Multiplex Amplified Nominal Tandem-Repeat Analysis (MANTRA), a Rapid Method for Genotyping *Mycobacterium tuberculosis* by Use of Multiplex PCR and a Microfluidic Laboratory Chip[▽]

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A variable-number tandem-repeat genotyping method for *Mycobacterium tuberculosis* was converted to run in a multiplex PCR format on a 12-well microfluidic laboratory chip. Epidemiologically and genotypically distinct isolate clusters of *M. tuberculosis* were identified. This rapid genotyping method has potential application in smaller clinical laboratories and public health field investigations.

In recent years, public health has come to depend on molecular methods such as IS6110 typing and spoligotyping to genotype *Mycobacterium tuberculosis* isolates from clinical samples. These results have been used to identify possible clusters of genetically related isolates and thus determine whether clustering of infections has taken place. Prompt epidemiological application of molecular typing methods has been hampered by the centralization of genotyping in distant reference centers with high workloads and slow turnaround time. An important addition to the *M. tuberculosis* genotyping repertoire is based on the detection of variable-number tandem-repeat (VNTR) sequences (also known as mycobacterial interspersed repetitive-unit [MIRU] sequences) (4, 7). An increasing number of Australian public health laboratories now offer a *M. tuberculosis* VNTR genotyping service and use an online VNTR data interpretation web browser (1). Public health laboratories with both PCR capability and subsequent gene fragment length analysis can now run this method but at the cost of limited sequencing capacity. An alternative version of the VNTR genotyping method was developed at the Centers for Disease Control and Prevention, using a microfluidic LabChip analyzer for endpoint analysis (3). The *M. tuberculosis* VNTR method has sparked limited interest since then, possibly due to the multiple PCR products that need fragment analysis (9). More recently, VNTR genotyping that combines a multiplex PCR format with LabChip analysis has been applied to *Staphylococcus* species (5, 6). In this note, we describe MANTRA (multiplex amplified nominal tandem-repeat analysis), a 15-target, fully multiplexed version of VNTR genotyping for *M. tuberculosis* using the 2100 bioanalyzer in conjunction with the DNA 1000 laboratory chip in order to achieve

more immediate throughput than either monoplex format PCR or a DNA sequencer in GeneScan mode.

M. tuberculosis isolates from Lowenstein-Jensen slopes were suspended in buffer, sonicated, and heated at 100°C. The 34 tested isolates included three isolates from an epidemiological cluster, four isolates affected by a suspected cross-contamination event, and 27 unrelated isolates. The identities of the isolates and their genotypic and epidemiological relatedness were not divulged to either the molecular biologist or the interpreting pathologist until after examination of the laboratory chip results was complete. The mycobacterial suspensions were vortexed, centrifuged at 20,000 RCF for 1 min to pellet gross debris, and diluted to 1:100 to provide template DNA. Multiplexed VNTR amplicons were produced using a Qiagen multiplex PCR kit (Qiagen GmbH, Hilden, Germany). Fifteen VNTR primer pairs (Table 1) were incorporated into the master mix at a final concentration of 0.2 μM each. Each reaction consisted of 10 μl of Qiagen 2× master mix, 2 μl of Qiagen Q reagent, 2 μl of 10× primer stock, and 6 μl of DNA template for a final reaction volume of 20 μl in a 0.2-μl thin-wall PCR tube. PCR was performed on an Applied Biosystems (ABI, Scoresby, Victoria, Australia) 2720 thermal cycler using the following protocol: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 60°C for 90 s, and 72°C for 60 s. A final extension step of 72°C for 10 min was performed before the reaction mixture was cooled to 4°C. Amplicons were resolved on an Agilent 2100 bioanalyzer (Agilent Technologies, Forest Hill, Victoria, Australia) using a DNA 1000 kit (Agilent), according to the manufacturer's instructions, and analysis was performed using the DNA 1000 series II assay script with default settings. At the completion of analysis, all 34 tested samples from three separate analysis files were combined using the comparison feature of the analysis software. The composite gel-like image of the 34 sample set and sizing ladder was saved as a tagged image file format (TIFF) image (Fig. 1). Every sample was compared to every other sample in a pairwise comparison approach by overlaying electropherograms, using the upper and lower markers as key reference points. Six groups of sam-

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TABLE 1. Primers used in this study

