------------|-----------------------|
| O                    | 0                     |
| R                    | 6                     |
| E                    | 1                     |
| H                    | 26                    |
| Z                    | 0                     |
| Subtotal             | 33                    |
| OR                   | 0                     |
| OE                   | 0                     |
| OH                   | 1                     |
| OZ                   | 0                     |
| RE                   | 1                     |
| RH                   | 70                    |
| RZ                   | 0                     |
| EH                   | 0                     |
| EZ                   | 0                     |
| HZ                   | 0                     |
| Subtotal             | 72                    |
| ORE                  | 0                     |
| ORH                  | 9                     |
| ORZ                  | 1                     |
| OEH                  | 0                     |
| OEZ                  | 0                     |
| OHZ                  | 0                     |
| REH                  | 32                    |
| REZ                  | 0                     |
| RHZ                  | 14                    |
| MHZ                  | 1                     |
| Subtotal             | 57                    |
| OREH                 | 13                    |
| OREZ                 | 0                     |
| OEHZ                 | 0                     |
| ORHZ                 | 4                     |
| REHZ                 | 28                    |
| Subtotal             | 45                    |
| OREHZ                | 43                    |
| Total                | 250                   |

O, ofloxacin; R, rifampicin; E, ethambutol; H, isoniazid; Z, pyrazinamide.

contained the 68 Trp → Leu change, representing 25% of the isolates harbouring \textit{pncA} mutations.

In the present study, previously unreported rare mutations were also widely observed in the \textit{gyrA}, \textit{rpoB}, \textit{embB}, \textit{inhA}, \textit{katG} and \textit{pncA} genes (Table S2). A total of 4 and 11 rare mutations (mutation types G2, 7, 9 and 10 and R4, 6, 8, 15, 16, 17, 18, 20, 23, 24, 25, Table S2) in the \textit{gyrA} and \textit{rpoB} genes were, respectively, recovered. For the \textit{katG} gene, 38 out of 47 recovered mutation types (80%) were novel and not previously reported in the literature. In summary, a total of 102 novel mutations, which accounted for 80% of all changes detected in the seven resistance genes, were recovered in this study (Table S2).

\textbf{Correlation between resistance phenotypes and mutation profiles}

Analysis of correlation between the resistance phenotypes and mutation data suggested that a resistance phenotype was not always associated with the detection of a mutation in the corresponding resistance gene. In fact, for each phenotype/resistance gene combination, strains that exhibited a significant resistance phenotype without detectable mutations in the corresponding resistance gene were recovered (M4, M15, M51, M95, 710; Table 3). Among the isolates that were resistant to ofloxacin, only 78% were found to harbour mutations in \textit{gyrA} (Table 1). For those resistant to rifampicin, the rate was 93%. Among the strains that exhibited resistance to ethambutol, isoniazid and pyrazinamide, the proportion of isolates that harboured mutations in the respective genes was 83%, 66% and 80%, respectively. This phenomenon was also observed among some of the strains that were resistant to all the five test drugs, where mutations were often found in only two or three of the resistance genes (strains M4 and 1020 respectively; Table 3). For instance, among the 43 strains that exhibited resistance to all five drugs, as many as 29 (67%) did not have \textit{katG} mutations.

