Discrimination of Multidrug-Resistant *Mycobacterium tuberculosis* IS6110 Fingerprint Subclusters by *rpoB* Gene Mutation Analysis

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The resurgence of tuberculosis was exacerbated by an alarming emergence of *Mycobacterium tuberculosis* strains resistant to one or more antimicrobial agents, particularly among patients infected with human immunodeficiency virus.

In recent years, several powerful molecular strategies that allow rapid and accurate bacterial strain characterization for molecular epidemiology have been formulated. The typing methods most successful in describing the genetic diversity of *M. tuberculosis* complex are based on the detection and distribution of specific repetitive DNA sequences in the bacterial genome (13). IS6110 restriction fragment length polymorphism (RFLP) analysis has been found to be particularly useful for *M. tuberculosis* isolate differentiation. In general, most of the strains carry 10 to 15 copies of IS6110 randomly distributed along the chromosome. This standard DNA fingerprinting technique has been largely used in epidemiological studies (9, 15).

Patients infected by strains with identical RFLP fingerprints are usually considered to be epidemiologically related (15). However, it has been observed that IS6110-based RFLP analysis may distort our estimate of the recentness of transmission by grouping strains that are distantly related in time with fingerprints that remain identical for an enduring period (14). The epidemiological relatedness of strains is also assumed if their fingerprints differ in one or two IS6110 fragments. These differences could be due to an IS6110 transposition to other sites in the chromosome. Chevrel-Dellagi et al. (3), analyzing independent colonies from five isolates, have shown that small variations in RFLP patterns can arise during the strains’ cultivation. Moreover, Yeh et al. (16) found changes in DNA fingerprinting in 29% of sequential isolates, separated by at least 90 days. In spite of this high level of instability, meaning that the requirement for identical matching may underestimate the frequency of epidemiological links among patients, they suggest that strains with identical genotypes are likely to be epidemiologically linked. Similar observations were previously made (4) in an analysis of *M. tuberculosis* strains isolated from close contacts or in the context of outbreaks. Because of the RFLP fingerprinting instability, some authors assert the requirement of a second genetic marker to confirm epidemiological links (2, 7, 14).

Nevertheless, in other studies, where sequential genotypes of *M. tuberculosis* isolates were analyzed, a very low percentage or no change in RFLP patterns was found (1, 13).

In a previous study, we characterized by RFLP-IS6110 analysis (12) 43 multidrug-resistant (MDR) *M. tuberculosis* strains recovered from patients in Lisbon hospital units (6). Based on the RFLP results, three clusters of MDR strains were identified. A cluster was defined as two or more *M. tuberculosis* strains exhibiting identical molecular weight IS6110 fragments. RFLP fingerprints differing in one or two additional IS6110 fragments were also considered. The largest cluster, cluster A, includes 72% of the MDR tuberculosis (MDR-TB) strains. A retrospective epidemiological study was conducted with 27 patients with cluster A strains and revealed epidemiological links in 24 cases, suggesting the occurrence of a large outbreak (6). In this cluster, RFLP patterns either matched or differed by one or two IS6110 fragments. According to the number of IS6110 fragments (11, 12 or 13) cluster A was subdivided into three subclusters (Fig. 1).

MDR-TB strains were defined as strains resistant to at least rifampin (RIF) and isoniazid (INH). All isolates were tested for their susceptibilities to INH, RIF, streptomycin (STP), and ethambutol (ETH) by using the BACTEC 460 TB SIRE methodology (Becton Dickinson, Microbiology Systems, Cockeysville, Md.). Three antibiotic resistance patterns were found: resistance to (i) RIF, INH, and STP, (ii) RIF, INH, STP, and ETH, and (iii) RIF, INH, and ETH.

Recently, 56 more MDR *M. tuberculosis* strains, isolated from the same number of patients, were typed by RFLP-IS6110 analysis. An additional cluster was identified. Seventy-five of the 99 strains studied so far belong to cluster A and are spread throughout several Portuguese geographic regions. In this cluster, 37 strains were found to belong to subcluster A1, 22 to subcluster A2, and 16 to subcluster A3. Subcluster A1 contained isolates from five different hospital units, but 25 strains came from hospital A, a prison hospital that receives prisoners from the entire country. Strains from subcluster A2 were isolated in six different hospital units, but the majority of the patients had been admitted to hospital M. By tracking patients across health institutions we found epidemiological links among patients from hospital A (Table 1). Patients with strains in subcluster A2 were mainly found in hospital M.
The alarming increase in the number and scattering of a particular cluster of strains (cluster A) in several Portuguese regions suggested the occurrence of an outbreak of enormous proportions. In order to know whether or not these strains with similar but nonidentical fingerprints belonged to a single outbreak, we investigated the small variations in the RFLP fingerprints of cluster A strains. Indeed, these differences could point to different strains and, consequently, different outbreaks. Because IS6110 is the most frequently used genetic marker for *M. tuberculosis* strain differentiation, it is of critical importance to know whether strains with similar fingerprints belong to a single outbreak but were recently disseminated in a clonal way. To approach this problem and considering that all the MDR strains were resistant to RIF, sequencing of the *rpoB* gene was used as a second genetic marker.

