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Double-Repetitive-Element PCR Method for Subtyping *Mycobacterium tuberculosis* Clinical Isolates

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We describe a rapid method for subtyping *Mycobacterium tuberculosis* based on PCR amplification of segments located between two distinct DNA repetitive elements. This method, double-repetitive-element PCR, classified 46 clinical isolates as having 25 distinct patterns; the conventional restriction fragment length polymorphism analysis classified the same isolates as having 23 distinct patterns. The double-repetitive-element PCR is a rapid subtyping method that has a discriminating power similar to that of the restriction fragment length polymorphism method.

Currently, one standardized genetic subtyping method for *Mycobacterium tuberculosis* relies on restriction fragment length polymorphism (RFLP) analysis based on the organism's repetitive element IS*6110* (1, 3, 8). However, this RFLP typing method has several limitations. One major disadvantage is the time and labor required to perform the procedure. Because the method requires a sufficient amount of extracted DNA, the primary culture must have abundant growth or must be subcultured to yield enough organisms. After culture growth, the procedure involves DNA extraction, restriction endonuclease treatment, electrophoresis, and Southern blot hybridization, which require multiple reagents and steps. Hence, the entire RFLP procedure may take 3 to 4 weeks to yield interpretable results.

We describe a novel rapid subtyping method based on PCR amplification of *M. tuberculosis* DNA segments located between two copies of repetitive elements. The repetitive elements are IS*6110* and the polymorphic GC-rich repetitive sequence (PGRS). IS*6110* is a repetitive element described by Thierry et al. and is found in members of the *M. tuberculosis* complex in numbers ranging from 0 to more than 20 copies randomly distributed in the chromosome (7). Ross et al. described another repetitive element, the PGRS present on the recombinant plasmid TBN12, which is found in *M. tuberculosis* strains as well as in several other *Mycobacterium* species (6, 9). In *M. tuberculosis* PGRS appears to be present in at least 30 copies, varying in number and distribution from strain to strain.

Primers corresponding to the terminal sequences of the insertion elements IS*6110* and PGRS were designed so that the 3′ ends of the primers were directed downstream from the elements to amplify segments between the repetitive-element sequences. The primers and their sequences are as follows: Ris 1, 5′-GGC-TGA-GGT-CTC-AGA-TCA-G; Ris 2, 5′-ACC-CCA-TCC-TTT-CCA-AGA-AC; Pntb 1, 5′-CCG-TTG-CCG-TAC-AGC-TG; and Pntb 2, 5′-CCT-AGC-CGA-ACC-CTT-TG. Figure 1 is a schematic representation of the double-repetitive-element PCR (DRE-PCR) procedure. The rationale for this procedure is based on the fact that the distances between the repetitive elements and the copy numbers of IS*6110* and PGRS vary from strain to strain. These variations allow different sizes and numbers of DNA fragments to be amplified, yielding unique banding patterns for different *M. tuberculosis* strains (Fig. 1).

We applied DRE-PCR to examine clinical *M. tuberculosis* isolates obtained from the New York Hospital TBNetwork surveillance system. Of 167 isolates that had been analyzed by the standard IS*6110* RFLP method described by van Embden et al. (8), we examined 46 isolates that represented most of the RFLP patterns identified in our previous study (4). Two or more study isolates with RFLP patterns identical to one another, or with RFLP patterns identical to isolate patterns archived at the Tuberculosis Center of the Public Health Research Institute, were considered to have cluster patterns and therefore possibly to be epidemiologically related.

To perform DRE-PCR, we extracted DNA from a loopful of bacterial growth on Lowenstein-Jensen slants by boiling the sample in 1 ml of sterile water for 10 min and then freeze-thawing it overnight. The PCR amplification mixture contained a 10-μl aliquot of extracted DNA solution and a reaction mixture containing 0.5 pM primers (the sequence data for the primers to PGRS were obtained from the GenBank database under the accession number m95490), 200 μM deoxynucleoside triphosphate (Pharmacia Biotechnology, Piscataway, N.J.), 50 mM Tris-HCl, 50 mM KCl (pH 8.8), 2.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.5 U of *Taq* polymerase (Boehringer GmbH, Mannheim, Germany). The reactions were performed with an automated thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). DNA samples were denatured by incubation at 95°C for 10 min before amplification for 30 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 2 min, and primer extension at 72°C for 1 min. The amplification products were analyzed by gel electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under UV illumination. As with the RFLP analysis, the agarose gel electrophoresis patterns that were identical for two or more strains among the 46 isolates were considered cluster patterns.

