Evidence of transmission of *Mycobacterium tuberculosis* by random amplified polymorphic DNA (RAPD) fingerprinting in Taipei City, Taiwan

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Abstract

Aims—To determine, by strain identification of *Mycobacterium tuberculosis*, whether transmission has occurred between individuals or whether new strains are present.

Methods—A rapid protocol for random amplified polymorphic DNA (RAPD) analysis was developed. This protocol was applied to 64 strains of *M tuberculosis* that had been confirmed by culture and microbiological methods.

Results—There are five groups of *M tuberculosis* prevalent in Taipei city, Taiwan. The major types are groups I and III. Groups I and II had been prevalent until the end of last year when, according to our group analysis, they had been eradicated. However, group III was continuously present from the middle of 1995 to the middle of 1996, and group IV was present at the end of both years, which indicated that both groups were transmitted continuously. These clustered strains had demographic characteristics consistent with a finding of transmission tuberculosis. Also, there were 13 of 64 strains with unique RAPD fingerprints that were inferred to be due primarily to the reactivation of infection. In the drug resistance analysis, the major type represented included group III and part of group IV.

Conclusions—Our preliminary data imply, not only that the prevalence of *M tuberculosis* in Taipei city is due to transmission rather than reactivation, but that drug resistance also may play a role in tuberculosis transmission.

(*J Clin Pathol* 1997;50:505–508)

Keywords: *Mycobacterium tuberculosis*; random amplified polymorphic DNA (RAPD); fingerprint; transmission; reactivation

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Accepted for publication 25 February 1997

A reliable method for differentiating strains within *Mycobacterium tuberculosis* would be useful for tracing the transmission route of tuberculosis infection, and would make it possible to determine whether secondary or relapsed tuberculosis develops from exogenous reinfection or by endogenous reactivation. Molecular typing by fingerprinting is an attractive option in clinical microbiology. Most typing systems, including the one that has been accepted as the standard technique, are based on the insertion sequence IS6110. This sequence is integrated at random chromosomal sites in different copy numbers, and thus restriction fragments carrying this marker are highly polymorphic. Unfortunately, IS6110 restriction fragment length polymorphism (RFLP) analysis requires the preliminary growth of cultures to produce microgram quantities of pure unsheared DNA, followed by a lengthy blotting and hybridisation protocol. Therefore, there has been considerable interest in the development of polymerase chain reaction (PCR) based typing systems.

Random amplified polymorphic DNA (RAPD), which is also referred to as arbitrary primer PCR,[1] is a DNA fingerprinting method that has been used successfully to type various bacteria.[1-4] This technique uses PCR to amplify DNA fragments but, in place of primers directed at a specific target, a single randomly selected primer is used. This bands at multiple sites along the genome at low annealing temperatures and products are produced between primers binding in closed proximity to opposite DNA strands. Compared with RFLP and other recently described molecular typing methods, such as mixed linker PCR, RAPD is quicker, requires no previous genetic knowledge about the target organism, and is technically less demanding to perform.

The purpose of this study was to report the use of both RAPD analysis for the typing of strains of *M tuberculosis* and DNA fingerprinting in conjunction with traditional epidemiological methods, to investigate the pathogenesis of resurgent tuberculosis in Taipei City.

Materials and methods

BACTERIAL STRAINS

Sixty four strains of *M tuberculosis* isolated at the General Veterans Hospital in Taipei, Taiwan during a 16 month period (June 1994 to September 1995) were selected for RAPD fingerprinting. These isolates were grown from clinical specimens. The patients admitted to this hospital were likely to be heterogeneous with regard to their geographical origins. Only strains isolated from apparently unrelated patients were included in the present study, and isolates obtained from close contacts (family contacts) of patients known to have tuberculosis were excluded to avoid bias in the estimation of the number of *M tuberculosis* strains. Of the 64 strains, 42 were isolated from

sputum, eight were isolated from lymph nodes, six were isolated from urine, and eight were isolated from cerebrospinal fluid. Two strains were isolated from sputum specimens from the same patient on two separate occasions, six months apart. All isolates were identified as *M tuberculosis* by growth rate, optimal growth temperature, colony morphology, and biochemical testing (niacin accumulation, nitrate reduction, 68°C semiquantitative catalase, thiophen-2 carbocylic acid hydrazide susceptibility, and pyrazinamidase). Susceptibility testing was performed with the Centers for Disease Control and Prevention (Atlanta, Georgia, USA) version of the proportion method.[5]