Locus	Repeat length (bp)	Product size range (bp)	Primer sequence (5'-3')
ETR-A ^a	75	270–945	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGCGATT
ETR-B ^a	57	178–691	GCGAACACCAGGACAGCATCATG GGCATGCCGGTGATCGAGTGG
ETR-C ^a	58	102–624	GTGAGTCGCTGCAGAACCTGCAG GGCGTCTTGACCTCCACGAGTG
MIRU-2 ^b	53	524–1,057	CATCGAATTGGACTTGCAGCAAT CGACGTCGTAGAGAGCATCGAAT
MIRU-4 ^b	77	161–854	GTCAAACAGGTCACAACGAGAGGAA CCTCCACAATCAACACACTGGTCAT
MIRU-10 ^b	53	273–750	ACCGTCTTATCGGACTGCACTATCAA CACCTTGGTGATCAGCTACCTCGAT
MIRU-16 ^b	53	422–899	CGGGTCCAGTCCAAGTACCTCAAT GATCCTCCTGATTGCCCTGACCTA
MIRU-20 ^b	77	298–991	GCCCTTCGAGTTAGTATCGTCGGTT CAATCACCGTTACATCGACGTCATC
MIRU-23 ^b	53	130–607	CGAATTCTTCGGTGGTCTCGAGT ACCGTCTGACTCATGGTGTCCAA
MIRU-24 ^b	54	447–915	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA
MIRU-26 ^b	51	297–756	GCGGATAGGTCTACCGTCGAAATC TCCGGGTCATACAGCATGATCA
MIRU-27 ^b	53	330–807	TCTGCGTGCCAGTAAGAGCCA CTGATGGTGACTTCGGTGCCTT
MIRU-39 ^b	53	712–1,189	CGGTCAAAGTTCAGCACCTTCTACATC CTCGGTGTTCCTTGAAGGTGGTTT
MIRU-40 ^b	77	284–770	GATTCCAACAAGACGCAGATCAAGA TCAGGTCTTTCTCTCACGCTCTCG

^a Described by Frothingham and Meeker-O’Connell (7).
^b Described by Cowan et al. (4). The smallest size is 1 copy; the largest size is 10 copies (11 for MIRU-2).

ples with highly similar patterns were obtained by this method. These were downselected from the full 34 sample set, and a second composite gel image was generated (Fig. 2). The first group (group 1) shown in lanes 4, 5, 6, and 9 of Fig. 2 contains isolates from a group of international students residing in housing on the same street. Isolate 9 (Fig. 2, lane 9) was obtained from a patient with no obvious epidemiological connection other than a similar geographic origin. Groups 2

FIG. 1. Gel-like image of MANTRA *M. tuberculosis* genotyping results, showing 34 analyzed samples and a DNA fragment size ladder. Upper and lower size markers are present in ladders and all *M. tuberculosis* isolate lanes. The vertical scale represents migration time.

FIG. 2. Gel-like image of MANTRA *M. tuberculosis* genotyping results, showing a DNA fragment size ladder and 16 downselected samples. Isolate designation is shown at the top of each lane; group assignment is shown at the bottom. Upper and lower size markers are present in ladders and all *M. tuberculosis* isolate lanes. The vertical scale represents migration time.

to 5 contained pairs of isolates that had very similar patterns but no known epidemiological link. Group 6 contained four samples that were implicated in a laboratory contamination event, resulting in a single isolate being introduced to multiple samples. A number of other isolates were less closely related, differing by 2 or more electropherogram peaks. All results were stored as Agilent run files (denoted as .XAD files) and subsequent comparison files and then downloaded as TIFF files for onward transmission.

Tuberculosis is one of the most common fatal infections worldwide. Its ease of transmission, ability to remain dormant in apparently healthy people, and the difficulty of effective treatment present a unique challenge to public health authorities. The impact of *M. tuberculosis* genotyping on public health has been limited by slow turnaround time, high running cost, and possibly the centralization of genotyping services. The method described here is fast, is relatively inexpensive, and can be operated in small clinical microbiology laboratories using equipment employed for a range of other molecular microbiology procedures. It successfully distinguished an epidemiological cluster and a possible additional isolate of similar geographic origin previously unknown to the molecular biologist and interpreting pathologist. Additionally, a second cluster of isolates was implicated in a laboratory contamination event. In MANTRA, multiple products are produced without any intention of allocating specific alleles in subsequent analysis. No fluid handling was required to combine PCR products because the amplification step was a true multiplex PCR. As with all clinical molecular biology methods, there are caveats. The MANTRA method is intended for comparative use, as indicated by the "N" (for "nominal"), a reminder that the technique does not aim to be a stand-alone genotyping method. This may be unimportant when no more than a comparative analysis of current isolates is required but could cause difficulty when attribution of a specific allele number is needed for comparison with a national or international isolate collection. A recent comparison of 15-target and 24-target VNTR typing of *M. tuberculosis* highlighted the inability of VNTR-based typing methods to detect all strain lineages and emphasized the

importance of restricting application of VNTR methods to isolates from a single epidemiological lineage (2). Although a number of less closely related isolates were recognized, no attempt was made to determine a measure of isolate similarity since this would be beyond the intended scope of the method. The other important caveat is the need to seek further confirmation of apparent genotypic clustering by a distinct second method such as spoligotyping or IS6110 typing. Spoligotyping is the preferred method for combination with VNTR-MIRU genotype results in the previously noted web browser (1). This combination of methods is generally restricted to large reference centers and may take weeks or months to return definitive results. The methods we describe here allow a preliminary assessment of *M. tuberculosis* phylogeny during the early stages of a public health investigation when results are of most use in guiding disease control. The equipment required for *M. tuberculosis* MANTRA is the same as that used previously for field-work overseas (8), raising the possibility of inserting genotyping capability closer to the main burden of disease. This method may therefore be suitable in the future as close support for tuberculosis control programs. In addition, the alternative laboratory chip result format provided in the bioanalyzer software can be used to compare samples analyzed on different chips or on different analyzers located in geographically separated laboratories.

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