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Bacteriological and Molecular Analysis of Rifampin-Resistant *Mycobacterium tuberculosis* Strains Isolated in Australia

LILLY K. W. YUEN,[1]* DAVID LESLIE,[2] AND PETER J. COLOE[3]

Victorian Infectious Diseases Reference Laboratory, North Melbourne, 3051,[1] 4 The Avenue, Balaclava, 3183,[2] and Applied Biology and Biotechnology Department, RMIT University, Melbourne, 3000,[3] Australia

To develop a better understanding of the epidemiology and molecular biology of rifampin-resistant *Mycobacterium tuberculosis* strains in Australia, 50 clinical isolates (33 rifampin-resistant and 17 rifampin-sensitive strains) cultured between 1990 and 1997 were analyzed by a number of bacteriological and molecular techniques. Examination of the drug resistance profiles of the 33 rifampin-resistant isolates revealed that 91% were resistant to rifampin in combination with resistance to isoniazid, 88% were resistant to rifampin on first isolation, and 81% showed cross-resistance with rifabutin. On the basis of the demographic data provided for the patients infected with the rifampin-resistant strains, 90% of the patients were born overseas. Of these patients, 64% developed clinical symptoms within 5 years of residence in Australia. On a molecular level, analysis of the *rpoB* gene revealed that 97% of the rifampin-resistant isolates had missense mutations within a conserved region of the gene, and eight types of missense mutations were detected. Of the 31 rifampin-resistant isolates that were typed by restriction fragment length polymorphism (RFLP) analysis, 28 distinct patterns were obtained by RFLP analysis with IS*6110*, and three clusters of genetically related isolates were identified. All isolates within the clusters were from patients who were born overseas and who had the same country of origin. The results from this study provide an overview of the current situation of rifampin resistance in Australia and can serve as a basis for continued monitoring of drug-resistant *M. tuberculosis* strains isolated within the country.

Tuberculosis (TB) is a disease of major public health concern worldwide. Despite the availability of highly effective anti-TB drugs and widespread efforts to implement prevention programs, about a third of the world's population (1.7 billion people) are infected with *Mycobacterium tuberculosis* and are at risk of developing clinical TB (17). Each year, approximately 8 million new active cases and 3 million deaths associated with the disease are reported from around the world.

In the 1990s, control of TB has been further complicated by an increase in the incidence of drug-resistant strains of *M. tuberculosis* which have been found in both developing and industrialized countries (1). The most common causes of acquired drug resistance in *M. tuberculosis* are inadequate chemotherapeutic regimens and noncompliance by patients during therapy (20). In the United States, multidrug-resistant (MDR) strains of *M. tuberculosis* have been responsible for a large number of institutional outbreaks, resulting in more than 200 cases of clinical diseases, and over 80% of these cases have occurred in people infected with the human immunodeficiency virus (HIV) (6, 7). In this setting, infections with MDR *M. tuberculosis* are often associated with extraordinarily high mortality rates and rapid progression from diagnosis to death (7).

In Australia, the incidence of TB is low by international standards. Since 1989, the annual incidence of laboratory-confirmed cases has remained between 3 and 4 cases per 100,000 population (2–5), and the rate of drug resistance is less than 18%. The majority of the drug-resistant infections involved either streptomycin or isoniazid alone or in combination with other antibiotics excluding rifampin. In 1996, the rates of resistance to rifampin alone and to rifampin in combination with isoniazid were 0.1 and 2.0%, respectively (3). However, according to Australian Communicable Diseases Intelligence figures (8), approximately 75% of the new TB cases reported in 1996 were among patients born overseas, particularly those born in countries where drug resistance among *M. tuberculosis* strains is an increasing problem. If this trend continues, emergence of MDR *M. tuberculosis* is likely to become a major public health risk in Australia.

This paper represents the results of a study of 33 rifampin-resistant *M. tuberculosis* strains that were isolated in Australia between 1990 and 1997. Rifampin is an important antibiotic used in the treatment of TB, and efficacy of chemotherapy can be markedly reduced when infections are caused by *M. tuberculosis* strains that are rifampin resistant (22). The goals of the present study were (i) to evaluate the drug resistance profiles of these isolates, (ii) to analyze the demographics of the patients from whom the rifampin-resistant isolates were cultured, (iii) to analyze a region within the isolates' *rpoB* gene that had been associated with rifampin resistance (31), and (iv) to evaluate the genetic polymorphisms of these isolates.