The molecular basis of RIF resistance in *M. tuberculosis* was identified (11), and it was ascribed to 15 distinct codon mutations in a 69-bp region of the *rpoB* gene. In this study we sequenced and checked for RIF resistance mutations in the *rpoB* genes of 45 randomly chosen MDR *M. tuberculosis* strains representing each of the three identified subclusters (A1, A2, and A3).

The 45 strains were isolated in 11 different hospitals of Lisbon. Twenty-four strains belong to subcluster A1, 13 to subcluster A2, and 8 to subcluster A3. These strains can be divided into two antibiotypes. Twenty-two strains from subcluster A1, three strains from subcluster A2, and six strains from subcluster A3 were resistant to INH, RIF, and STP. All remaining strains in this selected group were resistant to INH, RIF, STP, and ETH.

*rpoB* was obtained by PCR directly from genomic DNA of *M. tuberculosis* strains. Genomic DNA extraction was performed as described by van Embden et al. (12). A 350-bp fragment of *rpoB* (GenBank accession no. L27989) was amplified by PCR with the following synthetic oligonucleotide primers: forward, RPOB-1 (5’-GGGAGGGGATGACCACCC-3’).
TABLE 2. \textit{rpoB} mutations found in MDR-TB strains

<table>
<thead>
<tr>
<th>Subcluster</th>
<th>\textit{rpoB} mutation(s) detected</th>
<th>No. of strains analyzed</th>
</tr>
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<tbody>
<tr>
<td>A1</td>
<td>Asp\textsubscript{516} \rightarrow Val</td>
<td>24</td>
</tr>
<tr>
<td>A2</td>
<td>Ser\textsubscript{531} \rightarrow Leu</td>
<td>13</td>
</tr>
<tr>
<td>A3</td>
<td>Ser\textsubscript{531} \rightarrow Leu, His\textsubscript{526} \rightarrow Asp, Leu\textsubscript{533} \rightarrow Pro</td>
<td>8</td>
</tr>
</tbody>
</table>

3'); and reverse, RPOB-2 (5'-GCGGTACGGCGTTTCGAT GAAC-3'). The DNA was amplified in a PCR mixture containing 50 ng of template DNA, 20 pmol of each primer, 2 U of \textit{Taq} polymerase (Gibco-BRL, Grand Island, N.Y.), 10 mM Tris-HCl (pH 8.4), 2 mM MgCl\textsubscript{2}, and 200 \mu M concentrations of deoxynucleoside triphosphates. After a denaturation at 94°C for 4 min, the reaction mixture was subjected to 30 cycles of amplification (denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 15 min). The amplified DNA was separated in a low-melting-point agarose gel (2%) and purified with the QIAquick gel extraction kit (Qiagen).

Sequencing was performed by using the RPOB-1 and RPOB-2 forward and reverse sequencing primers to initiate the dideoxy-mediated chain-termination reaction (8).

Forty-four of the 45 RIF-resistant strains analyzed revealed point mutations in the \textit{rpoB} gene region. We did not find insertions or deletions in the DNA fragments studied. We found in codon 516 of all 24 strains in subcluster A1 a change from Asp (GAC) to Val (GTC) (Table 2). The 13 strains of subcluster A2 all exhibited, in codon 533, a change from Ser (TCG) to Leu (TTG). In the eight strains of subcluster A3, four mutation types were detected: a change from Ser (TCG) to Leu (TTG) in codon 531, a change from His (CAC) to Asp (GAC) and a change from His (CAC) to Arg (CGG) in codon 526, and a change from Leu (CTG) to Pro (CCG) in codon 533. In this subcluster we found one strain without any mutation within the sequenced \textit{rpoB} gene region.

We examined the effects of the mutations in the \textit{rpoB} gene on the MICs of rifampin (data not shown) for the MDR strains. Our results are in agreement with those of other authors (5, 10) and showed that for strains exhibiting mutations in codons 516, 526, and 531 rifampin MICs are \( \geq 64 \) \( \mu \)g/ml.

These results show that \textit{rpoB} sequencing analysis can subdivide an IS6110-RFLP-based \textit{M. tuberculosis} cluster of strains into distinct subclusters. Indeed, all the subcluster A1 strains exhibit the same \textit{rpoB} mutation, which is different from the RIF resistance mutation detected in all subcluster A2 strains. These mutational differences in A1 and A2 subclusters suggest the occurrence of two independent outbreaks. As strains from subclusters A1 and A2 were found in different hospital units, not only in Lisbon but also throughout the country (reference 6 and data not shown), there must be epidemiological links between these different units. The results for subcluster A3 may suggest the occurrence of evolutionary divergence at the clonal level.

Some studies support the use of RFLP analysis for tracking transmission, considering it appropriate to include one-band differences in IS6110 genotype-defined clusters. Nevertheless, our results demonstrate that DNA fingerprinting based merely on IS6110 is sometimes insufficient and stress the importance of using additional genetic markers for the unambiguous confirmation of the identities of strains. In this study we were able to differentiate what was supposed to be a large outbreak into at least two different outbreaks in two hospital units, although the strains are genetically related. Further studies that correlate epidemiological data with the fingerprint data are necessary to confirm the epidemiological links.

REFERENCES