

Characterization of rifampicin-resistant *Mycobacterium tuberculosis* in Taiwan

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Sixty-three rifampicin-resistant (Rif^r) isolates of *Mycobacterium tuberculosis* from Kaohsiung, Taiwan, were analysed for mutations in the core region (69 bp, codons 511–533) of the *rpoB* gene. Some 84.1% (53/63) of the resistant isolates showed mutations in this region, especially in codons 531 (41.5%), 526 (18.9%), 516 (15.1%) and 533 (7.5%). Five novel alleles of a total of 16 different types of mutations were identified in Rif^r isolates. Ten Rif^r isolates (15.9%) exhibited no mutations in the core region of *rpoB*. Also, they did not show mutations in another 365 bp fragment (codons 99–220) of *rpoB*. The agar proportion method was used to determine the relationship between the degree of rifampicin resistance and alterations in the core region of *rpoB*. The results revealed that the mean MIC was 92.38 µg ml⁻¹ for the 53 isolates with a mutation in the core region, whereas the mean MIC of the other 10 isolates without mutations was only 24.8 µg ml⁻¹. This indicates that the isolates with mutations in the core region had higher levels of resistance than those without mutations in this region. IS6110 restriction fragment length polymorphism (RFLP) was used for typing of 55 Rif^r *M. tuberculosis* isolates. Isolates contained two to 19 copies of IS6110, with sizes ranging from 600 to 16 000 bp. The majority (85%) contained six to 16 copies. No strains lacking IS6110 were found. A total of 54 of 55 RFLP types were defined at the 90% similarity level. The observation of varied IS6110-associated banding patterns indicates that an outbreak of drug-resistant tuberculosis did not occur in this area.

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INTRODUCTION

Among infectious diseases, tuberculosis (TB) is one of the most frequent causes of death in the world, with more than 2 million TB-related deaths reported each year (Dye *et al.*, 1999). In 1997, an estimated 8 million new cases were reported and approximately 2 billion people, one third of the world's population, were infected with *Mycobacterium tuberculosis* (Dye *et al.*, 1999). TB is one of the most important communicable diseases in Taiwan. It was the twelfth most common cause of death in 1998. The rates of incidence and mortality of TB per 100 000 population were respectively 64.89 and 6.93 in the same year (Kan, 2000). In recent years, the control of TB has been impeded by the emergence of drug-resistant *M. tuberculosis* strains (Kan, 2000).

Rifampicin has proven to be an effective antituberculosis

agent and its use has greatly shortened the duration of chemotherapy for the treatment of TB. Rifampicin resistance heralds higher rates of treatment failure and death for the patient and a poor outcome if the isolate is also resistant to isoniazid (Goble *et al.*, 1993). The action of rifampicin is believed to interfere with transcription in bacteria by binding to the β subunit of RNA polymerase (the product of the *rpoB* gene) (Jin & Gross, 1988). Mutations in certain highly conserved codons encoded by *rpoB* account for 'single step' high-level resistance to rifampicin in *M. tuberculosis* (Telenti *et al.*, 1993). More than 90% of rifampicin-resistant (Rif^r) *M. tuberculosis* strains from different countries appear to harbour specific point mutations located in a 69-bp (core) region of *rpoB* (codons 511–533) (Mani *et al.*, 2001; Matsiota-Bernard *et al.*, 1998; Ohno *et al.*, 1996; Williams *et al.*, 1998).

Since Rif^r strains are a matter of great concern in Taiwan (Chiang *et al.*, 1998; Wang & Lin, 2001), it is of interest to study the molecular basis of rifampicin resistance in these local resistant isolates. One recent report concerned with the genotype of *rpoB* of Rif^r *M. tuberculosis* isolates from northern Taiwan reported four substitutions and one insertion (Qian *et al.*, 2002). The mutation patterns among large

Abbreviations: RFLP, restriction fragment length polymorphism; TB, tuberculosis; UPGMA, unweighted-pair group method using average linkage.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the novel *M. tuberculosis rpoB* alleles found in this study are AF312232–AF312236.

numbers of isolates from Kaohsiung, in southern Taiwan, acquired in this work should allow a better understanding of any hot-spot regions on this gene for suitable rapid diagnosis and proper control of TB in Taiwan.

