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Molecular Typing of *Mycobacterium tuberculosis* by Mycobacterial Interspersed Repetitive Unit–Variable-Number Tandem Repeat Analysis, a More Accurate Method for Identifying Epidemiological Links between Patients with Tuberculosis

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IS6110 fingerprinting of *Mycobacterium tuberculosis* is the standard identification method in studies on transmission of tuberculosis. However, intensive epidemiological investigation may fail to confirm transmission links between patients clustered by IS6110-restriction fragment length polymorphism (RFLP) typing. We applied typing based on variable numbers of tandem repeats (VNTRs) of mycobacterial interspersed repetitive units (MIRUs) to isolates from 125 patients in 42 IS6110 clusters, for which thorough epidemiological data were available, to investigate the potential of this method in distinguishing epidemiologically linked from nonlinked patients. Of seven IS6110 clusters without epidemiological links, five were split by MIRU-VNTR typing, while nearly all IS6110 clusters with proven or likely links displayed conserved MIRU-VNTR types. These results provide molecular evidence that not all clusters determined on the basis of multibanded IS6110 RFLP patterns necessarily reflect transmission of tuberculosis. They support the use of MIRU-VNTR typing as a more reliable and faster method for transmission analysis.

IS6110 restriction fragment length polymorphism (RFLP) typing of *Mycobacterium tuberculosis* has been used extensively in studies on tuberculosis transmission and is one of the most widely applied and standardized molecular typing methods (1, 6, 10, 31, 34, 35). *M. tuberculosis* isolates from epidemiologically linked patients generally show identical IS6110 RFLP patterns, thus comprising transmission clusters. Consequently, the finding that a substantial proportion of tuberculosis cases in industrialized countries is clustered by DNA fingerprinting is interpreted as a reflection of a high rate of recent transmission (2, 5, 8, 14, 22, 27, 28, 32, 39). However, IS6110-based RFLP fingerprints are not always reliable indicators of epidemiological linkage between tuberculosis patients (7, 33). Even the most meticulous analysis of all available data on possible contacts between clustered patients does not reveal epidemiological links in all cases (33). Furthermore, because in many settings tuberculosis often results from casual contacts, the majority of the links can be identified only after combining the genotyping of the *M. tuberculosis* isolates with intensive epidemiologic investigation. In addition, opportunities for early and thus more-efficient prevention and intervention are limited by the fact IS6110 RFLP typing is labor intensive and requires weeks for culturing *M. tuberculosis*.

Typing methods based on mycobacterial interspersed repetitive unit (MIRU)-VNTR analysis offer a potential solution to the drawbacks faced using IS6110 RFLP typing. MIRU-VNTR analysis determines the number of tandem repeats at multiple independent loci (12, 29). This PCR-based method is highly reproducible and much faster than IS6110-RFLP typing and displays a discriminatory power close to that of IS6110-RFLP, especially in low-incidence areas (9, 15, 18, 23, 25, 30). Previous studies have demonstrated the ability of MIRU-VNTR typing to split certain IS6110 clusters, suggesting that the use of IS6110 alone may overestimate the existence of transmission clusters (4, 9, 13, 15, 19, 21, 23). Therefore, MIRU-VNTR typing has been proposed as an efficient first-line typing method, to be followed by IS6110-RFLP subtyping of the resulting MIRU-VNTR clusters (9, 25). However, all but one of these observations were made on the basis of *M. tuberculosis* isolates harboring few copies of IS6110, for which IS6110 RFLP typing is known to have limited discriminatory power. Furthermore, epidemiological information on the clustered cases was limited.

To further evaluate the usefulness of MIRU-VNTR typing for conducting population-based studies of recent transmission, we applied secondary MIRU-VNTR typing and spoligotyping (17) to isolates from patients in IS6110 RFLP clusters selected from a previous population-based study, for whom extensive and well-
structured epidemiological data were available (33). Our aim was to determine in a representative manner the potential of MIRU-VNTR typing to distinguish epidemiologically linked patients from unlinked patients in IS610 RFLP clusters.

