JOURNAL OF CLINICAL MICROBIOLOGY. Nov. 1995. p. 3037-3039
0095-1137/95/$04.00+0
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Vol. 33. No. 11

Restriction Fragment Length Polymorphism Typing of Clinical Isolates of *Mycobacterium tuberculosis* from Patients with Pulmonary Tuberculosis in Madras, India, by Use of Direct-Repeat Probe

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Received 20 January 1995/Returned for modification 21 March 1995/Accepted 3 August 1995

Large numbers of *Mycobacterium tuberculosis* isolates that were obtained from patients' sputa on diagnosis and during follow-up after short-course chemotherapy in Madras, India. have either no copy or only a single copy of IS6110. This poses a limitation for DNA fingerprinting with an IS6110-based probe to determine the frequency of exogenous reinfection versus that of endogenous reactivation. In the present study, we overcame this limitation by using an alternate probe, the direct-repeat element. Comparison of pre- and posttreatment isolates by direct-repeat restriction fragment length polymorphism analysis indicated a high degree of endogenous reactivation among patients who have relapses after the successful completion of chemotherapy.

The pandemic of human immunodeficiency virus (HIV) infection has resulted in an increase in the incidence of tuberculosis in many countries. Evidence indicating an increased rate of recurrence of tuberculosis in HIV-infected patients treated with certain regimens has been reported (7). Controversy prevails regarding the relative importance of endogenous reactivation and exogenous reinfection as the cause of recurrent pulmonary tuberculosis. While epidemlological data obtained from south Indian *Mycobacterium bovis* BCG trials have suggested that the former is more important the latter cannot be ruled out because of the high degree of endemicity of tuberculosis in countries like India. Hence. monitoring the control of tuberculosis by epidemiological investigation is of the utmost importance. Fingerprinting by restriction fragment length polymorphism (RFLP) analysis has been a useful tool for studying the epidemiology of tuberculosis.

RFLP with the widely reported IS6110-based probe has been considered an excellent tool for tracing the transmission of particular strains of *Mycobacterium tuberculosis* during outbreaks (4, 5). Recently, IS6110 was successfully used to trace exogenous reinfection with multidrug-resistant *M. tuberculosis* In patients with advanced HIV infection (10). Although most of the *M. tuberculosis* strains carry multiple copies of IS6110. strains with a single copy or no copy have been reported in many countries (6, 13). Hence, it becomes imperative to use an alternate probe like the direct-repeat (DR) probe. which is a DR element from *M. bovis* BCG, to overcome the limitations of IS6110. We have used the DR probe to fingerprint isolates obtained from patients pretreatment and after a relapse to determine the frequency of exogenous reinfection versus the rare of endogenous reactivation in Madras in southern India.

The pretreatment and relapse isolates of *M. tuberculosis* used in the study were obtained from patients with pulmonary tuberculosis included in controlled clinical trials conducted at the Tuberculosis Research Centre, Madras. The isolates originated from 52 patients who had undergone short-course chemotherapy for 6 or 8 months. The initial isolate was obtained before starting treatment. and the subsequent isolate was obtained after stopping treatment. Patients with quiescent disease at the end of chemotherapy were followed for as long as 60 months by monthly sputum examination for up to 24 months from the start of treatment and at 3-month intervals thereafter in order to determine the stability of bacteriological quiescence and the relapse rates. A patient was classified as having had a bacteriological relapse (true relapse) if two or more positive cultures were obtained during a 6-month period. A culture was considered to be an isolated positive culture if there was no other positive culture in the previous 6 months or in the succeeding 6 months (11).

The isolates were grown in Middlebrook 7H9 medium containing 0.05% Tween 80 and 5% albumin-glucose complex at 37°C. DNA was prepared as described by Baess (1). The DNAs extracted from the isolates were subject IO restriction digestion overnight at 37°C with AluI. The fragments were separated electrophoretically, transferred to charged nylon membranes. and hybridized with the probe. The experiments were conducted twice to confirm the results. The 36-mer DR sequence (DR-r: 5' -GTTTCCGTCCCCTCTCGGGGTTTTGGGTCT GACGAC-3') of *M. bovis* BCG (8) was synthesized (Applied Bio System). This probe was labelled with fluorescein-dUTP by using ECL-3' oligo labelling and detection system (Amersham International plc, Buckinghamshire, United Kingdom).

Coded samples of 52 pairs of clinical isolates obtained pretreatment and titer a relapse were used in the analysis. Eight clinical isolates from these 52 pairs (either pretreatment or posttreatment) were omitted from the analysis because of partial digestion of DSA. Hence, a total of 44 pairs of clinical isolates or 96 [(44 X 2) - 8)] individual clinical isolates were analyzed by RFLP with DR. On analysis of these 96 individual clinical isolates with the DR probe. 30 different patterns were observed, and the number of bands rangedfrom two to seven.

