An overview of the phenotypic and genotypic characteristics of multidrug-resistant \textit{Mycobacterium tuberculosis} isolates from four Asian countries

Due to the HIV epidemic and the emergence of multidrug-resistant (MDR) strains, tuberculosis (TB) poses a public health threat globally after its initial decline decades ago. In Asia, the majority of TB patients live in highly populated countries such as India, China, Indonesia, the Philippines, Vietnam, Myanmar and Cambodia. In the year 2004, the World Health Organization estimated the TB incidence per 100 000 of the population as 103 in Malaysia, 176 in Vietnam, 245 in Indonesia and 293 in the Philippines. Vietnam has the highest percentage (2.3 \%) of drug-resistant cases as compared to the Philippines and Indonesia (1.5 \%) and Malaysia (0.1 \%) (WHO, 2006). Mortality is highest in the Philippines, with 48 deaths per 100 000 of the population per annum, and co-infection of TB in HIV patients is becoming a growing concern in this part of the world (Iyawoo, 2004; WHO, 2006).

Timely diagnosis of both TB and MDR-TB is important for containing the disease. Diagnostic delay and improper treatment may lead to acquired drug resistance and increased transmission events as observed in Indonesia and elsewhere in the world (Anonymous, 1998; Streicher \textit{et al.}, 2004). The conventional method of drug susceptibility testing (DST) is time-consuming, and standardization of the test for certain drugs poses a problem due to instability of the drugs in different culture media (Zanetti \textit{et al.}, 1997). Various liquid culture-based systems for assessment of drug resistance, such as the BACTEC 460 TB, MGIT 960 (Becton Dickinson), MB/BACT System (bioMérieux), MB Redox (Biotest AG) and ESP Culture System II (AccuMed International), reduce the turnaround time to 1–2 weeks. However, these methods impose a considerable high running cost. It is now known that drug resistance in \textit{Mycobacterium tuberculosis} is due to mutations in genes of the bacterium associated with specific drugs and that drug-sensitive isolates lack these corresponding gene mutations (Johnson \textit{et al.}, 2006). This observation forms the basis for molecular detection of drug resistance by a variety of techniques. By using these methods, it has been shown that more than 90 \% of rifampicin-resistant clinical isolates have mutations in the \textit{rpoB} gene (van Rie \textit{et al.}, 2001). In addition, other fingerprinting methods, such as IS6110 RFLP and spoligotyping, have been used extensively to understand the strain dynamics in a given population (Kamerbeek \textit{et al.}, 1997; Streicher \textit{et al.}, 2004; van Embden \textit{et al.}, 1993; Victor \textit{et al.}, 1999).

This multicentre study focused on Indonesia, Malaysia, the Philippines and Vietnam, which have a high incidence of TB and where little is known about the variation in drug-resistant strains of \textit{M. tuberculosis}. Standardized phenotypic and genotypic methods were used to give an overview of the characteristics of MDR isolates.

A total of 103 MDR \textit{M. tuberculosis} isolates (resistant to at least rifampicin and isoniazid) originating from these four Asian countries, comprising 51, 22, 18 and 12 from the Philippines, Vietnam, Indonesia and Malaysia, respectively, were included in this study. These isolates were chosen from culture collections of each of the participating countries, dating from 1996 to 2006. The isolates originated from government state-owned hospitals and private clinics of each of the countries. \textit{M. tuberculosis} identification was confirmed by using the niacin and nitrate strips method (Indonesia, the Philippines and Vietnam) or the AccuProbe Culture Confirmation kit (GenProbe) (Malaysia). Phenotypic DST was carried out by the LJ proportional method (Indonesia and Vietnam), the LJ absolute concentration method (Malaysia) and by the disc submerged proportion method using Middlebrook 7H10 agar (the Philippines). The following critical concentrations were used: 0.2 \mu g and 1.0 \mu g isoniazid ml\(^{-1}\), 1.0 \mu g rifampicin ml\(^{-1}\), 5.0 \mu g ethambutol ml\(^{-1}\) and 2.0 \mu g streptomycin ml\(^{-1}\). DNA templates for genotyping were prepared from scraped colonies in 400 \mu l water and boiled for 30 min. Drug-resistance genotyping was done by a probe-based method (Victor \textit{et al.}, 1999) and direct DNA sequencing, whereas spoligotyping (Kamerbeek \textit{et al.}, 1997) was used for strain differentiation.

About one-third of the isolates (31.1 \%) belonged to the Beijing strain family (Table 1). This finding was expected since it is known that the Beijing strain family dominates in Asian countries (Brudey \textit{et al.}, 2006). At the same time, the predominance of the EAI family (39.8 \%; 41/103) was evident in this study and most of these isolates (29 out of the 41) came from the Philippines (data not shown). The LAM family was present in the three countries except Vietnam (data not shown). Three of the isolates belonged to the Haarlem family, which is less-commonly seen in Asia (Brudey \textit{et al.}, 2006). Other known strain families that were less represented in this study included MANU, T and U. There were 15 isolates that could not be classified according to the international SpolDB4 database (Brudey \textit{et al.}, 2006).

Mutations in the cluster 1 region of the \textit{rpoB} gene that are commonly associated with rifampicin resistance were found in 91.3 \% (94/103) of the isolates. The majority of the isolates had mutations at codons 531 (53.4 \%), 526 (25.2 \%) and 516 (4.9 \%). Two of the isolates had a double mutation (codons 511 and 512 and codons 531 and 516). Less-frequent mutations were identified at codons 513 and 522. Isoniazid-resistance-related mutations in the \textit{katG} and \textit{inhA} genes were detected in 77 (74.8 \%) of the MDR isolates: 75 isolates had mutations in the \textit{katG} gene.
Table 1. Specific mutations to rifampicin and isoniazid

<table>
<thead>
<tr>
<th>Genotype family designation</th>
<th>Total number of strains (%)</th>
<th>Rifampicin [n=94 (91.3%)]</th>
<th>Number of strains with resistance mutations</th>
<th>Isoniazid [n=77 (74.8%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rpoB 531</td>
<td>rpoB 526</td>
<td>rpoB 516</td>
</tr>
<tr>
<td>EAI</td>
<td>41 (39.8)</td>
<td>26</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Beijing</td>
<td>32 (31.1)</td>
<td>14</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>U</td>
<td>5 (4.9)</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>LAM</td>
<td>4 (3.9)</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Haarlem</td>
<td>3 (2.9)</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T</td>
<td>2 (1.9)</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Manu2</td>
<td>1 (1.0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unclassified</td>
<td>15 (14.6)</td>
<td>9</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>103</td>
<td>55 (53.4%)</td>
<td>26 (25.2%)</td>
<td>5 (4.9%)</td>
</tr>
</tbody>
</table>

 codon 315 and only 2 isolates had mutations in the inhA promoter region. There was no correlation between mutation pattern and spoligotype in this study. Further studies need to be done in each of these countries to provide an in-depth characterization of MDR-TB. The correlation between drug resistance gene mutations and phenotypic resistance is congruent with what has been published (van Rie et al., 2001). The rpoB gene, specifically the rifampicin resistance-determining region, remains a good marker for rapid diagnosis of MDR-TB and can be incorporated into the current MDR-TB detection strategies in TB control.

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