CULTURE AND GENOMIC DNA PREPARATION

Culture of mycobacteria strains and DNA extraction was performed as described by Salfinger *et al*, with modification.[6] Briefly, mycobacteria strains were grown for three weeks at 37°C in 20 ml of 7H9 Middlebrook medium supplemented with albumin dextrose catalase (Difco, Detroit, Michigan, USA). Cycloserine (1 mg/ml) was added for the last 24 hours of culture. Bacteria were pelleted by centrifugation and resuspended in 500 µl of TE buffer (50 mM Tris, pH 8.1, containing 50 mM EDTA and sucrose 25% wt/vol) and 500 µg/l of lysozyme. Finally, 500 µl of 100 mM Tris (pH 8) containing 400 µg/ml of proteinase K and 1% sodium dodecyl sulphate (SDS) was added followed by 80 µl of cetyltrimethylammonium bromide (CTAB). Samples were vortexed briefly and incubated for 30 minutes at 65°C. An equal volume of phenol and chloroform-isoamyl alcohol was added and precipitated with ethanol using a standard method. The concentration of DNA was determined by spectrophotometric analysis at an optical density of 260 nm.
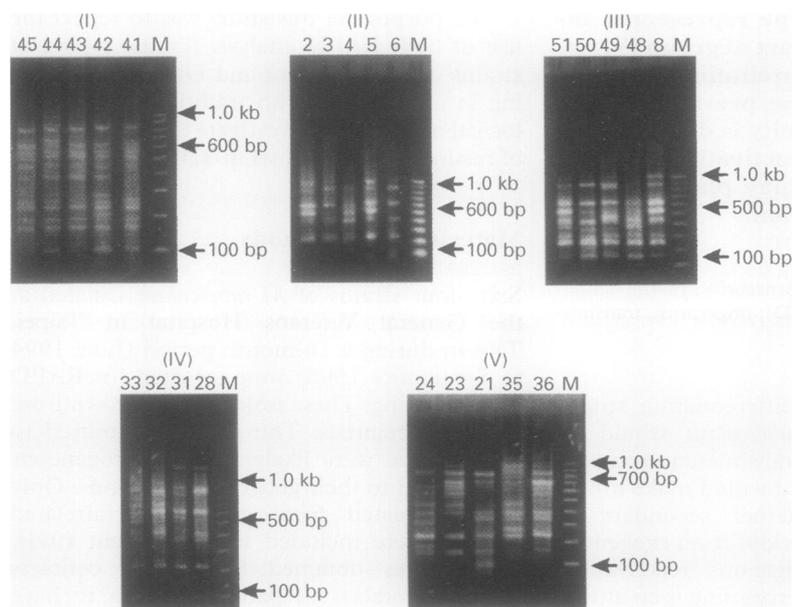
POLYMERASE CHAIN REACTION AMPLIFICATION

Three primers (DKU43, AATCGGGCTG; DKU44, CCGGGGCGGTTC; DKU49, CCGCCGACCGAG) were used either alone or in combination (DKU44 and DKU49 or DKU49 and DKU43). The primer sequences are according to Prasit *et al*.[4] Amplification was carried out using 1 ng of template DNA, 1.5 µM of each primer, and 2.3 units of Taq polymerase in a total volume of 50 µl made up with reaction buffer (Promega, Madison, Wisconsin, USA) containing 10 mM Tris HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.01% wt/vol gelatin, and 200 µM of each dNTP. The reaction mixture was overlaid with 50 µl of mineral oil, incubated for three minutes at 94°C and heated in a DNA thermal cycler 480 (Perkin Elmer, Norwalk, USA) for 35 cycles consisting of: 30 seconds at 94°C, 15 seconds at 40°C and 30 seconds at 72°C. The products were visualised by ethidium bromide staining after electrophoresis in a 2% agarose gel. PCR was carried out in triplicate, at least, on all samples to ensure the reproducibility of the results. Samples with and without *M tuberculosis* DNA were used as positive and negative controls, respectively, in each RAPD experiment.

COMPUTER ANALYSIS AND STATISTICS

Dendron software was used to compare *M tuberculosis* fingerprints. The RAPD patterns were scanned with a Shaper Scanner into the Dendron data file in a Macintosh II computer using a direct illumination adapter. The lanes were corrected for angle distortion, separated, and scanned for pixel density. The RFLP patterns were normalised by equating molecular weight markers between the films. The similarity values (Sab) were calculated between each pair of RAPD fingerprints on the basis of band positions alone by the formula: Sab = (number of bands shared between A and B)/[(number of bands in A) + (number of bands in B) − (number of bands shared between A and B)]. This is a simplification of the formula used by Schmid *et al*[7] and ignores differences in intensity of hybridisation. Identical isolates have a Sab value of 1.0, whereas isolates with no bands in common have a Sab value of 0.0. Sab values between 1.0 and 0.6 were assumed to belong to the same cluster.

