Variable-number tandem-repeat (VNTR) typing with a panel of 24 loci is the current gold standard in the molecular typing of Mycobacterium tuberculosis complex isolates. However, because of technical problems, a part of the loci often cannot be amplified by multiplex PCRs. Therefore, a considerable number of single-locus PCRs have to be performed for the loci with missing results, which impairs the laboratory work flow. Therefore, the original in-house method described by Supply et al. in 2006 was reevaluated. We modified seven primers and the PCR master mixture and obtained a strongly optimized in-house 24-locus VNTR typing method. The percentage of instantly complete 24-locus VNTR patterns detected in the routine flow of typing activities increased to 84.7% from the 72.3% obtained with the typing conducted with the commercially available Genoscreen MIRU-VNTR typing kit. The analytical sensitivity of the optimized in-house method was assessed by serial dilutions of M. tuberculosis in bronchoalveolar lavage fluid. A 1:10 dilution of the different strains tested was the lowest dilution for the detection of a complete 24-locus VNTR pattern. The optimized in-house 24-locus VNTR typing method will reduce the turnaround time of typing significantly and also the financial burden of these activities.

Given the common use worldwide of the standardized VNTR typing method, we have attempted to improve the original in-house VNTR method described by Supply et al. (1). In addition, we have determined the minimum amount of DNA required for successful VNTR typing of Mycobacterium tuberculosis in clinical material.

**MATERIALS AND METHODS**

**Samples.** For optimization of the in-house 24-locus VNTR typing technique, we used the DNA of two different M. tuberculosis strains, control strain H37Rv and a strain from the National Tuberculosis Reference Laboratory (NLA000901369). For the final quality check of the optimized in-house method, we used the panels used in the first (3) and second (6) proficiency studies on VNTR typing.

To detect the effect of the implementation of the optimized in-house technique rather than the commercial method used, we included the results of routine typing of M. tuberculosis isolates as part of the national surveillance in The Netherlands conducted at the National Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. The percentage of complete 24-locus VNTR patterns obtained with the commercial typing kit
from January 2010 to November 2011 was compared with that obtained with the optimized in-house method from November 2011 to July 2013.

**DNA isolation.** DNA isolation was performed with the QIAamp DNA Minikit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol for DNA purification from blood and body fluids. From a positive culture medium, 1 ml was centrifuged for 15 min (11,800 × g). The pellet was used as the input for the DNA isolation procedure. From a solid medium, 1 colony was suspended in MilliQ water to serve as the starting material. The DNA was eluted from the column in 30 μl of elution buffer and diluted to a final concentration of 10 ng/μl.

**Twenty-four-locus VNTR typing by the commercial kit.** The commercial 24-locus VNTR typing kit from Genoscreen (Lille, France) was used in accordance with the manufacturer’s instructions. This technique was used in a diagnostic setting until November 2011.

**Optimized in-house method for 24-locus VNTR typing.** The original set of primers described by Supply et al. was evaluated by the optimized in-house method for 24-locus VNTR typing. Seven primers affecting the amplification of 4 loci of the complete 24-locus VNTR set were replaced with new primers designed with Primer3Plus (3). The sequences of these new primers are in bold in Table 1; the other primers used are identical to those described by Supply et al. (1). The final concentrations of the forward and reverse primers for a particular locus were identical.

Three different commercially available PCR master mixes were used to amplify the DNA control samples, i.e., PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, United Kingdom), Multiplex PCR 5× Master Mix (Westburg Benelux Office, Leusden, The Netherlands), and AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA).

A DNA input of 2 μl was used to perform the amplification reactions. The PCR products obtained with the optimized in-house method of 24-locus VNTR typing were visualized by gel electrophoresis (2% agarose).

All VNTR analyses were performed according to standard laboratory procedures by three experienced technicians.

We considered implementation only when a specific combination of PCR master mixture and primers met the following criteria. (i) Twenty-four loci in monoplex reactions had to produce the expected amplicon sizes for the DNA controls used, and (ii) triplex PCRs had to produce identical amplicon sizes, as detected with a capillary electrophoresis (CE) DNA analyzer (ABI 3730).

**Implementation in a diagnostic setting.** For final implementation in a diagnostic setting, national surveillance, the lengths of PCR products sizes expected for all of the 24 loci. Even in a multiplex reaction, the amplicons were clear in the analysis of the results in the CE DNA analyzer.