On the basis of the number and the molecular sizes of the bands, similar RFLP patterns were grouped. After decoding the samples, 29 of 44 posttreatment isolates were classified as true relapse isolates, while 15 isolates were classified as iso-

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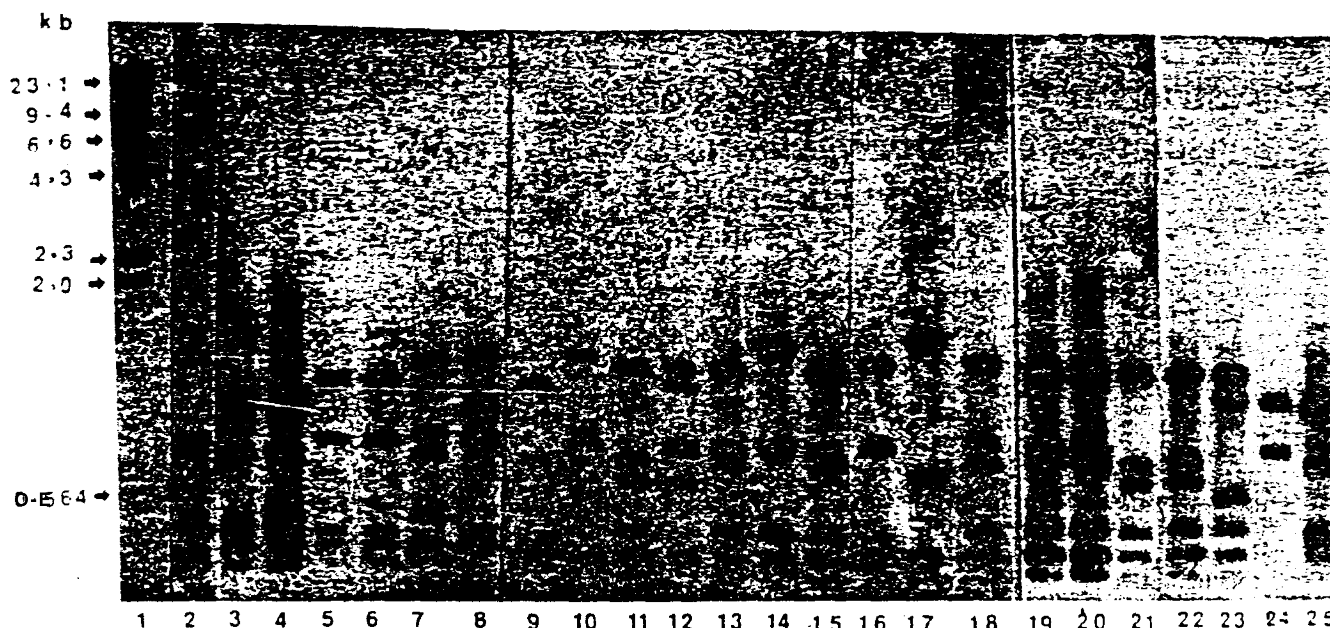

FIG. 1. DNAs from strains with a single copy of IS6110 digested with Alu1 and probed with DR showing multiple bands. Lane 1, bacteriophage lambda HindIII marker: lanes 2 to 25, clinical isolates

lated positive isolates. Among the 29 true relapse isolates, the banding patterns of 20 isolates matched those of their corresponding pretreatment isolates. and the remaining 9 true relapse isolates showed patterns different from those of their pretreatment counterparts. Of the 15 isolated positive isolates, 2 isolates showed patterns that matched those of their pretreatment counterparts and the remaining 13 isolates showed patterns dissimilar to those of their original pretreatment counterparts (Table 1). Among the isolated positive patients, the infecting strain was new in a greater percentage than that noted among the patients with true relapses. The difference between the pretreatment and posttreatment isolates among patients with relapses could arise because of laboratory cross contamination or mixed infections. In order to rule out mixed infections, we fingerprinted tie clones cultured from each of the 10 randomized sputum samples by using the DR probe. All five clones of each isolate showed similar patterns.

In a study being submitted separately, we report that more than 40% of the clinical isolates of *M. tuberculosis* from Madras have either no copy or a single copy of IS6110 (3). Thirty such clinical isolates that had only a single copy of IS6110 were fingerprinted by using DR. DR-RFLP showed two to seven bands, whereas a single band was found with the IS6110 probe (Fig. i). Among these 30 isolates, 17 different patterns were observed. Similarly, four isolates which were negative for IS6110 showed multiple bands with the DR probe. van Soolingen et al. (12) have reported that multiple bands could be resolved for strains with a single copy of IS6110. Our results are similar, thus confirming the observations of van Soolingen et al. (12).

TABLE 1. Comparisons between initial and true relapse isolates or isolated positive isolates by RFLP with the DR probe

Patient group	No. (%) of isolates:		
	Same	Different	Total
True relapse	20 (69)	9 (31)	29
Isolated positive	2 (13)	13 (87)	15

The controversy regarding exogenous reinfection in previously infected patients has existed for several decades (9). However, it was thought to occur rarely because of the immunity conferred by the initial infection. RFLP studies conducted in Hong Kong showed that the patterns for 88% of the isolates from patients with relapses matched those for their pretreatment counterparts. indicating a high frequency, of occurence of infections caused by endogenous reactivation of *M. tuberculosis* (2). Our present study showed a similar trend in that the patterns for 695 of the isolates from patients with relapses matched those for pretreatment isolates.

Small et al. (10) have reported that among the *M. tuberculosis* strains from 11 HIV-positive patients who were infected by strains which developed resistance to antimicrobial agents, the RFLP patterns of six strains remained essentially unchanged, despite the development of drug resistance. Our observations also indicate that the RFLP patterns obtained with the DR probe have no correlation with drug susceptibility or resistance.

In summary, the present study was designed to show the utility of RFLP with the DR probe in characterizing the rate of *M. tuberculosis* reinfection versus the rate of reactivation. These data suggest that among the patient population studied, the rate of relapse caused by reactivation exceeds the rate of relapse caused by reinfection.

R. Sahadevan acknowledges the Council of Scientific and Industrial Research, Government of India, for providing a senior research fellowship during the period of study.

The technical assistance of G. Kubendran of the Bacteriology Department and the secretarial assistance of Shanthi Viswanathan of the Immunology Department, Tuberculosis Research Centre, are gratefully acknowledged.

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