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Optimization of Standard In-House 24-Locus Variable-Number Tandem-Repeat Typing for *Mycobacterium tuberculosis* and Its Direct Application to Clinical Material

Jessica L. de Beer,[a] Onno W. Akkerman,[b] Anita C. Schürch,[a,c,d] Arnout Mulder,[a] Tjip S. van der Werf,[b,e] Adri G. M. van der Zanden,[f] Jakko van Ingen,[g] Dick van Soolingen[a,d,h]

National Tuberculosis Reference Laboratory (IDS), Centre for Infectious Disease Control (CIB), National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands[a]; University of Groningen, University Medical Center Groningen, Department of Pulmonary Diseases & Tuberculosis, Groningen, The Netherlands[b]; Radboud University Medical Centre/NCMLS, Centre for Molecular and Biomolecular Informatics, Nijmegen, The Netherlands[c]; Erasmus Medical Center, Department of Viroscience, Rotterdam, The Netherlands[d]; University of Groningen, University Medical Center Groningen, Department of Internal Medicine, Infectious Diseases, Groningen, The Netherlands[e]; Laboratory for Medical Microbiology and Public Health, Enschede, The Netherlands[f]; Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen, The Netherlands[g]; Department of Pulmonary Diseases, Radboud University Medical Centre, Nijmegen, The Netherlands[h]

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.

Since the introduction of standardized variable-number tandem-repeat (VNTR) typing in 2006 by Supply et al. (1), laboratories worldwide have implemented this method. Compared to the level of discrimination of the formerly used restriction fragment length polymorphism (RFLP) typing method, that of 24-locus VNTR typing has proven to be sufficient to trace the transmission of tuberculosis (TB) in low-burden settings (1, 2). The advantages of VNTR typing over RFLP typing include simplified comparison of the results and applicability to small amounts of DNA, by which the turnaround time decreased from an average of 44 days to 15 days at our laboratory. However, in the first worldwide proficiency study of VNTR typing, the level of interlaboratory reproducibility was only 60% and intralaboratory reproducibility was only 72%. A second worldwide proficiency study revealed important improvements after the adjustment of some technical elements in the methodology and a higher degree of standardization (6). Still, laboratories applying VNTR typing face several technical challenges. First and foremost, some of the 24 loci may not be amplified in the multiplex PCRs and have to be amplified with a single-locus PCR; this holds true both for the commercially available MIRU-VNTR typing kit (Genoscreen, Lille, France) and for the in-house methods. In practice, this involves a significant increase in the workload and turnaround time.

The need for an optimized, fast, and high-quality VNTR typing method is high, especially for municipal health services and clinicians. The results of typing are used to steer the direction of source case finding and eventually to support the activities of the elimination of TB transmission. For the clinician, the most important information extracted from the results of typing is whether the patient has a TB relapse or an exogenous TB reinfection.

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

Received 10 December 2013 Returned for modification 19 December 2013 Accepted 28 January 2014

Published ahead of print 5 February 2014

Editor: S. A. Moser

Address correspondence to Jessica L. de Beer, jessica.de.beer@rivm.nl.

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doi:10.1128/JCM.03436-13

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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 mixtures 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 were detected with a CE DNA analyzer (ABI 3730) with the addition of an internal lane size standard, the GeneScan 1200 Size Standard (Applied Biosystems, Foster City, CA).

Samples used for analytical sensitivity testing. In a recent publication by Akkerman et al. (4), 14 molecular assays were compared for *M. tuberculosis* complex detection in pooled bronchoalveolar lavage fluid (BALF). Serial dilutions of four *M. tuberculosis* strains with various numbers of copies (1, 5, 10, and 20) of the IS*6110* element were used to determine the analytical sensitivity of the optimized 24-locus VNTR typing method in BALF. All VNTR analyses were performed in triplicate.

Statistical analysis. The statistical significance of differences between the two tests performed was determined by Pearson's chi-square test on a two-by-two table of method versus result.

RESULTS

Optimization of the in-house 24-locus VNTR typing method. The results of amplification by PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, United Kingdom) did not meet the criteria because multiple nonspecific amplicons with unexpected lengths were obtained. The results of amplification by the Multiplex PCR 5× Master Mix (Westburg Benelux Office, Leusden, The Netherlands) also did not meet the expectations. In this case, the optimum annealing temperatures for the different loci were too far apart to be useful in a multiplex PCR.

The amplification results of the AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA) yielded the amplicon 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 IS*6110*, one of the three VNTR analyses yielded results for only 22 loci instead of 24; for the other three strains with higher IS*6110* 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 IS*6110*, 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 IS*6110* copies but three times showed an incomplete VNTR pattern with

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TABLE 1 Primer sequences used in the optimized 24-locus VNTR typing method[a]