Nucleotide sequencing was repeated for resistant isolates that harboured no mutation in the corresponding gene, with results confirming the absence of mutations in these isolates. On the other hand, however, the vast majority of isolates that harboured mutations were found to be resistant to the corresponding drug, although such correlation was not absolute. All but one of the 56 \textit{gyrA} mutants were ofloxacin-resistant, and 206 out of 208 isolates that contained \textit{rpoB} mutations were resistant to rifampicin (Table 1). For strains harbouring mutations in the \textit{embB}, \textit{katG} and \textit{pncA} genes, resistance rates were 93%, 100% and 80%, respectively. Nevertheless, our data also show that there is discrepancy between the phenotypes of strains carrying the same mutation. For example, an isolate (M62) that carried a \textit{gyrA} mutation (94 Asp → Ala, mutation type G6; Tables 3 and S2) was found to be susceptible to ofloxacin, whereas another isolate (M59) carrying the same mutation was resistant to this drug (Table 3). Likewise, one strain (M14) carrying an \textit{rpoB} mutation (516 Asp → Tyr, mutation type R10; Tables 3 and S2) was susceptible to rifampicin, whereas three other strains carrying the same mutation were found to be resistant to the drug. In addition, we observed that identical mutations were occasionally associated with varied resistance phenotypes. For instance, isolates that carried the codon 306 mutations (Met → Ile and Met → Val) in the \textit{embB} gene were found to be associated with resistance levels ranging from 2 to >4 mg/L (M58, M59, M62, M64, M77, M80, M98, 710, TB5 and 1064; Table 3). In summary, however, the vast majority of the mutation types found in this study were found to be consistently associated with a significant resistance phenotype (Table S2).

\textbf{Discussion}

\textbf{Overview}

Drug resistance of MTB is generally believed to be caused by point mutations in several key resistance genes within the MTB genome. Although hot-spot mutations have been identified in these resistance genes, few studies have managed to reveal...
Table 3. Phenotypic and mutational profiles of representative isolates

| Strain number | MIC (mg/L)$^a$/Phenotype | Mutations in specific resistance genes | Remarks (see footnote) |
|---------------|---------------------------|----------------------------------------|-----------------------|
|               | O  | R  | E  | H  | Z  | gyrA | rpoB | embB | katG | pncA |
| M4            | 4.8 (R) | >64 (R) | >4 (R) | 1 (R) | (R) | —    | —    | —    | —    | —    | 531 Ser → Leu | 306 Met → Val | —    | 68 Trp → Leu | A |
| M14           | 0.6 (S) | 16 (S) | 2 (S) | >1 (R) | (S) | —    | —    | 516 Asp → Tyr | —    | 315 Ser → Thr | —    | B |
| M15           | >4.8 (R) | >64 (R) | >4 (R) | >1 (R) | (R) | —    | —    | 531 Ser → Leu | 306 Met → Val | —    | 68 Trp → Leu | A |
| M45           | 2.4 (S) | 16 (S) | >4 (R) | 0.2 (S) | (S) | —    | —    | —    | 306 Met → Val | —    | —    | C |
| M47           | >4.8 (R) | >64 (R) | 2 (S) | 0.2 (S) | (R) | 94 Asp → Tyr | 516 Asp → Val | —    | —    | —    | D |
| M51           | 1.2 (S) | >64 (R) | 2 (S) | >1 (R) | (S) | —    | —    | —    | 315 Ser → Thr | —    | —    | E |
| M58           | 1.2 (S) | >64 (R) | >4 (R) | >1 (R) | (R) | —    | 531 Ser → Leu | 306 Met → Ile | —    | —    | F |
| M59           | >4.8 (R) | >64 (R) | >4 (R) | >1 (R) | (R) | 94 Asp → Ala | 531 Ser → Leu | 306 Met → Val | —    | 162 Gly → Asp | F, G |
| M62           | 0.6 (S) | >64 (R) | >4 (R) | >1 (R) | (R) | 94 Asp → Ala | 531 Ser → Leu | 306 Met → Val | —    | 162 Gly → Asp | F, G |
| M64           | 0.6 (S) | >64 (R) | 2 (S) | >1 (R) | (S) | —    | 505 Phe → Leu | 306 Met → Ile | 315 Ser → Thr | —    | F |
| M77           | 1.2 (S) | >64 (R) | 2.8 (S) | >1 (R) | (S) | —    | 526 His → Tyr | 306 Met → Ile | 315 Ser → Thr | —    | F |
| M80           | 1.2 (S) | >64 (R) | 2.8 (S) | >1 (R) | (S) | —    | 526 His → Tyr | 306 Met → Ile | 315 Ser → Thr | —    | F |
| M82           | (S) | (S) | (R) | (R) | (R) | —    | 574 Ser → Leu | —    | 311 Asp → Gly | —    | B |
| M95           | 4.8 (R) | >64 (R) | 2.8 (S) | >1 (R) | (S) | —    | 531 Ser → Leu | 306 Met → Ile | —    | —    | A |
| M98           | 1.2 (S) | 16 (S) | 2 (S) | >1 (R) | (S) | —    | —    | 306 Met → Val | —    | —    | F |
| 710           | 1.2 (S) | >64 (R) | 2 (S) | >1 (R) | (R) | —    | 531 Ser → Leu | 306 Met → Ile | —    | —    | F |
| TB5           | 0.6 (S) | >64 (R) | 2.8 (S) | >1 (R) | (S) | —    | 526 His → Tyr | 306 Met → Val | 315 Ser → Thr | —    | F |
| 1020          | 4.8 (R) | >64 (R) | 4 (R) | >1 (R) | (R) | —    | 531 Ser → Leu | 306 Met → Ile | —    | —    | A |
| 1064          | >4.8 (R) | >64 (R) | 2.8 (S) | >1 (R) | (R) | 94 Asp → Gly | 531 Ser → Leu | 306 Met → Val | 315 Ser → Thr | 75 Met → Thr | F |