The agarose gel electrophoresis patterns generated by the DRE-PCR method were compared with the IS*6110*-based RFLP patterns. The RFLP method yielded 23 distinct banding patterns among the 46 isolates. Eight of these were cluster patterns, and 15 were noncluster patterns. The DRE-PCR method gave 25 distinct banding patterns among the same 46 strains. Seven of these were cluster patterns, and 18 were noncluster patterns. Overall, DRE-PCR yielded 42 results concordant with those of the RFLP method. Three of the four

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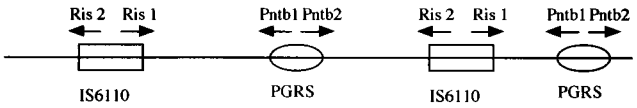

FIG. 1. DRE-PCR. Locations of the PCR primers Ris 1, Ris 2, Pntb 1, and Pntb 2 in relation to the insertion elements IS*6110* and PGRS are shown. Arrows indicate the direction of priming on the target DNA sequence.

discordant results were obtained with isolates that had cluster patterns by RFLP and noncluster patterns by DRE-PCR. One discordant result was from an isolate that had a noncluster pattern by RFLP and a cluster pattern by DRE-PCR. Therefore, the positive predictive value of DRE-PCR compared with the RFLP method was 96%.

Results for several isolates typed by the DRE-PCR method are presented in Fig. 2. Some strains known to be epidemiologically related by their RFLP patterns and clinical histories showed identical DRE-PCR patterns. For example, the commonly isolated cluster strain designated C yields three bands by the IS*6110* RFLP method. The same cluster strain isolated from different patients at different times generated identical patterns by the DRE-PCR method (Fig. 2A, lanes 1 through 3). By this new method we were also able to distinguish the multidrug-resistant strain referred to as the W strain from other W-variant patterns that differed by just one band (Fig. 2B, lanes 1 and 2).

A previous study used the terminal regions of the insertion element IS*6110* to divergently amplify DNA segments between two copies of this insertional sequence (5). For strains with a low copy number of IS*6110*, this method may not generate enough bands for the investigator to be able to discriminate them. When we attempted to amplify the DNA of the C strain by the method described above, we were unable to amplify any DNA fragments. The DRE-PCR method consistently generated six bands (Fig. 2A, lanes 1 through 3).

In determining cluster RFLP patterns we included strains with three or more bands. RFLP analysis generated patterns with 3 to 17 bands. For analysis of the isolates by DRE-PCR all banding patterns were included. The DRE-PCR method generated patterns with one to six bands. Four of the 46 isolates had DRE-PCR patterns with only one band. These four PCR products were compared with each other by using a standard molecular weight marker (*Hae*III digest of ϕX174), were determined to have different molecular weights, and were considered to have noncluster patterns. However, one would have to be cautious in making any diagnostic decisions based on one-band PCR patterns. As a screening test for epidemiologic studies, such an amplification may direct investigators to repeat the subtyping of such strains with the standard IS*6110* RFLP method.

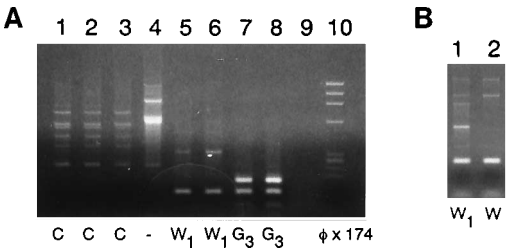

FIG. 2. DRE-PCR applied to clinical isolates. (A) Lanes 1 through 3, RFLP-defined cluster strain C; lane 4, a noncluster strain; lanes 5 and 6, multidrug-resistant *M. tuberculosis* strain W1; lanes 7 and 8, RFLP cluster strain G3. (B) Lanes 1 and 2, W1 and W, respectively (two multidrug-resistant variants with RFLP patterns that differ by just one band).

The IS*6110* RFLP pattern has been shown to be quite stable over time for most patterns in previous studies (2). To assess the stability of DRE-PCR patterns over time, we analyzed two sets of isolates collected from same patients at different intervals. The isolates retained the same DRE-PCR patterns and the same RFLP patterns over a period of at least 4 to 14 months (data not shown).

DRE-PCR is a rapid subtyping method that can be performed directly from the primary growth of *M. tuberculosis* isolates and can be completed in less than 2 days. Because this method requires less DNA, it eliminates the need to subculture the slowly growing mycobacterial strains in liquid media for 3 weeks. Therefore, it may even be possible to carry out this method on nonviable organisms or, if there are enough organisms, directly from sputum samples. DRE-PCR also eliminates the need to use the radioactive labeling techniques which are sometimes employed with RFLP typing methods.

DRE-PCR may serve as a rapid screening method to classify a large number of isolates into clusters for further subtyping by RFLP methods. The RFLP method requires a sophisticated, research- or reference-type laboratory setting. The DRE-PCR method is simpler to perform than RFLP and may not require such a highly sophisticated laboratory setting. This procedure can be used to create a database of agarose gel electrophoresis patterns of the *M. tuberculosis* isolates from a given institution or a community. Such a database may provide a rapid reference source to identify, for instance, drug-resistant strains. Hence, this simplified strain-typing method has clinical as well as epidemiologic applications.

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