Thus, the final PCR mixture of the optimized 24-locus VNTR typing method consisted of 12.5 μl of AmpliTaq Gold 360 Master Mix for each reaction with a total volume of 25 μl. Table 1 depicts the loci combined in the multiplex PCR mixtures and the final primer concentrations. The PCR program used was 10 min at 96°C; 40 cycles of 1 min at 96°C, 1 min at 60°C, and 1 min at 75°C; and a final step of 7 min at 72°C.

**Quality of the optimized in-house 24-locus VNTR typing method.** The interlaboratory reproducibility of the optimized in-house 24-locus VNTR typing method was tested before the implementation of this method in the daily routine. The results obtained with the panels used in the first and second worldwide studies of VNTR typing proficiency (5) organized by the RIVM were good; the interlaboratory reproducibility was 100%, and the intralaboratory reproducibility was 97%.

**Implementation of the optimized in-house VNTR typing method.** In a 20-month period (November 2011 to June 2013) after the introduction of the optimized in-house 24-locus VNTR typing method, 1,401 *M. tuberculosis* strains were typed by this method. The percentage of instantly complete VNTR patterns detected was 84.7% (n = 1,186). The performance of the commercial 24-locus VNTR typing method was mapped retrospectively. In a 22-month period (January 2010 to October 2011), 1,638 *M. tuberculosis* strains were typed in the daily routine. The percentage of complete 24-locus VNTR patterns detected by this test was 72.3% (n = 1,184). The percentage of initial complete results of the optimized in-house 24-locus VNTR typing method was shown to be significantly higher (P < 0.001) and was related to better yields for the loci for which new primers were designed.

The incomplete VNTR patterns detected were caused by missing results for one or more alleles because of amplification failure or because of the false detection of two different numbers of repeats for one or two alleles. Of the incomplete patterns detected by the commercial 24-locus VNTR method, 91.6% (n = 416) were due to missing results and 8.4% (n = 38) were due to the detection of double alleles. By the optimized 24-locus VNTR method, these were 80.9% (n = 174) and 19.1% (n = 41), respectively.