The correlation between the level of resistance to rifampicin and different mutational sites in the 69-bp core region of *rpoB* seemed to vary in different regions (Mani *et al.*, 2001; Williams *et al.*, 1998; Ohno *et al.*, 1997). Thus, the MICs of rifampicin for Rif^r strains of *M. tuberculosis* with known alterations in *rpoB* were determined.

Resistance to rifampicin has previously been associated with mutations in the early region of *rpoB* in addition to mutations in the middle or end regions of this gene in *Escherichia coli* (Jin & Gross, 1988; Lisitsyn *et al.*, 1984). Recently, Heep *et al.* (2000) also reported a single, novel amino acid mutation at codon 149 in a clinical isolate of *Helicobacter pylori* that developed rifabutin resistance during therapy. A homologous mutation was also found in some Rif^r *M. tuberculosis* isolates with wild-type sequences in the 69-bp region (Heep *et al.*, 2000). Therefore, isolates without mutations in the core region of the *rpoB* gene were examined for potential mutations in the early part of *rpoB*.

The genetic polymorphism of Rif^r *M. tuberculosis* isolates was evaluated by IS6110 DNA fingerprinting (van Embden *et al.*, 1993). The 1355-bp insertion sequence IS6110, belonging to the IS3 family of insertion elements of enterobacteria, has been found exclusively in members of the *M. tuberculosis* complex (Thierry *et al.*, 1990). It is widely used as a probe for strain differentiation (Cave *et al.*, 1991), confirming suspected cases of transmission (Edlin *et al.*, 1992; Zaza *et al.*, 1995), detection of laboratory contamination (Ramos *et al.*, 1999; Dunlap *et al.*, 1995) and distinguishing between exogenous re-infection or endogenous reactivation (Shafer *et al.*, 1995; Small *et al.*, 1993). In this work, we compared the RFLP patterns of Rif^r *M. tuberculosis* isolates from patients in Kaohsiung by using IS6110 as a probe in order to examine the epidemiological relatedness of the isolates.

METHODS

***M. tuberculosis* strains.** Clinical Rif^r isolates of *M. tuberculosis* were obtained from the Taiwan Provincial Kaohsiung Chronic Disease Prevention Center, the Kaohsiung Medical University Hospital and the Kaohsiung Chang Gung Memorial Hospital during 1996–1998, representing at least one-third of all cases in Kaohsiung. They were identified by conventional methods that included routine microscopy, culture and positive nitrate and niacin tests (Kent & Kubica, 1985). The standard *M. tuberculosis* strains H37Rv^T (ATCC 27294^T) and Mt.14323 (van Embden *et al.*, 1993) and rifampicin-susceptible strains were employed as controls.

Drug-susceptibility testing. The test followed the modified agar proportion method using Middlebrook 7H10 agar plates to determine the susceptibility of *M. tuberculosis* clinical isolates (Kent & Kubica, 1985; Inderlied & Nash, 1996). The rifampicin concentration was 1.0 µg ml⁻¹. If the number of colonies that grew on the rifampicin-containing plate was < 1% of the number of colonies that grew on a drug-free medium, the isolate was defined as susceptible to rifampicin. The isolate was resistant if the number was > 1%.

To determine the MIC of rifampicin of each isolate, serial twofold dilutions of rifampicin were incorporated in 7H10 agar at concentrations that ranged from 0 to 256 µg ml⁻¹. Sets of quadrant Petri dishes (one quadrant in each plate contained drug-free medium) were inoculated with each isolate. Plates, tested in duplicate, were incubated at 37 °C in the presence of 5% CO₂. Each plate was checked weekly and results were recorded after weeks 3 and 4. The MIC was defined as the lowest concentration of drug that inhibited growth of the bacterial population by more than 99%.