MATERIALS AND METHODS

Bacterial samples. A total of 50 clinical isolates of *M. tuberculosis* were selected for this study (Table 1). All isolates were selected on the basis of their rifampin susceptibility, which was determined by reference laboratories. Thirty-three rifampin-resistant isolates were selected from among the 50 clinical isolates received from the mycobacterial reference laboratories of the following institutions in Australia: Victorian Infectious Diseases Reference Laboratory, Melbourne, Victoria, Queensland Diagnostic and Reference Laboratory for Mycobacterial Diseases, The Prince Charles Hospital, Brisbane, Queensland; Institute of Clinical Pathology and Medical Research, Westmead, New South Wales; Western Australian Centre for Pathology and Medical Research, Perth, Western Australia; and Institute of Medical and Veterinary Science, Adelaide, South Australia. Seventeen rifampin-susceptible *M. tuberculosis* isolates were selected as controls from the reference laboratory at the Victorian Infectious Diseases Reference Laboratory. All patient demographic data were collected and were kindly provided by the reference laboratories and the Department of Human Services, Melbourne, Victoria.

* Corresponding author. Mailing address: Victorian Infectious Diseases Reference Laboratory, 10 Wreckyn St., North Melbourne, 3051, Australia. Phone: 61-3-9342-2618. Fax: 61-3-9342-2665. E-mail: lilly.yuen@nwhcn.org.au.

TABLE 1. Number of *M. tuberculosis* isolates selected from the five state mycobacterial reference laboratories in Australia

State	No. of isolates	
	Sensitive	Resistant
Victoria	17	17
New South Wales	0	8
Queensland	0	6
South Australia	0	1
Western Australia	0	1
Total	17	33

On the basis of reports from the Australian Mycobacterium Laboratory Network (2, 3, 5), a total of 4,735 clinical isolates of *M. tuberculosis* were isolated in the country between 1990 and 1996. Of these, 73 isolates were resistant to rifampin. Since 29 of the 33 rifampin-resistant *M. tuberculosis* isolates selected in this study were cultured within the same period, this study has evaluated approximately 40% of all the rifampin-resistant *M. tuberculosis* isolates that have been cultured in Australia.

Drug susceptibility testing. In the early 1990s, the resistance ratio method, as described by the Centers for Disease Control and Prevention, U.S. Department of Health and Human Services (16), was used by the mycobacterial references laboratories in Queensland, New South Wales, and Victoria to determine the first-line drug susceptibilities of all first-time *M. tuberculosis* isolates recovered from patients. Currently, all mycobacterial references laboratories in Australia use the BACTEC 460 (Becton Dickinson) radiometric method for drug susceptibility testing (28).

The Etest (AB BIODISK, Solna, Sweden) was used to determine the rifampin MICs for all the *M. tuberculosis* isolates selected. The test was performed by using a modification of the method described by Wanger and Mills (34). Briefly, McFarland no. 3 standard suspensions of *M. tuberculosis* were lawn cultured onto Middlebrook 7H10 agar plates (Difco, Detroit, Mich.) enriched with 10% Middlebrook oleic acid-albumin-dextrose-citrate supplement (Micro Diagnostics, Coorparoo, Queensland, Australia) and were incubated at 37°C in an atmosphere of 5% CO_2. After 24 h, a rifampin Etest strip was placed onto the center of each plate and the plate was reincubated under the same conditions until sufficient growth occurred (2 to 4 weeks) to determine the MIC.

All *M. tuberculosis* isolates resistant to rifampin were tested to determine their susceptibility to the remaining first-line anti-TB antibiotics (isoniazid, ethambutol, and pyrazinamide) and to rifabutin. Susceptibility to isoniazid and ethambutol was tested by the resistance ratio method, and susceptibility to rifabutin was tested by the absolute concentration method. Both of these methods were performed as described by the Centers for Disease Control and Prevention (16). To determine the susceptibilities of the selected isolates to pyrazinamide, the pyrazinamidase screening method described by Wayne (35) was used. All isolates which demonstrated pyrazinamidase activity were considered susceptible to pyrazinamide.

PCR. The oligonucleotide primers described by Telenti et al. (31), TR1 (5′-TACGGTCGGCGAGCTAT-3′) and TR2b (5′-TACGGCGTTTCGATGAAC C-3′), were used to amplify a 411-bp fragment from a conserved region within the *rpoB* gene. Chromosomal DNA was amplified in a 50-μl mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.75 mM MgCl₂, dimethyl sulfoxide, each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 0.5 μM, and 1.25 U of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, Md.). The reaction was performed in an automated thermal cycler (Hybaid, Middlesex, United Kingdom) by using a program consisting of an initial denaturation at 94°C for 3 min, followed by 25 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C and an additional extension step at 72°C for 5 min. The PCR products were then purified with Geneclean III (Bio 101, Inc., La Jolla, Calif.) according to the manufacturer's instructions.