The analytical sensitivity of VNTR typing by the optimized 24-locus VNTR method was tested with DNA from serial dilutions of the four *M. tuberculosis* strains in BALF. The 24-locus VNTR patterns of the four *M. tuberculosis* strains used to prepare dilutions are given in Table 2. Undiluted samples and 1:10 dilutions of all four strains yielded a complete 24-locus VNTR pattern by the optimized in-house method. With the 1:100 dilution of BALF with a strain with a single copy of IS6110, one of the three VNTR analyses yielded results for only 22 loci instead of 24; for the other three strains with higher IS6110 copy numbers, this dilution showed the complete VNTR pattern thrice. Also, with the 1:1,000 dilution of BALF with the strain with a single copy of IS6110, one of the triplicates showed an incomplete VNTR pattern of 23 loci. The 1:1,000 dilution of the other samples tested showed a triplicate of complete patterns for the strains with 5 and 10 IS6110 copies but three times showed an incomplete VNTR pattern with
<table>
<thead>
<tr>
<th>Locus</th>
<th>Genome position</th>
<th>Mixture no.</th>
<th>Final primer concn (nM)</th>
<th>Primer sequence (5’–3’ with labeling)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIRU 04, ETR-D</td>
<td>580</td>
<td>1</td>
<td>400</td>
<td>GCGGAGAGAGGGCTCACCTCG(FAM)’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GCGGAGAGAAAGCCGACCC</td>
</tr>
<tr>
<td>MIRU 26</td>
<td>2996</td>
<td>1</td>
<td>400</td>
<td>CATAGGCGACCAGGGCGCATTAG(VIC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TAGCTCATCGTGGAATCTGTGAC</td>
</tr>
<tr>
<td>MIRU 40</td>
<td>802</td>
<td>1</td>
<td>400</td>
<td>GGGTTGCTGGAATCGAAGCTGT(FAM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GGGTATCTCGGGAATCAGATA</td>
</tr>
<tr>
<td>MIRU 10</td>
<td>960</td>
<td>2</td>
<td>400</td>
<td>GCCACCTTGGTACAGCTACCT(FAM)</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>GTCATTGAACTTCAGGGTCC</td>
</tr>
<tr>
<td>MIRU 16</td>
<td>1644</td>
<td>2</td>
<td>320</td>
<td>CCGCTCGTGACCCCTGGTGAC(VIC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TCGGTATGGCTGAAATCTGGAATCTGTGAC</td>
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<td>3192</td>
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<td>ACTGATTTGGCTCATACGGAATCTGTGAC</td>
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<td></td>
<td>CTTGGGCCGCATCAAGGGCATTATT</td>
</tr>
<tr>
<td>ETR-C</td>
<td>577</td>
<td>3</td>
<td>320</td>
<td>CGAGAGTGGCGATGGCGGTTATCT(VIC)</td>
</tr>
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<td></td>
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<td></td>
<td>AATGACCTGAAACGGAATCTGTGAC</td>
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<tr>
<td>ETR-A</td>
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<td>3</td>
<td>400</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CGAAGCCTGGGGGTCCGATT</td>
</tr>
<tr>
<td>Mtub 30</td>
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<td>400</td>
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<td></td>
<td></td>
<td>ACTTGAAACCACCGAGCCATTAGTA</td>
</tr>
<tr>
<td>Mtub 39</td>
<td>3690</td>
<td>4</td>
<td>400</td>
<td>CGGTAGGGCGGATGACGTCTCTC(VIC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TAGACGCGGACGGGAAAGGTCGATT</td>
</tr>
<tr>
<td>QUB 4156</td>
<td>4156</td>
<td>4</td>
<td>400</td>
<td>GATGTGCTGGTGCTGTCACT(FAM)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>TGACCACGGAGATTGCTCTAGTC</td>
</tr>
<tr>
<td>QUB 11b</td>
<td>2163b</td>
<td>5</td>
<td>800</td>
<td>GTGCAAGTGATGGTGTCGAT(FAM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GTAAGGGGATGCGGAAAT</td>
</tr>
<tr>
<td>Mtub 21</td>
<td>1955</td>
<td>5</td>
<td>400</td>
<td>AGATCCCCAGTTGTCGTCGTC(VIC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CAACCGCTGTTCTGTGAAAT</td>
</tr>
<tr>
<td>QUB 26</td>
<td>4052</td>
<td>5</td>
<td>640</td>
<td>AAGCCTAGCTGGTCCGAT(NED)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GCCAGGTCTCTTAGGAT</td>
</tr>
<tr>
<td>MIRU 02</td>
<td>154</td>
<td>6</td>
<td>400</td>
<td>TACCTAGGGAGGCTCAGAAAT(FAM)</td>
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<td></td>
<td></td>
<td></td>
<td>CGACATTTGACAGAATCTGCAA</td>
</tr>
<tr>
<td>MIRU 23</td>
<td>2531</td>
<td>6</td>
<td>400</td>
<td>CTGTGATGGCGGACAAAGCG(VIC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AGCTGCAAGGGTTGGGATCTGTGAC</td>
</tr>
<tr>
<td>MIRU 39</td>
<td>4348</td>
<td>6</td>
<td>400</td>
<td>CGGAAACGTCTACGCCCACACAT(NED)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CGCATGACAAAATGGACCCAAAC</td>
</tr>
<tr>
<td>MIRU 20</td>
<td>2059</td>
<td>7</td>
<td>400</td>
<td>GGAGAGATGCGCTCTGATGAT(FAM)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>GGAGACCCGAGACCGGTA</td>
</tr>
</tbody>
</table>

(Continued on following page)
>10 missing loci for the strain with 20 IS6110 copies. At dilution one step higher, 1:10,000, incomplete VNTR patterns were obtained for all of the strains. Table 3 shows the results in quantitation values of the M. tuberculosis detection tests for the analytical sensitivity of VNTR typing determined at a 1:100 dilution.

**DISCUSSION**

We have optimized the VNTR typing method. The combination of seven redesigned primers and the use of a suitable master mixture contributed to the high percentage (84.7%) of complete 24-locus VNTR typing profiles in the first multiplex PCR run. This practical improvement is considerable, as fewer strains have to be reamplified by single-target PCRs, and this reduces the workload and turnaround time.