DNA extraction. Genomic DNA was extracted as described by van Soolingen *et al.* (1994) with modifications. Bacteria were harvested from the Löwenstein–Jensen slopes, heat-killed and incubated with lysozyme (1 h, 37 °C) followed by digestion with 50 µg proteinase K in 10% SDS for 30 min at 65 °C. A further incubation with CTAB/NaCl for 10 min at 65 °C was followed by partition using chloroform/isoamyl alcohol (24:1, v/v). Genomic DNA was extracted with phenol/chloroform and precipitated with 100% ethanol.

PCR amplification of mycobacterial strains. Aliquots of purified mycobacterial DNA (10–20 ng) were added to PCR reagents. The 157-bp *rpoB* fragment (nt 1846–2002) was amplified by using the primers Tr8 (5'-TGCACGTCGCGGACCTCCA-3') and Tr9 (5'-TCGCC GCGATCAAGGAGT-3') as described previously (Telenti *et al.*, 1993). To target a 365-bp fragment (early part) of *rpoB* in *M. tuberculosis*, primers Tb176F (5'-CTTCTCCGGTTCGATGTCGTTG-3') and Tb176R (5'-CGCGCTTGTCGACGTCAAACCTC-3') were used (Heep *et al.*, 2000).

Purification of PCR products and DNA sequencing. Template DNA was purified from the PCR products by using QIAquick PCR purification kit (Qiagen). Nucleotide sequencing was performed with the ABI PRISM Dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) and the reactions were analysed on an ABI PRISM 373A DNA sequencer.

Restriction fragment length polymorphism (RFLP). Fifty-five Rif^r *M. tuberculosis* isolates were available for RFLP analysis. DNA fingerprinting was performed as described previously (van Embden *et al.*, 1993; van Soolingen *et al.*, 1994). Briefly, genomic DNA was digested with *Pvu*II for 4–6 h before being separated electrophoretically in a 1% agarose gel in 1× TBE running buffer at a constant voltage of 32 V for 16 h. Next, the separated DNA fragments were transferred onto Hybond nylon membrane (Amersham). Hybridization was then performed using a digoxigenin-labelled 245-bp fragment of IS6110 (25 ng ml⁻¹) as the DNA probe. Membranes were hybridized overnight under stringent conditions at 65 °C. Hybridized digoxigenin-labelled probe was detected with the DIG luminescent detection kit (Boehringer Mannheim) following the manufacturer's instructions.

As recommended previously (van Embden *et al.*, 1993), *Pvu*II-digested genomic DNA of *M. tuberculosis* reference strain Mt.14323 served as an external control of the hybridization conditions and a mixture of *Pvu*II-digested supercoiled DNA ladder and *Hae*III-digested φX174 DNA served as an internal marker.

Cluster analysis. DNA fingerprints were analysed by the GelCompar software (version 4.0; Applied Maths). A cluster was defined as two or more isolates with identical RFLP patterns when five or more copies of IS6110 were present. Autoradiograms were digitized by using a scanner with an optical resolution of 300 d.p.i. The sizes of IS6110 RFLP fragments were calculated by comparison of their mobilities with those of a set of internal markers of known molecular sizes (van Soolingen *et al.*, 1994). The accuracy of the normalization procedure was controlled by comparing the IS6110 fingerprint patterns of reference strain *M. tuberculosis* Mt.14323. The fingerprint patterns were analysed for similarity by using the Dice coefficient and a dendrogram was

calculated with the unweighted-pair group method using average linkage (UPGMA) according to the supplier's instructions. Band positions were determined by using the peak-finder function of the GelCompar software and were controlled manually by comparison with the original IS6110 autoradiogram.

Statistical analysis. The Mann–Whitney test was used for comparing mean MICs with the software package SPSS (SPSS Institute); $P < 0.05$ was considered significant.