Results

Of 67 patients who had culture proved tuberculosis during the study period, the medical records were reviewed for 64 patients with known RAPD fingerprints. All 64 patients were human immunodeficiency virus (HIV) negative. All samples were from Chinese patients who lived in Taipei city (31 men and 33 women). On the basis of their DNA fingerprints (fig 1) 51 of the 64 cases were classified as group I–V: 29.3% were group I, 17.6% were group II, 25.5% were group III, 17.6% were group IV, and 9.8% were group V. The remaining 13 cases were unclassified. The time course showed that group I and II accumulated around June 1994 to January 1995. In contrast, group III occurred from June 1994 to Septem-
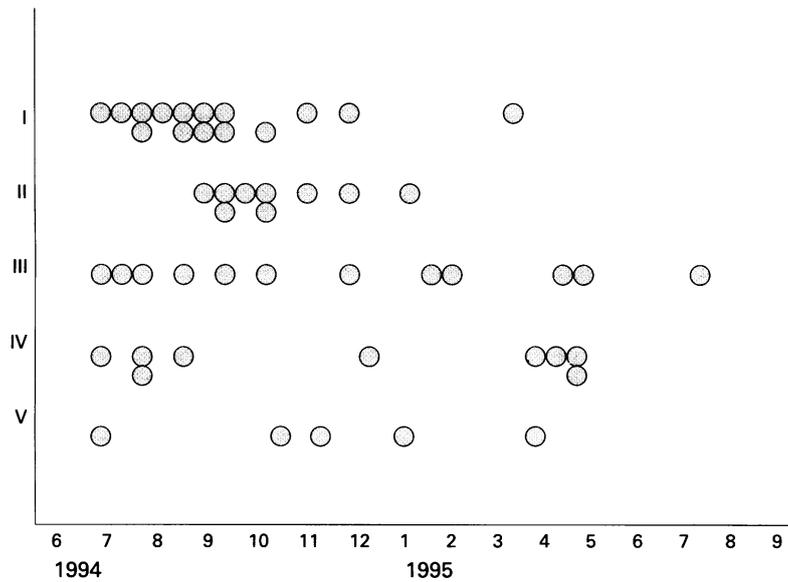

Figure 1 Using DKU44 and DKU49 as primers five groups of Mycobacterium tuberculosis could be distinguished. The number on top of each lane represents the different patients. Lane M, molecular weight markers.

Figure 2   *The time course showed that groups I and II were accumulated at one end (between June 1994 and January 1995), group III was spread over the whole year, group IV accumulated at both ends (July to September 1994 and May to June 1995), and group V was distributed in a sporadic manner.*
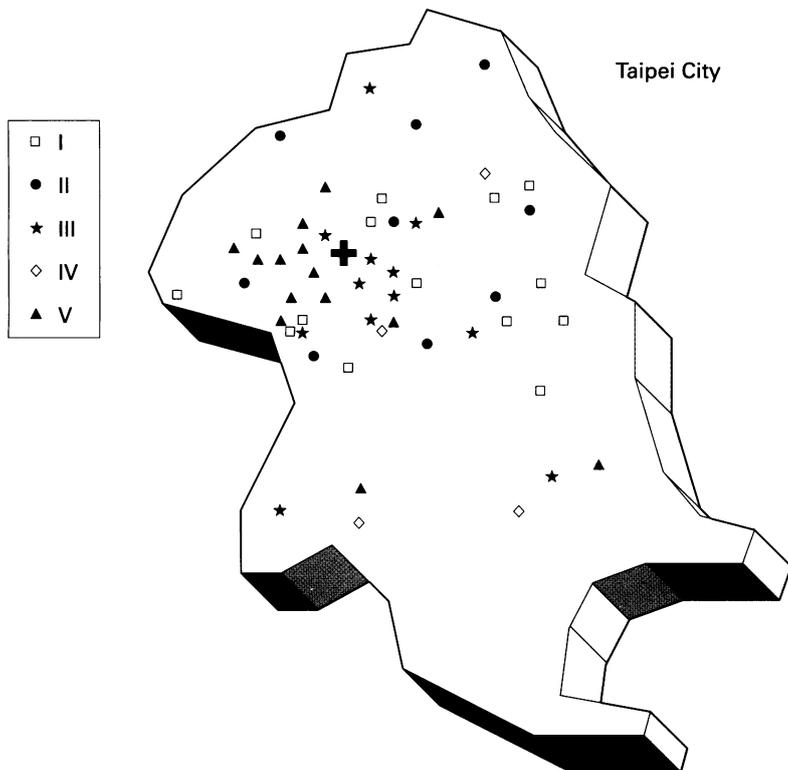

