Characterization of pyrazinamide and ofloxacin resistance among drug-resistant *Mycobacterium tuberculosis* isolates from Singapore

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Objectives: To evaluate rapid molecular approaches for the detection of pyrazinamide (PZA) and ofloxacin resistance, by screening 100 known drug-resistant *Mycobacterium tuberculosis* isolates.

Methods: *Mycobacterium tuberculosis* isolates were tested for phenotypic resistance to pyrazinamide and ofloxacin using the BACTEC 460 radiometric method and the E-test, respectively. Mutation screening was done by amplifying the *pncA*, *gyrA*, and *gyrB* genes by the polymerase chain reaction (PCR) and direct automated sequencing.

Results: Twelve isolates were PZA-resistant and 8 of 12 (66.7%) isolates had missense mutations or deletions at the *pncA* gene, suggesting that mutation or deletion at the *pncA* gene is the major molecular mechanism of PZA resistance among the Singaporean isolates. Using the E-test, 48 isolates were resistant to ofloxacin, with minimum inhibitory concentrations of 4 μg/mL or higher. No mutations were observed at the quinolone resistance-determining region (QRDR) of *gyrA* in all isolates. At the QRDR of *gyrB*, mutations were present in 1 of 48 ofloxacin-resistant isolates and 0 of 19 ofloxacin-susceptible isolates.

Conclusions: In Singapore, genotypic analysis of resistance to PZA and ofloxacin is inadequate and should be complemented by conventional methods.

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Pyrazinamide (PZA), an analogue of nicotinamide, is an important antituberculous drug. It is used together with isoniazid and rifampicin for the current short course (6 month) chemotherapy of *Mycobacterium tuberculosis* (MTB) infection.1 Owing to the emergence of multidrug-resistant tuberculosis, fluoroquinolones, including ofloxacin, are increasingly used for treatment of tuberculosis.2

In vitro susceptibility testing for PZA and ofloxacin have not been routinely available in Singapore and the region. The goals of this study were to determine the incidence of PZA and ofloxacin resistance among drug-resistant MTB isolates from Singapore and to determine the feasibility of rapid susceptibility testing by molecular methods. One hundred clinical isolates of MTB, known to be drug-resistant to at least one first-line antituberculous drug, were screened for susceptibility to pyrazinamide, using the BACTEC 460 radiometric method, and for susceptibility to ofloxacin using the E-test.

To investigate the molecular mechanisms for PZA resistance, the authors sequenced the entire *pncA* gene in both PZA-resistant and PZA-susceptible isolates. The *pncA* gene encodes the bacterial enzyme pyrazinamidase (PZase), which converts PZA to bactericidal pyrazinoic acid, and loss of PZase activity has been correlated with *pncA* mutations and PZA resistance.3-6

Currently, the only known target of fluoroquinolones in mycobacteria is the DNA gyrase, a tetrameric protein composed of two A subunits and two B subunits.7-10 Mycobacterial resistance to quinolones has been associated with mutations in the quinolone resistance-determining regions (QRDR) of *gyrA* at positions 90, 91, and 94 (*M. tuberculosis* numbering system).7-10 The authors sequenced the QRDR regions of *gyrA* and *gyrB* in clinical isolates of MTB from Singapore.

**MATERIALS AND METHODS**

**Drug susceptibility testing**

One hundred consecutive Singaporean clinical isolates of MTB, known to be resistant to at least one anti-tuberculous drug (isoniazid, rifampicin, streptomycin, ethambutol) were collected from the Central Tuberculosis Laboratory, Department of Pathology, Singapore General Hospital, and have previously been described.11
Clinical information about the subjects has been previously published. Of these 100 isolates, 53 were resistant to one drug, and the remaining isolates were resistant to two or more drugs. These were evaluated for phenotypic resistance to pyrazinamide using the BACTEC 460 radiometric method (Becton Dickinson, Towson, MD), with pyrazinamide at 100 µg/mL. For phenotypic resistance to ofloxacin, the E-test was used according to the manufacturer's instructions (AB Biodisk, Solna, Sweden).

DNA extraction

DNA was extracted from bacterial colonies grown on Löwenstein-Jensen slants by first, heat inactivating the bacteria at 80°C for 20 minutes, followed by digestion with lysozyme and proteinase K. The nucleic acids were precipitated with isopropanol, and the nucleic acid pellet was resuspended in tris-EDTA (TE) buffer.

Polymerase chain reaction

Purified DNA was amplified in a 50-µL polymerase chain reaction (PCR) mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 1.5 mM MgCl₂, 100 µM of each dNTP, 0.25 mM of each primer, and 1.25 U of Taq DNA polymerase (Promega, Madison, USA).

For mutation analysis of the entire pncA gene, oligonucleotide primers were designed from the complementary sequence of the pncA gene (GenBank Accession No. AL021899). Two overlapping fragments of 391 bp (nucleotide number 27578-27188) and 313 bp (nucleotide number 27251-26939) were amplified. Primers for the first fragment were 5'CAAAC-TGCCGGGGCAGTGCCTGCGC3' (pncAF1) and 5'TCAGCAGTGGGCGGTGGCTTG3' (pncAR1) and the primers for the second fragment were 5'GGTGCCTACACCGGACCGTA3' (pncAF2) and 5'TCCACCGCCGCACAACGCTTC3' (pncAR2). Oligonucleotide primers for the amplification of the gyrA and gyrB genes were as previously reported.

The PCR products were purified (Wizard PCR purification kit, Promega, Madison, WI, USA) and sequenced using the Applied Biosystems automatic DNA sequencer (model 377).