MATERIALS AND METHODS

Study population. In a prospective, population-based study conducted in the province of North Holland, The Netherlands (population, 2,500,000), we previously investigated tuberculous cases for which DNA fingerprinting clustering was an indication of epidemiological linkage and therefore of recent transmission (33). Furthermore, we determined which of these patients could have been detected earlier by contact tracing. The study included all tuberculosis patients residing in the study area and reported to the Municipal Health Services, as is mandatory in The Netherlands. Of 664 patients included from 1 July 1998 to 1 July 2000, 485 (73%) had a positive M. tuberculosis culture. Patients who were part of an IS610 cluster with one or more other patients from North Holland and diagnosed within the 2-year period preceding or following such patients' diagnosis (n = 155) were assigned to transmission groups (TG) according to the likelihood of epidemiological linkage between patients. The assignments were based on the results of extensive interviews of the patients before and after the results of RFLP typing became available, combined with sociodemographic and clinical data.

For the present study, the isolates obtained from 114 of the 155 patients in IS610 clusters were subtyped using MIRU-VNTR. Isolates to be subtyped were chosen based on the availability of DNA at the mycobacteria laboratory at the Diagnostic Laboratory for Infectious Diseases and Perinatal Screening of the National Institute of Public Health and the Environment; the 114 patients did not differ significantly from the other 41 patients with respect to age, sex, or Dutch versus non-Dutch ethnicity and nationality (t test and χ² test; P > 0.05).

In addition, isolates from 11 patients from outside the above-described study period were included when a cluster was represented by only a single isolate within the study period. In the resulting sample of 125, assignments into the various transmission groups differed slightly between this study and the previous one because of the inclusion of these 11 isolates. The number of isolates subtyped by MIRU-VNTR per TG and the description of the epidemiological link belonging to each TG are depicted in Table 1.

TABLE 1. Characteristics of the TG classified in this study

<table>
<thead>
<tr>
<th>TG</th>
<th>No. of patients</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44</td>
<td>Patients with a clear epidemiological link, confirmed by IS610-PGRS typing</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>Patients with links as clear as those in TG1, confirmed by IS610-PGRS typing, who should have been, but were not, detected by contact tracing</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>Patients with an epidemiological link which was initially unclear but became likely after IS610-PGRS typing and the second interview</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>Patients for whom meticulous analysis of all available data ruled out an epidemiological link but whose IS610-PGRS typing results suggested that they belonged to a certain cluster</td>
</tr>
</tbody>
</table>

RESULTS

The results of secondary MIRU-VNTR typing of the M. tuberculosis isolates from 125 patients, previously assigned to 42 IS610 RFLP clusters, are shown in Fig. 1 and Table 3.

Among the 24 IS610-PGRS clusters comprising 57 patients with a proven epidemiological link (TG1 and TG2), only one (cluster 8), including two patients, was subdivided by MIRU-VNTR typing. Of the 23 IS610-PGRS clusters comprising 54 patients with a likely epidemiological link (TG3), three (clusters 23, 32, and 34) were subdivided by MIRU-VNTR typing. In clusters 23 and 34, one isolate was distinguished from four and two other isolates, respectively, while cluster 32 included two isolates subdivided by MIRU-VNTR typing. In all cases where IS610-PGRS clusters of TG1, TG2, or TG3 were split by MIRU-VNTR typing, the differences were minor and always concerned only one MIRU locus.

In contrast, of the seven IS610 clusters without established epidemiological links (TG4), five (71.4%) were split by MIRU-VNTR typing. This difference in the proportion of subdivided clusters is highly significant (TG4 versus TG1, TG2, and TG3, P = 0.00097 [Fishers’ exact test]). MIRU-VNTR typing identified a total of 12 genotypes among isolates from 14 patients in these groups.

As an average, two MIRU-VNTR loci changed in each of the five TG4 clusters subdivided by MIRU-VNTR typing. This number was thus higher than the number of changes observed for the TG1, TG2, and TG3 clusters that were split by MIRU-VNTR typing (Fig. 1).