DNA sequencing. Sequencing of the PCR product was performed with the Sequenase DNA Sequencing Kit (Amersham) according to the manufacturer's instructions. Both DNA strands were sequenced in the presence of 10% dimethyl sulfoxide with either oligonucleotide primer TR9 (5′-TCGCCGCGATCAAGG AGT-3′) or oligonucleotide primer TR8 (5′-TGCACGTCGCGGACCTCC A-3′) (31).

RFLP typing. Chromosomal DNA extraction and Southern blot hybridization were performed as described by Ross et al. (26). To determine the patterns of the rifampin-resistant *M. tuberculosis* isolates by restriction fragment length polymorphism (RFLP) analysis with IS*6110* (RFLP-IS*6110* patterns), chromosomal DNA was digested with restriction endonuclease *Pvu*II and was hybridized with a probe prepared from a 247-bp PCR product of IS*6110* (26). The chromosomal DNAs of *M. tuberculosis* isolates with similar IS*6110*-RFLP patterns were redigested with *Alu*I and hybridized with a previously described polymorphic GC-rich repetitive sequence probe, *p*TBN12 (26). Both IS*6110* and *p*TBN12 probes

were labelled with digoxigenin with a nucleic acid labelling system supplied by Boehringer Mannheim GmbH (Mannheim, Germany).

Computer analysis. Southern blots were scanned at a resolution of 0.2 mm by using Molecular Analyst software and an imaging densitometer (model GS-670; Bio-Rad Laboratories, Richmond, Calif.). The images created were saved as TIFF files and were analyzed with Gelcompar (version 4.0) software (Applied Maths, Ghent, Belgium). RFLP patterns were normalized with an *Eco*RI-*Hin*dIII-digested bacteriophage DNA molecular weight marker (Boehringer Mannheim), which was used as an external size marker. Banding patterns were compared with the Gelcompar software by the unweighted pair group method with arithmetic means clustering method and the Jaccard coefficient method and were verified visually.

Definition of clustering. A cluster of *M. tuberculosis* isolates was defined as two or more isolates which either (i) exhibit the same number of copies of the IS*6110* fragment with identical molecular weights and identical *p*TBN12 RFLP patterns or (ii) showed one additional or one missing IS*6110* fragment and identical *p*TBN12 RFLP patterns. Genetic relatedness is considered a possibility when strains have a similarity coefficient of ≥75% (27).

RESULTS

Drug resistance profiles. (i) Rifampin MICs. The Etest was used to determine the rifampin MICs for 30 rifampin-resistant *M. tuberculosis* isolates (3 isolates were nonviable) (Table 2). Of the *M. tuberculosis* isolates tested, the MICs for 2 (isolates R1 and R9) were 32 μg/ml and the MICs for 28 were ≥256 μg/ml. For all rifampin-susceptible isolates of *M. tuberculosis* selected as controls, rifampin MICs were ≤1 μg/ml.

Twenty-nine (88%) of the *M. tuberculosis* isolates studied were resistant to rifampin on first isolation (primary resistance) and four acquired rifampin resistance within 3 to 6 months of the patients' commencement of chemotherapy. For the *M. tuberculosis* isolates with acquired rifampin resistance, the rifampin MICs increased from <1 μg/ml on primary isolation to >256 μg/ml within 3 to 6 months.

(ii) First-line drug susceptibilities. Analysis of the first-line drug susceptibility profiles for the 33 rifampin-resistant *M. tuberculosis* isolates revealed that only three were resistant to rifampin alone; all remaining isolates were also resistant to isoniazid (Table 2). Of the rifampin- and isoniazid-resistant isolates, 14 (42%) were susceptible to the remaining two first-line anti-TB drugs, 11 (33%) were also resistant to ethambutol, 4 (12%) were also resistant to pyrazinamide, and 1 (3%) isolate was resistant to all four of the first-line anti-TB drugs. Since *M. tuberculosis* is defined as MDR when it is resistant to both rifampin and isoniazid, approximately 91% of the rifampin-resistant *M. tuberculosis* isolates studied were MDR.

(iii) Rifabutin susceptibility. To assess the extent of cross-resistance between rifampin and rifabutin, rifabutin susceptibility was determined for 30 of the 33 rifampin-resistant strains of *M. tuberculosis* (3 strains were nonviable) (Table 2). Of the rifampin-resistant *M. tuberculosis* isolates tested, 6 were susceptible to rifabutin at concentrations of 1.0 μg/ml, while 24 showed cross-resistance with rifabutin at concentrations of >2 μg/ml. The rifabutin susceptibility of one of the nonviable rifampin-resistant strains of *M. tuberculosis* had previously been reported to be resistance. Hence, approximately 81% of *M. tuberculosis* strains with high levels of resistance to rifampin demonstrated cross-resistance to rifabutin. All rifampin-susceptible *M. tuberculosis* control strains were also susceptible to rifabutin.