Another important aspect of this improvement is cost efficiency. The high cost of the commercially available VNTR kit and a lack of access to a CE DNA analyzer hamper its use in many laboratories. The optimized in-house method may be an important alternative, because the products of single-locus PCRs can be analyzed on a gel without the need of a sophisticated DNA analyzer, and the yield of complete VNTR profiles obtained by this inexpensive approach is better than that obtained by the commercial method. In a recent proficiency study of VNTR typing, the sizing of VNTR PCR products yielded almost the same degree of reliability as the commercial method (6).

For the daily practice of molecular epidemiological studies of TB and to reduce the laboratory turnaround time, an adequately

---

**TABLE 1** (Continued)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genome position (Mbp)</th>
<th>Mixture no.</th>
<th>Final primer concn (nM)</th>
<th>Primer sequence (5’−3’ with labeling)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIRU 24</td>
<td>2687</td>
<td>7</td>
<td>400</td>
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</tr>
<tr>
<td>MIRU 27</td>
<td>3007</td>
<td>7</td>
<td>400</td>
<td>GCGATGAGGGTGCCACCTCAA(NED) TCGAAGGCCCTCGTGCCAGTAA</td>
</tr>
<tr>
<td>Mtub 29</td>
<td>2347</td>
<td>8</td>
<td>400</td>
<td>GCCAGCCCGCCGATGCAACCT(FAM) AGGCCACCCGTTGCTTGTATGAC</td>
</tr>
<tr>
<td>ETR-B</td>
<td>2461</td>
<td>8</td>
<td>800</td>
<td>ATGGCCACCCGATACCGCTCAGT(VIC) CGACGGCCCATCTTGAGATGCTAC</td>
</tr>
<tr>
<td>Mtub 34</td>
<td>3171</td>
<td>8</td>
<td>320</td>
<td>GGTGCCACCTGGTCCAGATA(NED) GCTCTATTGCGAGGTTGAC</td>
</tr>
</tbody>
</table>

a Primers that differ from those used in the standardized method described by Supply et al. (1) are in bold.

b The final concentrations of the forward and reverse primers for a particular locus were identical.

c FAM, 6-carboxyfluorescein.

---

**TABLE 2** VNTR patterns of the four strains used to test the analytical sensitivity of the optimized in-house VNTR method

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genome position (Mbp)</th>
<th>No. of repeats in strain with following no. of IS6110 copies:</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIRU 04</td>
<td>580</td>
<td>1 2 2 2</td>
</tr>
<tr>
<td>MIRU 26</td>
<td>2996</td>
<td>2 5 6</td>
</tr>
<tr>
<td>MIRU 40</td>
<td>802</td>
<td>4 4 4 2</td>
</tr>
<tr>
<td>MIRU 10</td>
<td>960</td>
<td>4 4 4 3</td>
</tr>
<tr>
<td>MIRU 16</td>
<td>1644</td>
<td>3 3 3 3</td>
</tr>
<tr>
<td>MIRU 31</td>
<td>3192</td>
<td>3 3 2 3</td>
</tr>
<tr>
<td>VNTR 42</td>
<td>424</td>
<td>2 4 3 4</td>
</tr>
<tr>
<td>VNTR 43</td>
<td>577</td>
<td>3 3 4 4</td>
</tr>
<tr>
<td>VNTR ETR-A</td>
<td>2165</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>VNTR 47</td>
<td>2401</td>
<td>4 1 1 3</td>
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<td>VNTR 53</td>
<td>4156</td>
<td>1 3 2 2</td>
</tr>
<tr>
<td>VNTR QUB11b</td>
<td>2163b</td>
<td>3 3 1 5</td>
</tr>
<tr>
<td>VNTR 1955</td>
<td>1955</td>
<td>6 3 3 5</td>
</tr>
<tr>
<td>VNTR QUB-26</td>
<td>4052</td>
<td>6 5 6 8</td>
</tr>
<tr>
<td>MIRU 02</td>
<td>154</td>
<td>2 2 1 2</td>
</tr>
<tr>
<td>MIRU 23</td>
<td>2531</td>
<td>6 5 6 5</td>
</tr>
<tr>
<td>MIRU 39</td>
<td>4348</td>
<td>3 2 2 3</td>
</tr>
<tr>
<td>MIRU 20</td>
<td>2059</td>
<td>2 2 2 2</td>
</tr>
<tr>
<td>MIRU 24</td>
<td>2687</td>
<td>2 1 1 1</td>
</tr>
<tr>
<td>MIRU 27</td>
<td>3007</td>
<td>3 3 3 3</td>
</tr>
<tr>
<td>VNTR 46</td>
<td>2347</td>
<td>3 4 4 4</td>
</tr>
<tr>
<td>VNTR 48</td>
<td>2461</td>
<td>1 2 2 2</td>
</tr>
<tr>
<td>VNTR 49</td>
<td>3171</td>
<td>3 3 1 3</td>
</tr>
</tbody>
</table>