$^a$MICs of these drugs were determined by the absolute concentration method; (R) and (S) denote resistance and susceptibility, respectively, to specific drug.

A, ofloxacin-resistant isolate without gyrA mutations; B, rifampicin-susceptible rpoB mutant; C, ethambutol-resistant isolate that is susceptible to both rifampicin and isoniazid; D, isoniazid-susceptible isolate that is resistant to ofloxacin, rifampicin and pyrazinamide; E, rifampicin-resistant isolate without rpoB mutations; F, isolates exhibiting identical embB mutation but varied drug susceptibility levels; G, isolates exhibiting identical gyrA mutation but varied drug susceptibility levels.

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the range of phenotypic and mutational profiles that may be recovered in clinical MTB isolates. Among the 250 isolates in the present study, 213 isolates were MDR-TB (Table 2). To the best of our knowledge, these 213 isolates represent the largest collection of MDR-TB isolates ever reported in the literature; hence analysis of these isolates offers us a more comprehensive view on the scope of both phenotypic and genetic characteristics of these isolates.

Our data suggested that the majority of these isolates displayed multiple drug resistance phenotypes that involved two or more drugs and that only a small proportion of clinical isolates was resistant to only one agent (13%). It should be noted that, to our knowledge, the majority of the 250 strains in this study were isolated from patients who had previously received antituberculosis treatment. Although we lack the precise data on the relative proportion of isolates obtained from the first-time or re-treated patients, our finding that co-resistance to the first-line drugs, rifampicin and isoniazid, accounted for an overwhelming majority of all resistance phenotypes, tends to suggest that resistance in clinical MTB isolates is essentially ‘multiple’ in nature once it develops. It also appears that resistance to the other drugs develops on the basis of resistance to the first-line drugs of rifampicin and isoniazid, since resistance to other agents without involving at least one of these two drugs was extremely rare (only 1 out of 250 isolates, M45) and that over 50% of isolates remained susceptible to each of the other three test drugs (ofloxacin, ethambutol and pyrazinamide; Table 1). This phenomenon is probably related to the consecutive use of different anti-tuberculosis drugs for treatment, as well as to dissemination of the MDR strains. Nevertheless, detailed analysis of the spectrum of resistance phenotypes that was recovered in this study suggested that further development of resistance to drugs other than rifampicin and isoniazid exhibited some preferences towards certain antibiotic combinations such as rifampicin/isoniazid/ethambutol (32 isolates) and rifampicin/isoniazid/ethambutol/pyrazinamide (28 isolates; Table 2). Whether this phenomenon is related to the differential pattern of drug prescription among the patients from whom the isolates were collected remains to be investigated.