Patient demographics. (i) Age and sex distributions. Of the 33 patients from whom the rifampin-resistant *M. tuberculosis* isolates were cultured, 18 were men and 15 were women. The male-to-female ratio was 1.2:1. With the exception of one person whose age was not known, these patients were between 18 and 70 years of age, and the median age was 40.3 years. The age distribution was as follows: 5 (16%) patients, between 18 and 24 years of age; 14 (47%) patients, between 25 and 45

TABLE 2. Characteristics of rifampin-resistant *M. tuberculosis* strains isolated in Australia between 1990 and 1997

Strain no.	Country of birth	First-line drug to which strain is resistant[a]	Rifabutin susceptibility[b]	Rifampin MIC (μg/ml)	RFLP cluster	Mutated codon	Mutation
R1	Timor	I, R	S	32	A	522	TCG→CAG
R2	Timor	I, R	S	>256	A	522	TCG→CAG
R3	Timor	I, R, E	R	>256	B	526	CAC→GAC
R4	Timor	I, R, Pz	R	>256	B	526	CAC→GAC
R5	Timor	I, R, E (Int), Pz	R	Nonviable	B	526	CAC→GAC
R6	Turkey	I, R	R	>256		526	CAC→GAC
R7	Australia	R	R	>256		526	CAC→TAC
R8	Ethiopia	I, R	R	>256		526	CAC→TAC
R9	Vietnam	I, R, E	S	32		526	CAC→CTC
R10	Timor	I, R, Pz	S	>256		531	TCG→TTG
R11	Vietnam	I, R, E	R	>256		531	TCG→TTG
R12	Former Yugoslavia	I, R, E	R	>256		531	TCG→TTG
R13	Vietnam	R	R	>256		531	TCG→TTG
R14	Australia	I, R	R	>256		516	GAC→GTC
R15	Turkey	I, R	S	>256		516	GAC→GTC
R16	Vietnam	I, R, E	R	>256		572	ATC→TTC
R17	Wales	I, R	S	>256			
R18	Egypt	I, R, E	R	>256		526	CAC→CGC
R19	Philippines	I, R	R	>256		531	TCG→TTG
R20	Vietnam	I, R	Nonviable	Nonviable		526	CAC→TAC
R21	Unknown	I, R, E	R	>256		531	TCG→TTG
R22	Korean	I, R, Pz	R	>256		531	TCG→TTG
R23	Unknown	I, R	R	>256		531	TCG→TTG
R24	Unknown	I, R, E	R	>256		516	GAC→TAC
R25	Australia	I, R	R	>256		526	CAC→TAC
R26	Philippines	I, R, E	Nonviable	Nonviable		531	TCG→TTG
R27	Laos	I, R	R	>256		531	TCG→TTG
R28	Papua New Guinea	I, R	R	>256	C	531	TCG→TTG
R29	Vietnam	I, R	R	>256		531	TCG→TTG
R30	Papua New Guinea	R	R	>256	C	531	TCG→TTG
R31	Philippines	I, R	R	>256		531	TCG→TTG
R32	Korea	I, R, E	R	>256		531	TCG→TTG
R33	Vietnam	I, R, E	R	>256		531	TCG→TTG

[a] I, isoniazid; R, rifampin; E, ethambutol; Pz, pyrazinamide; Int, intermediate resistance.
[b] S, sensitive; R, resistant.

years of age; and 13 (41%) patients, between 46 and 70 years of age. Of the female patients, 12 (80%) were less than 45 years old and 3 were greater than 50 years old. Of the male patients, 7 were less than 45 years old and 10 were over 45 years old.

(ii) HIV infection status. The patient's HIV infection status is not usually provided to the laboratories. From the demographic details provided for the 33 patients, for only 1 was the HIV status disclosed, and that patient was HIV positive. The patient was a 42-year-old Caucasian male who had been born in Australia and who presented with disseminated TB. However, details of other illnesses and treatment for this patient were not available. The *M. tuberculosis* isolate cultured from the patient was resistant to rifampin, rifabutin, ciprofloxacin, clarithromycin, and cycloserine.

(iii) Country of origin. The countries of origin were known for 30 patients, and 27 of them were born overseas (Table 2). The countries from which these patients migrated include Timor (*n* = 6), Papua New Guinea (*n* = 2), Philippines (*n* = 3), Korea (*n* = 2), Laos (*n* = 1), Vietnam (*n* = 7), Egypt (*n* = 1), Ethiopia (*n* = 1), Turkey (*n* = 2), Wales (*n* = 1), and the former Yugoslavia (*n* = 1). Among the patients born overseas, three were members of the same Timorese family. Of the three patients born in Australia, one was a 55-year old woman and two were men ages 42 and 63 years, respectively, at the time of diagnosis.