Locus	Genome position	Mixture no.	Final primer concn[b] (nM)	Primer sequence (5′–3′ with labeling)
MIRU 04, ETR-D	580	1	400	GCGCGAGAGCCCGAACTGC(FAM)[c] GCGCAGCAGAAACGCCAGC
MIRU 26	2996	1	400	CATAGGCGACCAGGCGAATAG(VIC) TAGGTCTACCGTCGAAATCTGTGAC
MIRU 40	802	1	400	GGGTTGCTGGATGACAACGTGT(NED) GGGTGATCTCGGCGAAATCAGATA
MIRU 10	960	2	400	GCCACCTTGGTGATCAGCTACCT(FAM) GTTCTTGACCAACTGCAGTCGTCC
MIRU 16	1644	2	320	CCCGTCGTGCAGCCCTGGTAC(VIC) TCGGTGATCGGGTCCAGTCCAAGTA
MIRU 31, ETR-E	3192	2	400	GTGCCGACGTGGTCTTGAT(NED) ACTGATTGGCTTCATACGGCTTTA
Mtub 04	424	3	400	GGCAGCAGAGCCCGGGATTCTTC(FAM) CTTGGCCGGCATCAAGCGCATTATT
ETR-C	577	3	320	CGAGAGTGGCAGTGGCGGTTATCT(VIC) AATGACTTGAACGCGCAAATTGTGA
ETR-A	2165	3	400	AAATCGGTCCCATCACCTTCTTAT(NED) CGAAGCCTGGGGTGCCCGCGATTT
Mtub 30	2401	4	400	CTTGAAGCCCCGGTCTCATCTGT(FAM) ACTTGAACCCCCACGCCCATTAGTA
Mtub 39	3690	4	400	CGGTGGAGGCGATGAACGTCTTC(VIC) TAGAGCGGCACGGGGGAAAGCTTAG
QUB 4156	4156	4	400	**GATGTGCGGTACGTGCATC(NED)** **TGACCACGGATTGCTCTAGTC**
QUB 11b	2163b	5	800	**GTCGAAGTGAATGGTGGCAT(FAM)** **GTAAGGGGGATGCGGGAAAT**
Mtub 21	1955	5	400	AGATCCCAGTTGTCGTCGTC(VIC) CAACATCGCCTGGTTCTGTA
QUB 26	4052	5	640	AACGCTCAGCTGTCGGAT(NED) **GCCAGGTCCTTCCCGAT**
MIRU 02	154	6	400	TACTCGGACGCCGGCTCAAAAT(FAM) TGGACTTGCAGCAATGGACCAACT
MIRU 23	2531	6	400	CTGTCGATGGCCGCAACAAAACG(VIC) AGCTCAACGGGTTCGCCCTTTTGTC
MIRU 39	4348	6	400	CGGAAACGTCTACGCCCCACACAT(NED) CGCATCGACAAACTGGAGCCAAAC
MIRU 20	2059	7	400	**GGAGAGATGCCCTTCGAGTTAG(FAM)** **GGAGACCGCGACCAGGTA**

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TABLE 1 (Continued)

Locus	Genome position	Mixture no.	Final primer concn[b] (nM)	Primer sequence (5′–3′ with labeling)
MIRU 24	2687	7	400	GGGCGAGTTGAGCTCACAGAA(VIC) CGACCAAGATGTGCAGGAATACAT
MIRU 27	3007	7	400	GCGATGTGAGCGTGCCACTCAA(NED) TCGAAAGCCTCTGCGTGCCAGTAA
Mtub 29	2347	8	400	GCCAGCCGCCGTGCATAAACCT(FAM) AGCCACCCGGTGTGCCTTGTATGAC
ETR-B	2461	8	800	ATGGCCACCCGATACCGCTTCAGT(VIC) CGACGGGCCATCTTGGATCAGCTAC
Mtub 34	3171	8	320	GGTGCGCACCTGCTCCAGATAA(NED) GCTCTCATTGCTGGAGGGTTGTAC

[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.

>10 missing loci for the strain with 20 IS*6110* 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 2 VNTR patterns of the four strains used to test the analytical sensitivity of the optimized in-house VNTR method[a]

Locus	Genome position	No. of repeats in strain with following no. of IS*6110* copies:			
		1	5	10	20
MIRU 04	580	5	2	2	2
MIRU 26	2996	2	5	5	6
MIRU 40	802	4	4	4	2
MIRU 10	960	4	4	4	3
MIRU 16	1644	3	3	3	3
MIRU 31	3192	4	3	2	5
VNTR 42	424	2	4	3	4
VNTR 43	577	4	3	2	4
VNTR ETR-A	2165	6	3	2	4
VNTR 47	2401	1	4	1	4
VNTR 52	3690	4	3	2	3
VNTR 53	4156	1	3	2	2
VNTR QUB11b	2163b	3	3	1	5
VNTR 1955	1955	6	3	3	5
VNTR QUB-26	4052	6	5	6	8
MIRU 02	154	2	2	1	2
MIRU 23	2531	6	5	6	5
MIRU 39	4348	3	2	2	3
MIRU 20	2059	2	2	2	2
MIRU 24	2687	2	1	1	1
MIRU 27	3007	3	3	3	3
VNTR 46	2347	3	4	4	4
VNTR 48	2461	1	2	2	2
VNTR 49	3171	3	3	1	3

TABLE 3 Analytical sensitivity of VNTR typing performed with 1:100 dilution[a]

Assay	Cq with strain with following no. of IS*6110* copies:			
	1	5	10	20
In-house Roche 5	31.20	27.19	27.35	26.21
In-house TaqMan 5	30.43	27.50	26.44	25.30
In-house TaqMan 10	29.86	26.24	25.61	24.40
Lucron	30.31	27.29	26.92	25.57
Pathofinder	29.81	26.77	25.72	24.68
MP MTB	32.66	29.48	28.77	27.81
MP MTB/NTM	32.14	28.95	28.19	27.50
In-house ITS-HRM	24.62	28.33	29.24	28.22
Cobas	32.03	33.00	34.87	31.40
GeneXpert	20.90	24.77	24.57	19.43
Goffin MTB	32.66	29.48	28.77	27.81
Goffin MTB/NTM	32.14	28.95	28.19	27.47

[a] Associated with the quantitation cycle (Cq) values of the diagnostic tests performed in the study of Akkerman et al. (4).

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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 IS*6110* elements is unknown; therefore, the results of the 10-copy IS*6110* 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.

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