**Mycobacterium tuberculosis** isolates belonging to *katG gyrA* group 2 are associated with clustered cases of tuberculosis in Italian patients

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Fifty-one consecutive isolates of *Mycobacterium tuberculosis*, collected during a 2-year period in the north-east of Italy, were subjected to IS6110-RFLP analysis to detect the presence of clusters and assigned to one of the three genotypic groups delineated by single nucleotide polymorphisms in the genes *katG* and *gyrA*. All the isolates collected from the local population belonged to group 2 or 3, while group 1 isolates were found only in specimens collected from African immigrants. Clustered cases of tuberculosis, which are likely to be related to recently transmitted infection, were found to be significantly associated with *katG gyrA* group 2. In the local situation, strains belonging to this group may therefore present a higher risk of transmission.

**Introduction**

The recent development of reliable methods for molecular typing of *Mycobacterium tuberculosis* has provided powerful tools to unravel the epidemiology of tuberculosis, which is still a leading cause of morbidity and mortality all over the world. Molecular techniques can be used, among other things, to identify or confirm person-to-person transmission and to discriminate exogenous versus endogenous disease (van Soolingen, 2001). They have also been instrumental in demonstrating the successful spread of some strains, such as those belonging to the W-Beijing family (Bifani et al., 2002). A related potential use of molecular methods, still to be fully exploited, involves the identification of strains with increased virulence, for example, higher transmissibility, which would allow early recognition and improved containment of these strains. Sreevatsan et al. (1997) demonstrated that *M. tuberculosis* isolates can be divided into three genotypic groups, on the basis of single nucleotide polymorphisms at codon 463 of the *katG* gene and at codon 95 of the *gyrA* gene. These authors found that isolates belonging to groups 1 and 2 were more frequently associated with clustered cases of tuberculosis than isolates belonging to group 3. As clustering is considered a marker for transmission, the results were suggestive of a higher virulence of group 1 and group 2 organisms. However, a subsequent study (Rhee et al., 1999), which considered a more complex set of virulence characters, could not demonstrate a correlation between a specific genotypic group and virulence. The latter study also suggested that this correlation may depend on the composition of the population in which it is studied (Rhee et al., 1999).

In this study, we used DNA fingerprinting to detect the presence of clusters in a collection of *M. tuberculosis* isolates obtained from patients of an Italian region. The isolates were also characterized with respect to the three genotypic groups delineated by Sreevatsan et al. (1997), to investigate the possibility that strains belonging to specific genotypic group(s) may be more prone to form clusters.

**Methods**

**Isolates.** Fifty-one consecutive isolates of *M. tuberculosis* were collected in the Trieste district, in the north-east of Italy. They represented virtually all *M. tuberculosis* isolates recovered from patients in this district during 1999 and 2000. When multiple isolates were collected from the same patient, only the first one was included in the study. All but three isolates were derived from pulmonary infections. Three patients were HIV-positive. All isolates were studied without preliminary knowledge of possible epidemiological relationships among them.

**DNA fingerprinting.** All isolates were typed by IS6110-RFLP, according to the standardized procedure for *M. tuberculosis* (van Embden et al., 1993). Briefly, genomic DNA was digested with *Pvu*II, separated by agarose gel electrophoresis and subjected to Southern hybridization. The probe was obtained by amplification of a fragment of the insertion sequence IS6110 with primers INS-1 and INS-2 (van Embden et al., 1993), using genomic DNA from strain H37Rv (Bifani et al., 2000) as a template. The probe was labelled by incorporation of digoxigenin-11-dUTP during amplification (Lion & Haas, 1990). IS6110-RFLP fingerprints were analysed by using the GELCOMPAR II software (Applied Maths), using the Dice coefficient (Dice, 1945) to evaluate similarity. Two or more isolates were assigned to the same cluster when showing identical IS6110-RFLP profiles. Double-repetitive-element (DRE)-PCR (Friedman et al., 1995) was used as a secondary typing method to

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confirm the composition of the clusters obtained by IS6110-RFLP. DRE-PCR patterns were analysed visually.

Assignment of isolates to one of the three genotypic groups.