(iv) Period of residence in Australia prior to diagnosis. The date of arrival in Australia was known for 25 of the 27 patients

born overseas. The interval from the time of arrival in Australia to the time of isolation of *M. tuberculosis* for these 25 patients ranged between 0 months and 23 years. Of these patients, 10 received a diagnosis of TB within the same year of arriving in Australia (2 within the same month), 6 received a diagnosis of TB within 2 to 5 years, 4 received a diagnosis of TB within 6 to 10 years, and 5 received a diagnosis of TB after 11 or more years.

Genetic analysis. (i) *rpoB* gene analysis. A 69-bp region of the *rpoB* genes from all *M. tuberculosis* strains included in the study was analyzed by DNA sequencing. All sequence data obtained were compared with the sequence obtained from the GenBank database (accession no. L27989). Of the 17 rifampin-sensitive *M. tuberculosis* isolates sequenced, no mutations were detected within the 69-bp segment. On the other hand, eight different types of mutations, consisting of either one or two nucleotide substitutions, were detected within the same 69-bp segment in 31 of the rifampin-resistant *M. tuberculosis* (Table 2).

By using the *rpoB* codon numbering system described by Telenti et al. (31), all mutations were detected within codons 516, 522, 526, and 531 (Fig. 1). The most frequently encountered mutation was in codon 531 (52%), from TCG (Ser) to TTG (Leu). The next most frequently encountered mutation was in codon 526 (32%). The nucleotide substitutions found within this codon include CAC (His) to GAC (Asp), TAC (Tyr), CTC (Leu), or CGC (Arg). In strains R1 and R2, the
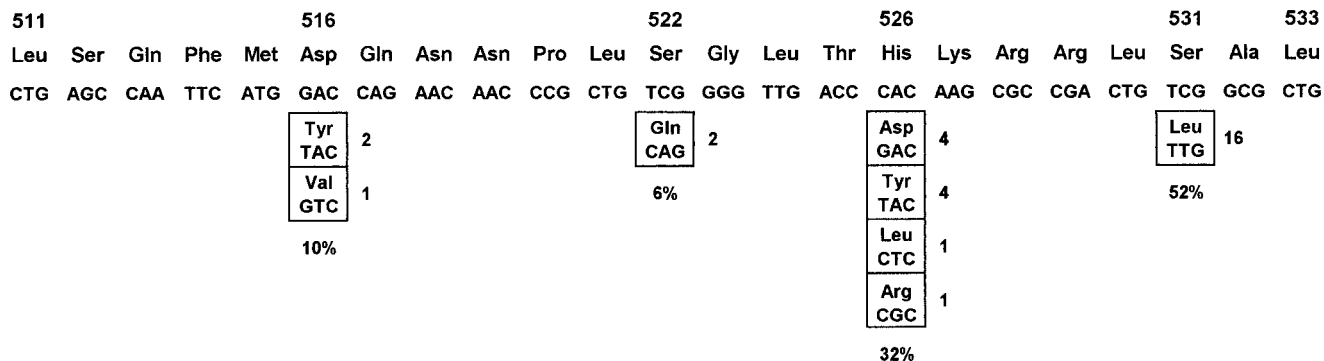
FIG. 1. Nucleotide substitutions detected within the 69-bp region of the *rpoB* gene in rifampin-resistant *M. tuberculosis* isolates. The wild-type sequence is shown across the top, and the positions of point mutations and substituted amino acids are shown in the boxes below. The numbers on the right of the boxes are the number of strains with the same mutation. The frequency of mutations detected within the codons is indicated below the boxes. The codons were numbered on the basis of the system used by Telenti et al. (32).

substitution of two nucleotide in codon 522 from TCG (Ser) to CAG (Gln) has not been reported previously.

Two rifampin-resistant *M. tuberculosis* isolates had no mutation within the 69-bp region. Of these two isolates, one (isolate R16) had a nucleotide substitution in codon 572 from ATC (Ile) to TTC (Phe), and one (isolate R17) had no mutation within the entire 411-bp fragment amplified.

(ii) RFLP analysis. To assess the genetic polymorphism of the rifampin-resistant *M. tuberculosis* strains, DNA was digested with *Pvu*II and was hybridized with the IS*6110* probe. The RFLP patterns produced were then analyzed with the Gelcompar software program, and a dendrogram was constructed to illustrate the genetic relatedness among the isolates. Two rifampin-resistant isolates (isolates R5 and R20) were nonviable and so could not be typed by RFLP analysis.