It should also be noted that our approach of drug susceptibility testing in this study, in which only a small number of susceptibility levels were tested, did not allow us to examine whether there were any enhancement effects on specific resistance phenotypes that could be attributable to accumulation of mutations in different resistance genes. However, we observed that single or double mutations (predominantly those of the *rpoB* and *katG* genes) were consistently associated with a significant resistance phenotype, hence it is likely that there are no additive effects on multiple drug resistance as a result of accumulation of multiple gene mutations. Our results also contradict previous reports that demonstrated some correlation between specific mutation types and resistance phenotypes.

### Resistance gene mutations

A large number of novel mutations were found in this study, suggesting that the range of mutations that may confer resistance to MTB can be much more extensive than those reported in the literature. Despite this finding, however, most mutations were still confined to only a few mutation hot spots in each gene. Our data also showed that the vast majority of mutations were associated with a single resistance level (Table S2). Exceptional cases include a *gyrA* mutation that gave rise to the 94 Asp → Ala change and an *rpoB* mutation that resulted in a 516 Asp → Tyr amino acid change, which were found to be associated with both susceptible and resistant phenotypes. Such findings prompted us to consider the need to investigate the direct effect of such hot-spot mutations on the basic structures and functional mechanisms concerning mycobacterial growth and survival. In fact, it is entirely possible that an identical mutation may be associated with different phenotypic characteristics of two bacterial strains if they simultaneously harboured different types of compensatory mutations, although mutations that lead to a reversal of resistance phenotypes have not been documented.

### Comparison with data in literature

A thorough review of data in the literature showed that well over 90% of rifampicin-resistant clinical isolates of MTB harboured mutations in the 81 bp core region of the *rpoB* gene. Nevertheless, this figure indicated that correlation between rifampicin resistance phenotype and *rpoB* mutations is not absolute. In our study, we recovered two resistant isolates that did not harbour any mutations (one of which is strain M51; Table 3); this is consistent with various past findings including a recent report by Suresh et al., who also showed that mutations were not present in a small proportion of resistant isolates. The phenomenon regarding discrepancy between phenotypic resistance and mutational data has also been consistently observed with other resistance genes in this and other studies.

We believe that some undefined resistance mechanisms or genetic mutations are responsible for this discrepancy. It is also possible that a small number of mutations might have been missed out by the current MPAC or SSCP mutation screening approaches. As for the rate and prevalence of hot-spot *rpoB* mutations, our results exhibit some discrepancy with those reported in the literature in both the types and frequency of hot-spot mutations. The most frequently recovered *rpoB* mutations in our study were those in codons 531 and 526 (Table S2), whereas only eight cases or 4% of mutations involved codon 516. These data are comparable with those reported in previous studies in China, especially codon 516 for which the rate of mutation was also reported to be 4% by Yue et al. However, these numbers are quite different from those of another study that covered MTB isolates collected from China, Taiwan, Korea and Japan, where the overall frequency of mutation at codon 516 was 17%. Significant variations in terms of the nature of genetic alteration and frequency of mutation at specific sites were also evident in other parts of the world. In Peru, the Leu-533 → Pro and the Asp-516 → Tyr amino acid changes were found to give rise to low-level resistance only. Although these two mutations were detected in the current work, both were found to be associated with a rifampicin MIC of 64 mg/L (Table S2). Another study that involved detailed analysis of the relationship between the degree of resistance to rifampicin and mutational sites of the *rpoB* gene suggested that *rpoB* mutations are mostly, but not necessarily, associated with rifampicin resistance of MTB and that the sites of mutations will affect the level of resistance to rifampicin. Locally, the Gln-513 → Arg and His-526 → Asn double mutation and a deletion at 518 Asn as reported by Yam et al. were also not detectable among our MTB isolates.
With regard to other resistance genes, a review of our own results and those in the literature showed that, with the exception of a few key mutation hot spots such as codon 315 in katG, both the types and frequency of mutations recovered in the katG, inhA and ahpC genes of isoniazid-resistant isolates differ quite significantly from each other among various reports.\textsuperscript{13,21,28–30} It is possible that isoniazid resistance in some isolates requires an elaborate cooperation of resistance mechanisms or mutations in several resistance genes, presumably including the kasA gene\textsuperscript{28} and the ethA gene,\textsuperscript{31} which were not included for investigation in this work. The data gathered so far also suggested that, apart from the key mutations in the katG gene, there is no firm evidence that allows us to correlate the other mutation types with the isoniazid resistance phenotype. Similarly, we observed both common and unique mutations when mutational data of the gyrA, embB and pncA genes are compared with those in the literature.\textsuperscript{19,20,31,32}