In total, 9 (21%) of the 42 IS610-PGRS RFLP clusters were subdivided by MIRU-VNTR typing. The numbers of IS610 RFLP bands in these isolates ranged from 5 to 15. Conversely, two examples (between clusters 9 and 17 and between cluster 16 and one isolate of cluster 32) were found in which isolates with distinct IS610-PGRS RFLP patterns (59.4 and 64.1% similarity, respectively) exhibited identical VNTR patterns (Fig. 1). These isolates also exhibited distinct spoligotyping patterns. For these isolates the discriminative power of IS610 RFLP typing and spoligotyping was thus higher than that of the MIRU-VNTR typing.

In contrast, all isolates within the same IS610-PGRS cluster exhibited identical spoligotyping patterns, except for isolates in cluster 38. One of the two TG4 isolates in cluster 38 contained one fewer direct repeat spacer than the other isolates of that
IS6110 cluster. This isolate had a MIRU-VNTR type identical to that of the two other isolates within the same IS6110 cluster.

Comparison of the spoligotypes and IS6110 fingerprints detected in this study with those in the international spoligotype and IS6110-RFLP databases revealed that the corresponding isolates belong to a wide variety of genotype families (11, 26). These families were distributed to identical degrees among the different “transmission groups.” All IS6110 clusters, except clusters 1, 15, 32, and 34, displayed IS6110 profiles consisting of six or more bands.

DISCUSSION

Molecular typing using IS6110 has generally been used as the standard method in studies of the transmission of tuberculosis, on the assumption that IS6110 RFLP-based clustering of cases is the result of recent transmission. However, even meticulous analysis of all epidemiological data fails to confirm transmission links among a number of patients within IS6110 clusters, except for IS6110-RFLP clusters in TG1, TG2, and TG3 that were split by MIRU-VNTR typing. In addition, in the few IS6110-PGRS clusters in TG1, TG2, and TG3 that were split by MIRU-VNTR typing, the MIRU-VNTR types differed only by a single MIRU locus, compared to differences in up to three loci for the TG4 clusters.

Failures to detect recent transmission chains indicated by IS6110-PGRS clusters are classically attributed to the limited sensitivity of conventional contact investigation, but our findings suggest that some reflect limitations in the resolution of IS6110-PGRS RFLP. In the present study, MIRU-VNTR typing was found to be the most accurate first-line method to exclude epidemiological links.

In our study, 71.4% (5/7) of the IS6110-PGRS clusters comprising patients for whom an epidemiological link was not detected (TG4) and whose isolates showed RFLP fingerprints with high copy numbers of IS6110 were subdivided by MIRU-VNTR typing. For these patients, meticulous analysis of all data, including the results of a second interview, indicated that they were unlikely to have met during the infectious period of the index patient. Examples include two immigrants from the same country, one with extrapulmonary tuberculosis diagnosed in 1998 after living in The Netherlands for 10 years and the other with infectious pulmonary tuberculosis that was diagnosed at immigration in 2000. Although a common but unidentified source for the two TG4 strains with matching MIRU-VNTRs cannot be ruled out completely, the thorough analysis of our epidemiological data makes this event less likely. The resolution of the other TG4 clusters by MIRU-VNTR typing fully corroborates the absence of a direct epidemiological link indicated by intensive contact investigation.

Conversely, in contrast to TG4, almost all patients in IS6110-PGRS clusters with an evident (TG1 and TG2) or likely (TG3) epidemiological link remained clustered after MIRU-VNTR typing. In addition, in the few IS6110-PGRS clusters in TG1, TG2, and TG3 that were split by MIRU-VNTR typing, the MIRU-VNTR types differed only by a single MIRU locus, compared to differences in up to three loci for the TG4 clusters split by MIRU-VNTR. TG1 and TG2 included relatives or close friends of an infective index patient who were identified by contact tracing (TG1) or who should have been—but were not—identified by that intervention (TG2). The latter group includes persons in close contact with an index patient but not mentioned by him or her, persons not reached for the contact investigation by the Municipal Health Service, and persons not complying with that investigation. Only one IS6110-PGRS TG1 cluster, including two patients, was subdivided by MIRU-VNTR typing out of the 24 clusters comprising a total of 57 patients in TG1 and TG2. This latter observation most likely