A total of 28 distinct RFLP patterns were obtained for the 31 rifampin-resistant isolates of *M. tuberculosis* tested (data not shown). Three groups of rifampin-resistant isolates exhibited very similar or identical IS*6110*-RFLP patterns (Fig. 2). To confirm their genetic relatedness, the chromosomal DNAs of the isolates were redigested with *Alu*I and were hybridized with the *p*TBN12 probe. With this GC-rich repetitive sequence probe, all *M. tuberculosis* isolates within the groups (designated clusters A, B, and C) produced identical RFLP patterns.

The isolates of *M. tuberculosis* in clusters A and B were initially cultured in Victoria. Cluster A consists of two isolates (isolates R1 and R2) with 92% similar IS*6110*-RFLP patterns. Direct comparison of the RFLP profiles revealed an extra 1.25-kb band in isolate R2 (Fig. 2). Both *M. tuberculosis* isolates were cultured from specimens from patients who originated in East Timor, and the same mutation was detected within codon 522 of their *rpoB* genes. Cluster B consists of three *M. tuberculosis* isolates (isolates R3, R4, and R5) cultured from specimens from members of the same Timorese family. However, chromosomal DNA was available for RFLP analysis of only isolates R3 and R4, and the IS*6110*-RFLP patterns produced were 94% similar according to the results obtained with the Gelcompar software. Direct comparison of the RFLP patterns revealed an extra 1.24-kb band in isolate R4 (Fig. 2). All three *M. tuberculosis* isolates had the same mutation within codon 526 of their *rpoB* genes. Cluster C consists of two *M. tuberculosis* isolates (isolates R28 and R30) initially cultured in Queensland (Fig. 2). It was the only cluster with isolates with identical IS*6110*-RFLP patterns. Both isolates were cultured from patients who originally came from Papua

New Guinea, and the same mutation was detected in codon 531 of their *rpoB* genes.

DISCUSSION

Rifampin is the most potent sterilizing antibiotic used for the treatment of TB. It has bactericidal activity against extracellular tubercle bacilli as well as the active and the near dormant bacilli that may reside within macrophages. In the case of drug-susceptible strains of *M. tuberculosis*, the combined use of the first-line anti-TB drugs, rifampin, isoniazid, ethambutol, and pyrazinamide, will most often result in successful cures. Rifampin and isoniazid are the most active of the first-line anti-TB drugs, and *M. tuberculosis* strains that are resistant to both of these antibiotics are considered MDR (13). In general, multidrug resistance is acquired in two steps, with the first step being the development of isoniazid resistance rather than rifampin resistance (23), suggesting that rifampin resistance can be used as a surrogate marker for the detection of MDR *M. tuberculosis* (9, 31). Since 91% of the rifampin-resistant strains examined in this study were also resistant to isoniazid, this indicates that rifampin resistance is a good predictor for multidrug resistance in Australia.

Analysis of demographic data revealed that 27 of 33 (90%) patients infected with the rifampin-resistant *M. tuberculosis* strains were born outside Australia. Twenty-five of the 27 patients born overseas were infected with MDR *M. tuberculosis* strains, and the majority of them (19 of 30 strains) were from Southeast Asia. Of the six Timorese patients with drug-resistant TB, contact tracings have shown that three of them are members of the same family, suggesting intrafamily transfer, and RFLP analysis has confirmed that the isolates cultured from specimens from these patients (cluster B) are genetically related (Fig. 2). In addition, RFLP analysis revealed that two other rifampin-resistant isolates cultured from specimens from Timorese patients (cluster A) are genetically related to each other.

