Comparison of random amplified polymorphic DNA with restriction fragment length polymorphism as epidemiological typing methods for *Mycobacterium tuberculosis*

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Abstract

Aim—To compare restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) methods for the epidemiological typing of *Mycobacterium tuberculosis*.

Methods—Thirty one *M tuberculosis* cultures originating from patients in the Canton of Berne in Switzerland, which had previously been typed by RFLP, were subjected to RAPD analysis. Cultures were coded so that the investigators were blind to the RFLP results until RAPD analysis was complete.

Results—The 31 cultures of *M tuberculosis* were divided into nine groups by RFLP and eight groups by RAPD. Generally there was good correlation between the groups identified by the two techniques, with the exception of strains that had only one copy of IS6110. Both methods subdivided isolates that were placed in a single group by the other method.

Conclusions—RAPD analysis is quick, simple, and useful for the comparison of small numbers of isolates. RFLP is more reproducible and therefore better suited for the accumulation of RFLP fingerprints for long term local surveillance and large epidemiological studies.

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Keywords: Random amplified polymorphic DNA, restriction fragment length polymorphism, *M tuberculosis*.

Tuberculosis is still a major cause of mortality, accounting for the deaths of three million people worldwide.¹ Although the prevalence of tuberculosis has declined dramatically in the last century in developed countries, there is some concern that this decline has now ceased.² Studies investigating the spread of *Mycobacterium tuberculosis* have been hampered by the paucity of methods available for the discrimination of isolates. Several genetic analysis techniques have been adapted for use with *M tuberculosis*, including restriction fragment length polymorphism (RFLP)^{3,4} and random amplified polymorphic DNA (RAPD).⁵

Typing by RFLP involves cutting purified genomic DNA at specific sites using a restriction enzyme. The DNA fragments generated can be compared after separation by electrophoresis. If these patterns are too complex certain bands can be selected by probing

for specific DNA sequences by Southern blot hybridisation. For *M tuberculosis* a probe specific to the small transposable element IS6110 (or 986) which is present in the majority of *M tuberculosis* strains in between one and 20 copies has proved particularly useful.^{3,4,6,7} Although relatively complex, requiring many steps over a number of days, this technique has gained widespread acceptance as a useful epidemiological tool, having been used successfully in investigations of the spread of tuberculosis in cities,⁸ hospitals,⁹ shelters for the homeless,¹⁰ prisons,¹¹ and for legal purposes.¹²

Random amplified polymorphic DNA analysis is a less specific method of producing DNA "fingerprints".^{13,14} This technique employs the polymerase chain reaction (PCR) to amplify DNA fragments but instead of the usual pair of primers directed at a specific target a single randomly selected primer is used. This binds at multiple sites along the genome at the low annealing temperatures used; products are produced between primers binding in close proximity to opposite DNA strands. Compared with RFLP and other recently described molecular typing methods, such as mixed linker PCR,¹⁵ RAPD is more rapid and less technically demanding to perform. RAPD has been used successfully to type a variety of different bacteria¹⁶⁻¹⁸ but its potential for typing *M tuberculosis* has only recently been reported.⁵ The purpose of this study was to compare RFLP with RAPD for the typing of *M tuberculosis*.

Methods

Strains of *M tuberculosis* were isolated from 165 patients in the Canton of Berne, Switzerland, between 1991 and 1992. All isolates were characterised by RFLP analysis at University of Berne using methods described previously.⁸ Briefly, DNA was extracted and purified from heat inactivated cells recovered from Middlebrook medium by centrifugation. After the DNA was cut using the restriction enzyme *PvuII*, fragments were separated by electrophoresis in 0.7% agarose and transferred to a nylon membrane by Southern blotting. Bands containing IS6110 were located by hybridisation to a digoxigenin labelled probe complementary to a 156 nucleotide section of IS6110 and visualised using a chemiluminescence kit (Boehringer Mannheim, Switzerland). The RFLP patterns were analysed and compared with epidemiological findings.⁸

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Comparison of *Mycobacterium tuberculosis* subgroups defined by restriction fragment length polymorphism and random amplified polymorphic DNA

Bern ID	Patient ID	IS6110 No ^a	RFLP group	RAPD group
MTB-10	MC	10	1	3
MTB-13	MC	10	1	3
MTB-28	MC	10	1	NT ^b
MTB-21	NL	10	1	3
MTB-4	MM	10	1	3
MTB-17	NM	10	1	3
MTB-30	AfA	11	2	4
MTB-24	HA	11	2	4
MTB-9	AvA	11	2	5
MTB-2	NA	11	2	5
MTB-15	TK	6	3	2
MTB-12	ST	6	3	2
MTB-7	VT	6	3	NT ^b
MTB-20	VT	6	3	2
MTB-27	VT	6	3	2
MTB-19	FF	13	4	6
MTB-29	AH	13	4	6
MTB-1	FA	1	5	2
MTB-31	ML	1	5	1
MTB-22	BA	1	6	8
MTB-16	PN	1	6	1
MTB-3	PN	1	6	2
MTB-18	PN	1	6	1
MTB-25	NS	4	7	1
MTB-6	FS	4	7	1
MTB-26	JT	4	7	1
MTB-11	WM	7	8	7
MTB-5	RS	7	8	7
MTB-14	WR	8	9	NT ^b
MTB-8	AS	8	9	NT ^b
MTB-23	AS	8	9	8

^aIS6110 No = number of insertion elements determined by Southern blotting.