---

**TABLE 3** Analytical sensitivity of VNTR typing performed with 1:100 dilution

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cq with following no. of IS6110 copies:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>In-house Roche 5</td>
<td>31.20</td>
</tr>
<tr>
<td>In-house TaqMan 5</td>
<td>30.43</td>
</tr>
<tr>
<td>In-house TaqMan 10</td>
<td>29.86</td>
</tr>
<tr>
<td>Lucron</td>
<td>30.31</td>
</tr>
<tr>
<td>Pathofinder</td>
<td>28.81</td>
</tr>
<tr>
<td>MP MTB</td>
<td>32.66</td>
</tr>
<tr>
<td>MP MTB/NTM</td>
<td>32.14</td>
</tr>
<tr>
<td>In-house ITS-HRM</td>
<td>24.62</td>
</tr>
<tr>
<td>Cobas</td>
<td>32.03</td>
</tr>
<tr>
<td>GeneXpert</td>
<td>20.90</td>
</tr>
<tr>
<td>Goffin MTB</td>
<td>32.66</td>
</tr>
<tr>
<td>Goffin MTB/NTM</td>
<td>32.14</td>
</tr>
</tbody>
</table>

* Associated with the quantitation cycle (Cq) values of the diagnostic tests performed in the study of Akkerman et al. (4).
performing 24-locus VNTR typing method is of the utmost importance. Failing loci hamper the interpretation of VNTR typing results in many countries, and this has introduced a bias into the international comparability of VNTR typing results. Failing loci are not the result of a natural absence of VNTRs, as often assumed, but merely the result of technical problems in the typing technique used (5).

In the meantime, the commercial 24-locus VNTR method was changed from an eight-reaction system to a six-reaction system. The manufacturer did not disclose information about the technical adjustments.

In this study, we also analyzed the analytical sensitivity of the improved in-house VNTR typing method. The detection of the 24-locus VNTR pattern of the *M. tuberculosis* complex in BALF showed an analytical sensitivity of 1:10 for the optimized in-house method. A slightly more lenient definition of analytical sensitivity changes the interpretation to an analytical sensitivity of 1:100. An analytical sensitivity of 1:100 can be used for VNTR typing. With the risk of missing a single locus, VNTR typing can still exclude the possibility of transmission and relapses.

In theory, this sensitivity provides the ability to perform typing of *M. tuberculosis* directly in BALF samples, even with a minimal bacterial load. This may increase the applicability of typing results in the early stage of examinations of TB outbreaks or, in an earlier diagnostic phase, permit better discrimination of whether a patient has a relapse or a new infection. The moment VNTR typing can be performed depends on the bacterial load of the sample, shown as quantitation cycle values, and can be read from Table 3 for different diagnostic tests. In practical diagnostics, the number of IS6110 elements is unknown; therefore, the results of the 10-copy IS6110 strain can be used and are most representative of the samples collected in The Netherlands. This study has some important limitations. First, we could not use a single set of samples to investigate the performance of the standard versus that of the improved VNTR methodology. Nonetheless, the sets used to assess the performance of both methods are representative of the epidemiology in The Netherlands and were similar with regard to genotype distributions. Second, analytical sensitivity should ideally be determined with prospectively gathered clinical specimens for which quantitative cultures are performed in parallel. In our national reference laboratory setting, these materials cannot be gathered.

In summary, optimization of the in-house 24-locus VNTR method resulted in 84.7% complete VNTR patterns in a diagnostic setting. This improves the laboratory work flow because of the reduction of the number of reamplification reactions. In addition, this technique is much cheaper than the commercial 24-locus VNTR method and is useful for laboratories without a CE DNA analyzer.

REFERENCES