Isolates of *M. tuberculosis* were assigned to one of the three groups delineated by Sreevatsan *et al.* (1997) on the basis of the combination of polymorphisms of the *katG* and *gyrA* genes (Fig. 1). Polymorphism at codon 463 of the *katG* gene was evaluated by PCR amplification of the relevant DNA fragment with *katG* primers (Rhee *et al*., 1999) followed by digestion with *MspI*. In the presence of the CGG variant of codon 463, a *MspI* recognition site is formed, so the two alleles are easily differentiated by their restriction patterns (Sreevatsan *et al*., 1997) (Fig. 1).

Polymorphism at codon 95 of the *gyrA* gene was detected by PCR amplification of a 229 bp DNA fragment with primers *gyrA* forward (Rhee *et al*., 1999) and *gyrA* reverse (5’-AGCATCTCCATCGCAAGCAGC-3’, this study). All amplicons were tested with digoxigenin-labelled probes specific for the two allelic variants, i.e. GYR-C (5’-TCTACGACACCTGACG-3’) and GYR-G (5’-TCTACGAGCCTGTCGGC-3’). Probe sequences are those described by Rhee *et al*., deprived of the arms. Amplicons were spotted onto a nylon membrane and hybridized with either probe for 3 h (Fig. 1). Hybridization temperatures of 45 °C for GYR-C and 50 °C for GYR-G were selected on the basis of preliminary experiments. Probe labelling by 3’-tailing and hybrid detection were carried out as described in the manufacturer’s manual (*The DIG System User’s Guide for Filter Hybridization*, Boehringer Mannheim).

**Results and Discussion**

**IS6110-RFLP typing**

Results of DNA fingerprinting by IS6110-RFLP are shown in Fig. 2. Forty-two different patterns were identified among the 51 isolates tested. Copy number of IS6110 ranged between five and 15 copies per isolate, with the exception of one isolate, which had only two copies of IS6110.

Thirty-six of 51 isolates (70%) showed a unique pattern, while the remaining 15 isolates could be divided into six clusters (named A–F), which included two to four isolates each. Double-repetitive-element (DRE-PCR) (Friedman *et al*., 1995) analysis was in agreement with the above results, and on no occasion could it discriminate among the isolates included in a cluster by IS6110-RFLP analysis (data not shown); therefore, the dendrogram in Fig. 2 was considered to depict the final subdivision in clusters of the isolates of the study.

Based on the fingerprinting data, approximately 18% of all tuberculosis cases could be attributed to recent transmission. This crude proportion, which was calculated by the method described by Small *et al.* (1994), is in overall agreement with a previous study performed on strains collected in southern

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**Fig. 1.** Polymorphism analysis of the *katG* and *gyrA* genes. The figure shows typical results obtained with three representative isolates. (A) Polymorphism at codon 463 of the *katG* gene. Amplicons (204 bp) from the *katG* gene were digested with *MspI* and the resulting fragments were separated by agarose gel electrophoresis. The CGG variant of codon 463 originates an additional *MspI* recognition site. (C) Polymorphism at codon 95 of the *gyrA* gene. Amplicons (229 bp) from the *gyrA* gene were spotted onto a nylon membrane and hybridized with either probe GYR-C or probe GYR-G. (B) Isolates were assigned to one of the three groups by combining the results of A and C.
Italy (Nastasi & Mammina, 1999), while it is lower than the one found by Moro et al. (2002) in the area of Milan. In the latter case, 28.1% of the tuberculosis cases were attributed to recent transmission. However, the studied population included a higher percentage of AIDS patients.

**Drug susceptibility of the clustered strains**

Clustered isolates were in most cases susceptible to all the drugs tested (Table 1). In the few cases in which antibiotic resistances were detected, these were generally shared by all members of a cluster, as in the case of the resistance to streptomycin in clusters A and D and of the resistance to isoniazide in cluster A (Table 1). Therefore, all isolates within a cluster had identical antibiograms, with the exception of isolate MT38, which differed from the other three components of cluster A in that it was resistant to rifampicin and rifabutin. However, this isolate was collected from a chronic patient and it may have developed resistances over time. The overall concordance of susceptibility patterns among isolates clustered in the same fingerprinting group is in accordance with the hypothesis that isolates within a group derive from the same chain of transmission.

**Characteristics of clustered patients**

Epidemiological relationships among clustered patients could not be investigated systematically, but in two cases a clear relationship was recognized: two of the three patients in cluster F were brothers and both patients of cluster C were Senegalese immigrants living in the same building.