An important factor that was observed in this study is the short period of time that patients with drug-resistant TB had resided in Australia prior to diagnosis; 40% of the patients presented with drug-resistant TB within the same year as their arrival in Australia (two patients presented with TB within a month of their arrival in the country). Given the short time in the country prior to clinical diagnosis, it is highly probable that these patients were already infected with the drug-resistant
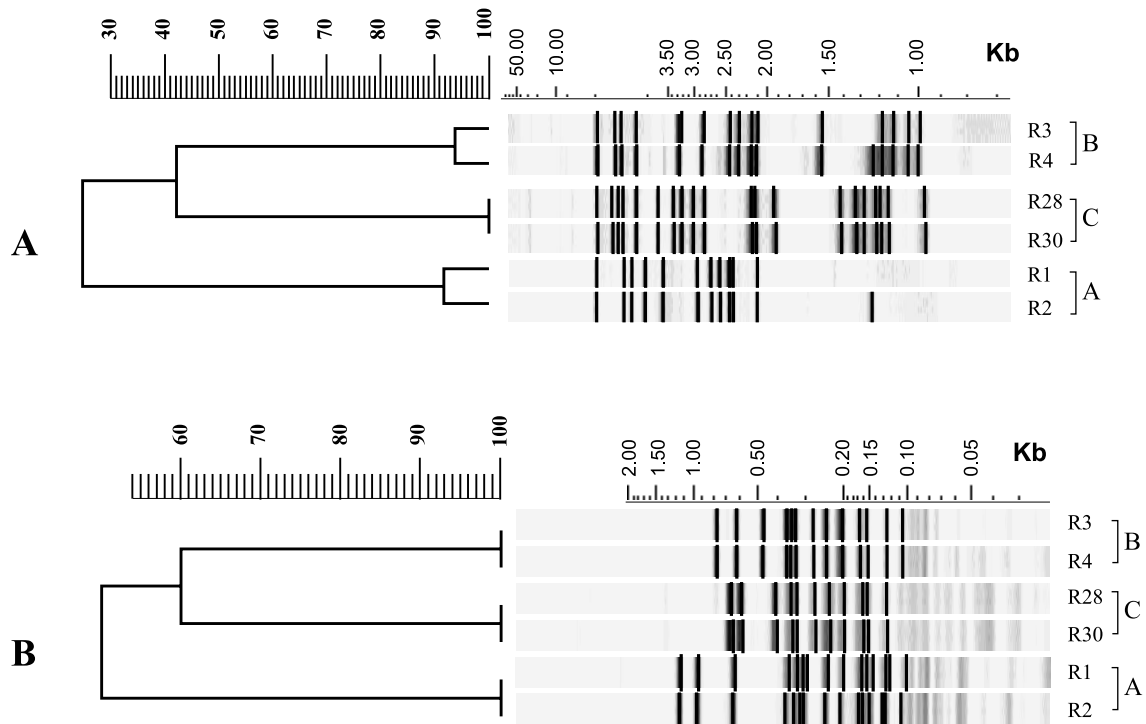
FIG. 2. Dendrograms of Southern blots produced with Gelcompar software showing the RFLP patterns and the percent similarities between the three clusters (designated clusters A, B, and C) of rifampin-resistant *M. tuberculosis* isolates that are epidemiologically related. The RFLP profiles were produced by digesting chromosomal DNA with *Pvu*II and hybridization with the IS*6110* probe (A) and by digesting chromosomal DNA with *Alu*I and hybridization with the *p*TBN12 probe (B).

strains on arrival. It is unfortunate that there are no adequate details on whether the patients had been given chemotherapy previously. Such data are important in determining whether the patients were initially infected with an MDR *M. tuberculosis* strain or whether the emergence of MDR *M. tuberculosis* was due to inadequate or inappropriate antibiotic treatment that resulted in acquisition of antibiotic resistance by the initial infecting isolate.

Of the 33 patients with rifampin-resistant TB studied, isolates from 29 (88%) were found to be resistant to rifampin on primary isolation. Although such patients represented only a very low proportion of the total number of the patients with TB nationwide, the possibility that drug-resistant strains were introduced into a highly susceptible population is a significant concern for public health. These data not only illustrate the need for a rapid and specific test for detection of MDR *M. tuberculosis* but also raise the question as to whether the present TB screening protocol for immigrants needs to be modified to include a wider group of people such as visitors who will reside in the country for long periods.

The HIV infection status of patients is not usually made available to the laboratories. On the basis of the data provided, only one case of rifampin-resistant TB was associated with HIV infection. Although the source of this drug-resistant *M. tuberculosis* strain is not known, to date there has been no evidence of further transmission of this strain into the HIV-infected population in Australia.

The molecular resistance mechanism for rifampin has been deciphered. On the basis of work conducted with *Escherichia coli*, rifampin resistance is the result of mutations within certain regions of the *rpoB* gene (clusters N, I, II, and III), which encodes the β-subunit of RNA polymerase (14, 18). Substitu-

tions of amino acids within any of these regions may result in either conformational changes to the rifampin binding site or a loss of affinity for the drug. In effect, rifampin can no longer bind to the enzyme to inhibit its function, hence resulting in resistance. Recently, the sequence of the *rpoB* gene of *M. tuberculosis* has been determined, and different types of mutations associated with rifampin resistance have been reported (24, 31, 32). The majority of mutations were detected within a 69-bp segment of the gene which is equivalent to the cluster I region in *E. coli* (30).