Figure 3   *In the area distribution analysis, groups III and IV were localised to the northwest area of Taipei, groups I and II tended to cluster in a northern direction, and group V showed a sporadic distribution pattern.*

Discussion

Molecular typing RAPD fingerprinting can distinguish strains of *M tuberculosis*. Compared with phage typing, this method yields greater polymorphism, is technically simpler and faster, and requires no radioactive materials. The degree of polymorphism obtained by RAPD was almost the same as that obtained by RFLP.[8] However, RAPD is faster and technically less demanding than most other molecular typing methods and, furthermore, no DNA sequence information is necessary. Also, much smaller amounts of purified DNA (< 25 ng) are required than for methods such as RFLP. In addition, since the bands visualised by RAPD are generally smaller than those in RFLP analysis, theoretically, RAPD can tolerate greater shearing of DNA. This is of practical importance as, in our experience, DNA isolation from mycobacteria is almost impossible to achieve without shearing.

Although RAPD is relatively simple and useful for epidemiological analysis, standardisation of the PCR mixture and conditions are very important for reproducibility. Owing to the presence of mucopolysaccharide in the cell wall of mycobacteria, purification of DNA by phenol chloroform and ethanol precipitation is not sufficient. In our experience, CTAB treatment is the simplest method of generating purified DNA, suitable for RAPD analysis, from boiled cell extracts. Also, it was necessary to standardise the amount of DNA in each RAPD reaction mixture (25 ng of DNA) to ensure that non-specific bands were not present. A range of primer concentrations from 0.5 to 1.5 µM in each reaction mixture is suggested, keeping the number of distinct bands constant. As the concentration of Taq polymerase was increased from 2.5 to 3.0 units, slight variations in the banding patterns were observed. In our experiments, less than 2.5 units of Taq polymerase were used. Finally, even with this standardisation, reproducible profiles were difficult to obtain, and it was necessary to perform triplicate analyses for the true profile differences to be differentiated from experimental variation.

By using a Sab value of 0.6 as the cutoff point, we classified *M tuberculosis* as either clustered or unique. Of the 64 cases of *M tuberculosis*, 51 (79.7%) grouped into five clusters and 13 (21.3%) had unique RAPD fingerprints. The substantial diversity of RAPD patterns among members of the study population suggests that the chance occurrence of identical RAPD fingerprints among unrelated cases would be unlikely. Therefore, we have inferred that cases of tuberculosis caused by strains with identical RAPD fingerprints are caused by recently transmitted disease, and that cases caused by strains with unique RAPD fingerprints are due primarily to the reactivation of infection. In our findings, the isolation of group I strains accumulated between July 1994 and January 1995, and group II accumulated between October 1994 and February 1995. However, group III was persistent throughout the whole year while group IV occurred both at the beginning of 1994 and end of 1995 (fig 2).

ber 1995. Group IV accumulated from July to September 1994 and May to June 1995 (fig 2). In the area distribution analysis, group III and group V were localised within a northwestern area of Taipei city. Whereas group I and II were distributed in a northerly manner, group IV appeared have a sporadic distribution pattern (fig 3). In the drug (isoniazid, refampin, ethambutol, and pyrazinamide) resistance analysis, most occurred in group III (68%) with ethambutol predominant (data not shown). No drug resistance was found in group II.

Groups III and IV represented a focal area of Taipei (fig 3) indicating that they are poorly controlled and may cause a "ping-pong" transmission. In contrast, for the unique (unclassified) RAPD fingerprint strains there was no limited time period tendency or area localisation. This is consistent with a finding of reactivated tuberculosis. The traditional opinion is that the majority of cases of tuberculosis in developed countries result from reactivation during adulthood of an infection contracted decades before[9 10]; it has been estimated that reactivation is responsible for up to 90% of cases in the United States.[11] Recently transmitted tuberculosis was thought generally to have a minor role.[12] However, investigations of institutional outbreaks caused by a single strain have clearly demonstrated that transmission and rapid progression to disease can occur.[13 14] Our findings suggest that the majority of tuberculosis cases in the Taipei city of Taiwan can be attributed to transmission rather than reactivation. Interestingly, our results showed that drug resistance was most common in groups III and IV (data not shown), which may explain, in part, the poor control of these groups.

This study confirms the potential use of RAPD fingerprinting techniques for the investigation of transmission and reactivation. Further research in this area should enable old questions about the relative importance of reactivation and reinfection in the pathogenesis of tuberculosis to be addressed anew.

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