It is commonly believed that younger individuals affected by tuberculosis should exhibit a higher ratio of recent infection to reactivation with respect to older patients (Vynnycky et al., 2001). In this study, the mean age of the clustered patients was considerably lower than the mean age of non-clustered ones (Table 2), in accordance with the hypothesis that clustered patients have a recently transmitted *M. tuberculosis* infection.

### Table 1. Antibiotic susceptibility of strains within clusters

All isolates of clusters B, C, E and F were sensitive to all five antibiotics tested (cluster B –isolates MT15 and MT16; cluster C –isolates MT22 and MT31; cluster E –isolates MT9 and MT39; cluster F –isolates MT1, MT33 and MT45).

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Isolate</th>
<th>SM</th>
<th>INH</th>
<th>RFP</th>
<th>EMB</th>
<th>RIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>MT38</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>MT41</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>MT49</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>MT48</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>D</td>
<td>MT4</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>MT17</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

*SM, Streptomycin; INH, isoniazid; RFP, rifampicin; EMB, ethambutol; RIF, rifabutin; R, resistant; S, sensitive.

### Table 2. Age distribution of patients

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Mean age in years (SD)</th>
<th>Median*</th>
<th>Range*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clustered patients</td>
<td>43.2 (13.0)</td>
<td>41</td>
<td>23–66</td>
</tr>
<tr>
<td>Non-clustered patients</td>
<td>62.8 (15.6)</td>
<td>66</td>
<td>20–88</td>
</tr>
</tbody>
</table>
Table 3. Distribution of the isolates among the three genotypic groups delineated by single nucleotide polymorphisms in katG and gyrA

<table>
<thead>
<tr>
<th>Isolate information</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>katG 463 CTG</td>
<td>katG 463 CGG</td>
<td>katG 463 CGG</td>
</tr>
<tr>
<td></td>
<td>gyrA 95 ACC</td>
<td>gyrA 95 ACC</td>
<td>gyrA 95 AGC</td>
</tr>
<tr>
<td>Clustered isolates*</td>
<td>2</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Sporadic isolates</td>
<td>0</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Number of isolates</td>
<td>2 (4%)</td>
<td>31 (61%)</td>
<td>18 (35%)</td>
</tr>
<tr>
<td>in the group (%) of</td>
<td>total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Clustered isolates are significantly (P < 0.001, $\chi^2$ test with Yates correction) associated with katG gyrA group 2.

Distribution of the isolates among the three genotypic groups delineated by polymorphisms of the katG and gyrA genes

All isolates could be classified unambiguously in one of the three groups delineated by the polymorphisms in the katG and gyrA genes (Sreevatsan et al., 1997). Typical results are shown in Fig. 1.

The majority of the isolates belonged to group 2 and group 3 (Table 3). Only two isolates were found to belong to group 1. Both of them were collected from the above-mentioned Senegalese immigrants who had recently arrived in Italy. The two isolates that made up cluster C were considered as imported from abroad. Overall, the results indicate that M. tuberculosis strains belonging to katG gyrA group 2 and group 3 are predominant among people living in the Trieste district, which is located in the north-east of Italy. However, group 1 strains, although detectable in foreign individuals, may be rare or even absent in the indigenous population. To our knowledge, this is the first study describing the distribution of Italian M. tuberculosis isolates among the three groups delineated by the katG gyrA polymorphisms (Sreevatsan et al., 1997). This distribution is not likely to be the same in different geographical areas and/or populations. For example, Rhee et al. (1999) found that group 1 strains are frequently associated with Asian individuals.

Noteworthy, with the exception of the isolates from Senegalese immigrants, all isolates included in IS6110-RFLP-based clusters belonged to group 2 (Table 3) and the association between clustered strains and group 2 was statistically significant (Table 3). No clustered isolates were found to belong to group 3, while the number of group 1 strains was too few to allow any consideration.

Our results are in contrast with those obtained by Rhee et al. (1999), who found that strains of the three genotypes did not differ for the rate of IS6110-based clustering. Moreover, as clustered cases are considered to be related to recent transmission (Vynnycky et al., 2001), our data support the hypothesis of a higher transmissibility of group 2 with respect to group 3 strains, as suggested previously by Sreevatsan et al. (1997).

The results reported in this study are based on a limited number of isolates and can be considered representative of the local situation, as all strains isolated in the district during a two-year period were included in the study. In this particular study, strains belonging to katG gyrA group 2 may present a higher risk of transmission with respect to strains belonging to group 3. Further studies are required to clarify if this observation can be extended to other Italian or European regions.

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References