Analysis of the conserved region within the *rpoB* gene of 33 rifampin-resistant *M. tuberculosis* isolates showed that 31 of them have missense mutations within the 69-bp segment of the gene, and a total of eight different types of mutations were detected within codons 516, 522, 526, and 531. These findings differed from those of previous studies in that the mutations are restricted to only four codon sites within the *rpoB* gene (15, 24, 31, 32). The lack of diversity could be due to the small number of rifampin-resistant strains that were studied and the limited population from which isolates were obtained. Interestingly, all rifampin-resistant *M. tuberculosis* isolates analyzed were resistant to high levels of rifampin ($\geq$32 μg/ml), and this finding is consistent with previous reports in which amino acid substitutions within codons 516, 526, and 531 have been associated with high-level rifampin resistance in *M. tuberculosis* (24, 31, 32). Since 94% of the rifampin-resistant *M. tuberculosis* isolates studied have missense mutations within a confined segment of the *rpoB* gene, molecular techniques could be used to analyze this segment of the gene for the detection of mutations associated with rifampin resistance in *M. tuberculosis*. However, techniques such as DNA sequencing, PCR–single-strand conformation polymorphism analysis, and PCR-hetero-

duplex formation analysis, have been used in previous studies to analyze this region of the *rpoB* gene, but their use is not practical for routine diagnostic laboratories. Recently, a line probe assay (Inno-LiPA-Rif.TB) from Innogenetics, Ghent, Belgium, has been released commercially as a diagnostic test that can rapidly identify clinical isolates as a member of the *M. tuberculosis* complex and determine the presence of point mutations within the *rpoB* gene. Evaluations performed with this kit showed that it is highly sensitive and specific and is a good means of identifying the rifampin susceptibility status of clinical isolates (10, 21). However, it was shown in this study that a small percentage of rifampicin-resistant *M. tuberculosis* strains still do not possess mutations within the 69-bp segment of the *rpoB* gene, and these resistant strains would not have been detected by the line-probe assay or a similar assay. This may be due to a mutation outside the segment of the *rpoB* gene analyzed or an entirely different mechanism of resistance. While genetic analysis of the *rpoB* gene is useful for rapid detection of rifampin resistance in *M. tuberculosis* isolates, particularly in outbreak situations, susceptibility testing results obtained should always be confirmed by conventional drug sensitivity testing methods.

Rifabutin is a derivative of the rifamycin group of antibiotics and has been reported to have some activity against rifampin-resistant *M. tuberculosis* (19). It is used as a second-line anti-TB drug for the treatment of drug-resistant strains of *M. tuberculosis*. However, rifabutin and rifampin have similar modes of action (12), and cross-resistance between the two antibiotics has been shown to occur (11). In the present study, 81% of the isolates showed cross-resistance between rifampin and rifabutin. This finding is very similar to that from a study conducted by Heifets and Iseman (11), in which 88% of rifampin-resistant *M. tuberculosis* strains examined had cross-resistance to rifampin and rifabutin. In a recent study, Williams et al. (36) demonstrated that mutations within codons 526 and 531 of the *rpoB* gene could contribute to rifamycin cross-resistance in *M. tuberculosis*. However, no particular type of mutation detected within the *rpoB* gene of rifampin-resistant isolates could be associated with rifabutin resistance in the present study. In addition, rifampin MICs for two *M. tuberculosis* isolates (isolates R9 and R10) were high, and isolates with mutations in codons 526 and 531 of the *rpoB* gene remained susceptible to rifabutin in vitro, but the clinical efficacy of rifabutin treatment in this setting is untested.

RFLP analysis is a powerful technique that enables epidemiological studies to be performed on a molecular level. The IS*6110* insertion sequence is a transposable element that is found only in species of the *M. tuberculosis* complex (33) and is widely used as a genetic marker for DNA fingerprinting of clinical *M. tuberculosis* isolates to monitor bacterial strain transmission and to confirm laboratory contaminations (25, 27, 29). In general, *M. tuberculosis* isolates that are genetically related demonstrate very similar or identical RFLP patterns, while nonrelated strains demonstrate significantly different patterns. By this technique, three clusters with very similar IS*6110*-RFLP patterns were identified among the rifampin-resistant *M. tuberculosis* isolates screened. Within each cluster, all the isolates were cultured from patients who were born overseas and who had the same country of origin. In addition, there was no evidence of transmission of MDR *M. tuberculosis* strains from individuals born overseas into the wider Australian population. The IS*6110*-RFLP patterns obtained for the rifampin-resistant strains of *M. tuberculosis* enabled a database to be established, and clinical isolates of *M. tuberculosis* with identical or very similar profiles could be deemed resistant to rifampin.

Rapid detection of rifampin resistance is very important for the timely and successful treatment of TB. The use of standard drug combination therapy against infections caused by MDR *M. tuberculosis* may result in prolonged or progressive disease and increased risk of transmission of drug-resistant strains of *M. tuberculosis* into the general community. The 33 rifampin-resistant *M. tuberculosis* isolates characterized in this study represented approximately 40% of the clinical rifampin-resistant *M. tuberculosis* isolates cultured nationwide between 1990 and 1997, and the data obtained can provide a general overview of the current situation of rifampin resistance in Australia. These results can also serve as a basis for continued monitoring of drug-resistant *M. tuberculosis* strains isolated in Australia.

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