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TABLE 2. Conditions for multiplex PCRs of 12 MIRU-VNTR loci

<table>
<thead>
<tr>
<th>Multiplex</th>
<th>Locus</th>
<th>VNTR length (bp)</th>
<th>MgCl2 concn (mM)</th>
<th>PCR primer pairs (5’ to 3’, with labeling indicated)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix A</td>
<td>VNTR 0580</td>
<td>77</td>
<td>3.0</td>
<td>GCAGCAGGAGACCCGAAGTTCAGCG (FAM) 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VNTR 2996</td>
<td>51</td>
<td>3.0</td>
<td>TACCTACGGGCGGCAGTGGCCG (NED) 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VNTR 0802</td>
<td>54</td>
<td>3.0</td>
<td>HCAAGACGTCGTTGAT (VIC) 30</td>
<td></td>
</tr>
<tr>
<td>Mix B</td>
<td>VNTR 0960</td>
<td>53</td>
<td>2.0</td>
<td>ACTTTGACGTTGATGTGCT (FAM) 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VNTR 1644</td>
<td>53</td>
<td>2.0</td>
<td>GCCCTGTCGACGCGCTGTG (VIC) 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VNTR 3192</td>
<td>53</td>
<td>2.0</td>
<td>AGTACAGCTGCTCGTCGTTT (VIC) 30</td>
<td></td>
</tr>
<tr>
<td>Mix F</td>
<td>VNTR 0424</td>
<td>51</td>
<td>1.5</td>
<td>GCAGCAGGAGACCCGAAGTTCAGCG (FAM) 38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VNTR 0577</td>
<td>58</td>
<td>1.5</td>
<td>TACCTACGGGCGGCAGTGGCCG (NED) 38</td>
<td></td>
</tr>
<tr>
<td>Mix G</td>
<td>VNTR 2401</td>
<td>58</td>
<td>3.0</td>
<td>ACTTTGACGTTGATGTGCT (FAM) 38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VNTR 3690</td>
<td>58</td>
<td>3.0</td>
<td>GCCCTGTCGACGCGCTGTG (VIC) 38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VNTR 4156</td>
<td>59</td>
<td>3.0</td>
<td>AGTACAGCTGCTCGTCGTTT (VIC) 38</td>
<td></td>
</tr>
<tr>
<td>Individual</td>
<td>VNTR 1982</td>
<td>78</td>
<td>1.5</td>
<td>GCCAGCAGGAGACCCGAAGTTCAGCG (FAM) 26</td>
<td></td>
</tr>
</tbody>
</table>
FIG. 1. IS6110-PGRS RFLP and MIRU-VNTR genotypes of the *M. tuberculosis* isolates from 125 patients in 42 IS6110-PGRS clusters, as assigned to four transmission groups (for explanation of transmission groups, see Table 1). Designations of MIRU-VNTR loci are given according to the position (in kilobase pairs) on the *M. tuberculosis* H37Rv chromosome. Alias designations are in parentheses. Spoligo, spoligotyping.
reflected a rare and stochastic MIRU-VNTR mutation event, possibly DNA replication or homologous recombination dependent, in clonal populations originating from recent transmission (30). This hypothesis is consistent with the low proportion of changes in MIRU-VNTR profiles observed among serial isolates from chronically infected patients (25).

In TG3, including 54 patients in 23 IS6110-PGRS clusters, an epidemiological link which was initially unclear became likely after IS6110 typing and a second interview. This group includes patients living in the same apartment complex or regularly using the same tram service as an index patient, as well as homeless persons who used housing facilities with an index patient before he or she was diagnosed and thereafter became untraceable. Only 3 of these 54 patients were not matched by identical MIRU-VNTR patterns within their three respective clusters. Notably, while one of these three clusters included isolates with 17 IS6110 copies (cluster 23), the two others (clusters 32 and 34) included isolates with only 5 IS6110 copies. Whereas the MIRU-VNTR difference in cluster 23 also probably reflects a rare MIRU-VNTR variation between clonally transmitted isolates (see above), another explanation may be possible for the differences observed in clusters 32 and 34. As noted earlier, a total of five IS6110 copies is considered the upper limit in defining the low-copy-number isolates. For such isolates, IS6110 RFLP is thought to be of limited specificity for detection of recent transmission even when complemented by spoligotyping and PGRS typing. Therefore, the possibility is not totally excluded that the patients’ whose low-copy-number isolates were distinguished by MIRU-VNTR typing were actually infected by as yet-unidentified contacts, different from the contacts of the other patients of clusters 32 or 34.