RESULTS

Of 100 Singaporean drug-resistant MTB isolates, 12 isolates were confirmed to be PZA resistant, 87 were PZA-susceptible, and the status of one isolate could not be determined as no growth in culture media was observed despite several attempts. All 12 PZA-resistant isolates and 20 PZA-susceptible isolates were screened for mutations at the pncA gene, by amplifying two overlapping fragments encompassing the entire pncA gene. The PCR products were directly sequenced using automated sequencing. Of the 12 PZA-resistant isolates, amino acid-altering mutations in pncA were identified in six isolates and deletions were identified in two isolates (Table 1). No deletions or amino acid-altering mutations were detected in any of the 20 PZA-susceptible isolates.

The minimum inhibitory concentration (MIC) for ofloxacin was determined using the E-test in the 100 clinical isolates of MTB (Table 2). Forty-eight isolates had MICs of 4 µg/mL or more and were considered resistant.

The QRDR region of the gyrA gene was sequenced for all 100 isolates, with no mutations detected. The QRDR of the gyrB gene was sequenced for 48 ofloxacin-resistant isolates for which the ofloxacin MIC was 4 µg/mL or more, and in 24 of 52 ofloxacin-susceptible isolates with ofloxacin MICs of less than 4 µg/mL. A mutation at codon 505 (GAC→GCC) was observed in one isolate with ofloxacin MIC of more than 32 µg/mL. No other mutations were observed.

DISCUSSION

Previous studies on PZA-resistant clinical isolates of MTD have detected mutations within the pncA gene at
frequencies of between 72% to 97%.

These mutations were observed throughout the entire length of the gene and included upstream mutations, misense changes, nucleotide insertions and deletions, and termination mutations. To characterize the molecular changes of \textit{pncA} among local isolates from Singapore, the entire \textit{pncA} gene was amplified and directly sequenced. Missense mutations and deletions were present in 67% of the Singaporean PZA-resistant clinical isolates of MTB, and these molecular changes spanned the entire \textit{pncA} gene. Interestingly, none of the mutations or deletions detected in the Singaporean isolates have been observed in other studies.\textsuperscript{4-6} One mutation at codon 63 (Asp→Ala) resulted in a different amino acid change than that previously reported by Scorpio and Zhang (Asp→His).\textsuperscript{3}

A common problem of current PZA susceptibility testing is the occurrence of false resistance, in which the isolates are susceptible upon retesting. Scorpio and colleagues reported that four PZA-resistant strains, as determined by the BACTEC method, were in fact susceptible.\textsuperscript{4} Differences in the susceptibility testing methods as well as the cutoff concentrations of pyrazinamide, ranging from 25 μg/mL to 400 μg/mL may contribute to the differences in the frequencies of mutation at \textit{pncA}. Sreevatsan and co-workers reported a frequency of \textit{pncA} mutations in only 72% of their isolates,\textsuperscript{3} similar to the frequency noted in the current study. Their isolates were tested for PZA susceptibility by the BACTEC method or the conventional proportion method with PZA concentration of 25 μg/mL. Hirano and colleagues detected mutations in \textit{pncA} in 97% of isolates with MICs of PZA at pH 6.0 of over 400 μg/mL.\textsuperscript{6} No mutations were present in three isolates with MICs of 100 μg/mL but only in five with MICs greater than 500 μg/mL.\textsuperscript{3} Hence, mutations in \textit{pncA} may be correlated with isolates with higher MICs, and isolates with lower MICs may be falsely resistant.

Mutation or deletion at the \textit{pncA} gene appears to be the major molecular mechanism of PZA resistance among the Singaporean isolates. However, a separate mechanism for PZA resistance may exist for the remaining one third of the PZA-resistant isolates for which no mutations or deletions were observed.

Mutations at the QRDR of the \textit{gyrA} gene at codons 90, 91, or 94 have been identified in 85% and 100% of fluoroquinolone (FQ) resistant isolates of MTB.\textsuperscript{7,8} In contrast to these studies, no mutations at \textit{gyrA} were detected in any of the Singaporean isolates. This suggests that alternative gene targets and mechanisms, such as impermeability attributable to alterations in outer membrane proteins and lowered levels of intracellular drug concentrations caused by energy-dependent efflux pumps may play a role in these isolates.\textsuperscript{3,5-10}

A low incidence of ofloxacin resistance has been reported by other investigators from Thailand (1.8%) and Taiwan (1.5%),\textsuperscript{17,18} which is similar to the incidence detected genotypically in the present study. The high incidence of ofloxacin resistance (48%) in Singapore, detected phenotypically using the E-test, was unexpected as ofloxacin has been shown to be effective in the treatment of patients with multidrug-resistant tuberculosis (MDR-TB) in countries in the region.\textsuperscript{19,20} The in vitro phenotypic testing was repeated on numerous occasions using different batches of E-test strips, with similar results. Further independent studies using the E-test for susceptibility testing of ofloxacin in MTB isolates is recommended.

This present study emphasizes the need for detailed genotypic analysis of "local" clinical isolates before strategies for rapid susceptibility determination are used, because mutations at a gene target may differ from one geographic region to another. The authors' previous report on genotypic analysis of isoniazid resistance highlighted differences in the prevalence of specific genotypes between different geographic regions.\textsuperscript{11} In that study, the targeted approach of analysis of the codon 315 region of the \textit{katG} gene and the promoter regions of \textit{inhA} and \textit{oxyR-ahpC} detected mutations in only 62.5% of isoniazid-resistant strains from Singapore, in contrast to 87% in Spain.\textsuperscript{21} A similar situation was also observed here for PZA. Finally, we report discrepancy between the commercially available phenotypic E-test and genotypic analysis of ofloxacin resistance using automated sequencing.

REFERENCES