^bNT = non-typable by RAPD.

Thirty one cultures, including representatives from each RFLP group and some triplicates, were provided for RAPD analysis at the Bristol Royal Infirmary, England. Cultures were coded so that RAPD analysis could be performed without prior knowledge of the RFLP groups. Each strain was inoculated on to Lowenstein-Jensen slopes and incubated at 37°C for two weeks; the resulting growth was tested for contamination by subculture on to blood agar plates incubated at 37°C for four days. DNA was extracted from a heat killed suspension of *M. tuberculosis* using Gene-Releaser (Cambio) according to the manufacturer's instructions (microwave method). The PCRs were performed in a total volume of 50 µl containing 0.25 U of Super TAQ poly-

merase (HT Biotechnology), buffer (10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 0.1% (vol/vol) Triton X-100, 0.01% (wt/vol) gelatin), 20 µM (each) deoxynucleoside triphosphate (Pharmacia), and 0.4 µM primer. The entire 12 µl of the GeneReleaser product was used for PCR. The reaction mixtures were overlaid with two drops of paraffin oil and then incubated for three minutes at 94°C. Forty five cycles of PCR were performed using a thermal cycler (Omnigene; Hybaid) consisting of a denaturing step for 20 seconds at 94°C, an annealing step for one minute at 36°C, and an extension step for one minute at 72°C. After the final cycle there was a step of seven minutes at 72°C. RAPD analysis was performed for each isolate using three different primers: IS986-FP (ACGCTCAACGCCAGAGACCA), IS986-RP (GATGAACCACCTGACATGAC) and INS-2 (GCGTAGGCGTCGGTGACAAA). Each analysis was run in triplicate to aid interpretation of the resulting RAPD patterns. After electrophoresis in a 2% agarose gel the PCR products were visualised by ethidium bromide staining. Gels were photographed and band patterns compared visually.⁵

On completion of RAPD typing, codes were broken and the results were compared with those obtained by RFLP analysis.

Results

The 31 *M. tuberculosis* cultures were separated into nine groups by RFLP; the correspondence between these groups and epidemiological data has been discussed previously.⁸ Twenty seven of 31 cultures were separated into eight RAPD groups as listed in the table; examples of RAPD patterns are shown in the figure. Four isolates (13%) were recorded as non-typable because the RAPD profile consisted of a single very bright band on the gel. In our experience single amplification products are not reliably differential; this was confirmed by the observation that three of the four cultures non-typable by RAPD were in different RFLP groups.

Generally there was good correlation of the groups identified by RFLP and RAPD, with the exception of one of the three sets of triplicate subcultures provided as controls (MTB 3, 16, 18) which was incorrectly divided into two groups by RAPD analysis. RFLP groups 1, 3, 4, 7, and 8 corresponded with the RAPD groups 3, 2, 6, 1, and 7 respectively. Both methods subdivided isolates that were placed in a single group by the other method. For example isolates in RAPD groups 1 and 2 were divided into four RFLP groups (3, 5, 6, and 7) and isolates in RFLP group 2 were subdivided into two RAPD groups (4 and 5) using the primer IS-FP.

Discussion

It is notable that most of the discrepancies between the two methods occurred with isolates which had only one copy of IS6110 (RFLP groups 5 and 6); with these exceptions the two methods correlated well. The limitation of RFLP in typing strains harbouring low copy

Random amplified polymorphic DNA patterns generated using the IS-FP primer for strains of *Mycobacterium tuberculosis* isolated from patients in the Canton of Berne. —ve = negative control; NT = non-typable; M = marker (100 bp DNA ladder; Gibco BRL).

numbers of IS6110, particularly single copy strains, has been well documented.^{8,19} Analyses using the RAPD protocol outlined were also less reliable for strains containing few copies of IS6110; strains with single copies did not form distinct RAPD groups but clustered with strains containing multiple copies. Furthermore the set of triplicate subcultures which was divided into two groups by RAPD analysis was from a strain with a single copy of IS6110. This may in part be explained by use of primers which are all complementary to regions of this insertion sequence.⁵ These primers were the most discriminatory of 40 tested, perhaps because, due to specific binding of these primers, they tend to amplify selectively regions around IS6110 elements, the location of which varies in an otherwise conserved genome.²⁰ Further investigation of RAPD using primers unrelated to IS6110 may produce more reliable results for single copy strains.

RAPD can be performed rapidly on primary isolates of *M. tuberculosis* and is a technique well suited for preliminary epidemiological studies on a small number of strains. It is, however, less reproducible than RFLP and triplicate RAPD analyses are vital in the interpretation of the banding patterns. Small numbers of isolates could be compared more easily by analysis in a single batch, with all the RAPD profiles run on the same gel, but difficulties were experienced when comparing RAPD profiles on different gels. These problems would make it difficult to establish a "library" of banding patterns for strains endemic to a particular community. The RFLP profiles are sufficiently stable for this to be possible: the IS6110 has been shown to remain stably inserted in the genome of *M. tuberculosis* for up to eight years,^{3,21} and we have documented a stable RFLP pattern for a period of six years (T Bodmer, unpublished data). It is probable that RFLP will remain the gold standard in laboratories with sufficient personnel and equipment resources.

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