Although the data in the literature as well as those gathered in this study suggested that the mutational and phenotypic patterns for multidrug resistance in MTB vary geographically, it is still possible that a significant proportion of multidrug resistance cases were due to clonal spread of certain types of resistant isolates, rather than intrinsic resistance development that involves spontaneous mutation and selection during the course of treatment. It remains to be seen whether clonal spread of resistant mutants accounts for the higher rate of multidrug resistance among re-treated cases, when compared with that of primary infections. This hypothesis may be tested through investigation of the epidemiological profiles of our collection of resistant isolates. Since high rates of acquired MDR-TB have also been reported in various parts of the world including Nepal (48.0%), Gujarat, India (33.8%), New York City, USA (30.1%), Bolivia (15.3%) and Korea (14.5%),\textsuperscript{33} future research should focus on analysing factors that determine dissemination of known resistant isolates.

**Predictive value of rpoB, katG and gyrA mutations**

Although it is now clear that there is no absolute correlation between a specific resistance phenotype and the presence of mutations in the corresponding resistance gene, our data confirm that almost all categories of drug-resistant MTB isolates in Hong Kong, including but not limited to the multidrug-resistant strains, exhibit resistance to either rifampicin or isoniazid, or both (see Table 2 for the distribution of resistance phenotypes, an exception being strain M45, which exhibited resistance to ethambutol but not to the other drugs including rifampicin and isoniazid, Table 3). This phenomenon may be related to the fact that rifampicin and isoniazid are the primary drugs of choice for treatment of MTB infections and that phenotypic resistance to these drugs is more readily detected in routine susceptibility testing.

On the other hand, resistance to either rifampicin or isoniazid (or both) was inevitably associated with mutations in either the rpoB or katG gene, or both. For example, a rifampicin-resistant strain that did not harbour an rpoB mutation (M51; Table 3) was found to carry a katG mutation, and all isoniazid-resistant strains that had no katG mutations were found to harbour rpoB mutations. In other words, we did not recover any strains that exhibited resistance to either rifampicin or isoniazid, without mutations in either or both of the rpoB or katG genes. In this regard, a molecular test that offers rapid and simultaneous detection of mutations in the rpoB and katG genes is highly desirable. Indeed, such test is available commericially.\textsuperscript{30} It would be advisable that the test may also include an assessment of mutations in the gyrA gene, as there is extremely good correlation between the presence of mutations in this gene and the fluoroquinolone resistance phenotypes. Positive detection of mutations in one or more of these three genes would infer a very high possibility of the presence of a multidrug resistance phenotype. A positive result should therefore be followed by either sequencing analysis of all known resistance genes or routine susceptibility testing, so as to reveal the entire drug susceptibility spectrum of the potentially multidrug-resistant isolate. On the other hand, we believe that a lack of mutations in all the three test genes infers that the test isolate is susceptible to all anti-tuberculosis drugs (the aforementioned strain M45 being an exception) and that no further standard susceptibility tests need to be performed. In addition, the molecular test should be carried out with smear-positive sputum specimens so as to speed up the susceptibility testing process. As a concluding note, we wish to stress that rapid and reliable identification of MDR-TB and their drug susceptibility phenotypes is instrumental in facilitating more effective treatment as well as control of spread of diseases involving this notorious pathogen. It is envisaged that such a goal may be attained through the development of new-generation molecular methods for MTB susceptibility testing.

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### Transparency declarations

None to declare.

### Supplementary data

Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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