A comparison of the overall discriminatory powers of MIRU-VNTR and IS6110-RFLP typing was beyond the scope of this study. It was designed to test the potential of MIRU-VNTR to split epidemiologically unlinked IS6110-PGRS clusters but not the potential of IS6110-PGRS to split epidemiologically linked MIRU-VNTR clusters. It is conceivable that if MIRU-VNTR typing had been used as initial typing method, a part of the MIRU-VNTR clusters would have been subdivided by IS6110-PGRS RFLP typing. In this study, two epidemiologically unlinked clusters with different IS6110-PGRS RFLP patterns had identical MIRU-VNTR patterns, consistent with the slightly lower resolution power of MIRU-VNTR typing compared to that of IS6110-RFLP typing (9, 25). Nevertheless, it is of considerable practical advantage to use MIRU-VNTR as the first-line method for transmission analysis, especially when combined with spoligotyping, as these two clusters had distinct spoligotypes, as has already been observed in previous studies (9).

In contrast to IS6110-PGRS-based RFLP typing, PCR-based MIRU-VNTR typing or spoligotyping can be applied with crude DNA extracts from heat-killed mycobacterial cultures as soon as they become positive, avoiding culture delays of several weeks. Depending upon the initial bacterial load, the total time needed to obtain the genotypes when starting from the clinical specimen can be reduced to a few days (3). Epidemiological investigations based on genotyping data can thus begin sooner, providing opportunities for the more rapid detection of secondary cases, latent tuberculosis infections, and sites of transmission (33). By combining MIRU-VNTR typing and spoligotyping, results can more easily be read and exchanged between laboratories than is the case with the complex IS6110-PGRS patterns. Therefore, the first-line use of MIRU-VNTR typing instead of IS6110-PGRS RFLP typing should provide a more rapid and more reliable insight into the dynamics of tuberculosis transmission both within and among communities or countries.

It can be concluded from this study that for a number of cases, estimated to be 14% in The Netherlands, the use of IS6110-PGRS RFLP typing will result in false clustering of tuberculosis patients, even if the isolates have high copy numbers of IS6110. The population-based study presented here included patients from both the general population and from tuberculosis risk groups in rural and urban areas representative of The Netherlands. Since the country’s sociodemographic and disease incidence characteristics are similar to those of most developed countries, we predict that our observations can be widely generalized.

Taking into consideration the technical advantages and the accuracy gained for reliable exclusion of epidemiological links, a combination of MIRU-VNTR typing and spoligotyping instead of RFLP-based methods would thus be recommendable in many settings for first-line screening of potential tuberculosis case clusters.

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REFERENCES


<table>
<thead>
<tr>
<th>Epidemiological link between clustered patients (transmission group)</th>
<th>IS6110 clusters involved*</th>
<th>No. of patients</th>
<th>No. of IS6110-PGRS clusters</th>
<th>No. (%) of IS6110-PGRS clusters split by MIRU-VNTR typing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evident link (TG1 and TG2)</td>
<td>1–23, 36</td>
<td>57</td>
<td>24</td>
<td>1 (4.2)</td>
</tr>
<tr>
<td>Likely link (TG3)</td>
<td>35–35, 37, 38</td>
<td>54</td>
<td>23</td>
<td>3 (13)</td>
</tr>
<tr>
<td>No link established (TG4)</td>
<td>36–42</td>
<td>14</td>
<td>7</td>
<td>5 (71.4)</td>
</tr>
</tbody>
</table>

* See Fig. 1 for designated cluster numbers.

b Isolates from a total of 125 patients were examined.


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