RESULTS

In this study, 63 Rif^r isolates and 12 rifampicin-sensitive (Rif^s) clinical isolates were first examined for mutations in a 157-bp fragment of *rpoB*. Of 63 Rif^r isolates, 84.1% (53/63) of the isolates showed mutations in this region. When compared with the published sequence, 15.9% (10/63) of the Rif^r isolates and 12 Rif^s strains exhibited no mutations in this region. The most prevalent mutation sites were in codons 531 (41.5%), 526 (18.9%), 516 (15.1%) and 533 (7.5%) (Table 1). The mutations found within the 69-bp core region of *rpoB* in 53 of the resistant isolates are shown in Fig. 1.

A total of 16 different mutations and five novel alleles were identified within a 157-bp region of *rpoB* of 53 Rif^r clinical isolates (Table 1; Fig. 1). Of the five novel alleles, one allele revealed changes of two bases in codon 516 (GAC→TTC; Asp→Phe) and the other four alleles showed point mutations

in two separate codons: codons 511 (CTG→CCG; Leu→Pro) and 516 (GAC→GGC; Asp→Gly); codons 526 (CAC→TAC; His→Tyr) and 521 (CTG→CTA; Leu→Leu); codons 526 (CAC→CGC; His→Arg) and 529 (CGA→CAA; Arg→Gln); and codons 516 (GAC→AAC; Asp→Asn) and 533 (CTG→CCG; Leu→Pro) (Table 1).

The agar proportion method was used to understand the relationship between the degree of resistance to rifampicin and the mutation site. Growth of the 12 sensitive strains was inhibited at rifampicin concentrations $\leq 0.25 \mu\text{g ml}^{-1}$, indicating the susceptibility of these strains. Isolates with mutations in the 69-bp core region of *rpoB* were highly resistant to rifampicin, with MICs ranging from 8 to 256 $\mu\text{g ml}^{-1}$, and the mean MIC was 92.38 $\mu\text{g ml}^{-1}$ (Table 1). The MICs for the remaining 10 resistant isolates were between 2 and 128 $\mu\text{g ml}^{-1}$ and the mean MIC was 24.8 $\mu\text{g ml}^{-1}$. Our results revealed that strains with mutations in the 69-bp core region had significantly higher MICs than those without mutations in this region ($P < 0.05$).

For the 10 Rif^r isolates without mutations in the 157-bp fragment, the possibility of mutations occurring in the early region (365-bp fragment) of *rpoB* was then examined. PCR-single-strand conformation polymorphism analysis and DNA sequencing exhibited patterns the same as that of *M.*

Table 1. Frequency of mutations in Rif^r *M. tuberculosis* isolates and their levels of rifampicin susceptibility

Mutated codon(s)	Specific mutation(s)	<i>n</i> (%) [*]	MIC ($\mu\text{g ml}^{-1}$) [†]
Resistant			
531	TCG(Ser)→TTG(Leu)	22 (41.5)	32 (1), 64 (9), 128 (11), 256 (1)
526	CAC(His)→GAC(Asp)	3 (5.7)	128
	CAC(His)→CGC(Arg)	2 (3.8)	32
	CAC(His)→TAC(Tyr)	5 (9.4)	32 (1), 128 (3), 256 (1)
516	GAC(Asp)→GTC(Val)	3 (5.7)	32 (1), 64 (2)
	GAC(Asp)→TAC(Tyr)	3 (5.7)	16 (1), 64 (2)
	GAC(Asp)→TTC(Phe) [‡]	2 (3.8)	128
533	CTG(Leu)→CCG(Pro)	4 (7.5)	8 (1), 64 (2), 128 (1)
511	CTG(Leu)→CCG(Pro)	1 (1.9)	32
522	TCG(Ser)→TTG(Leu)	1 (1.9)	8
511, 516	CTG(Leu)→CCG(Pro), GAC(Asp)→GGC(Gly) [‡]	2 (3.8)	64
	CTG(Leu)→CCG(Pro), GAC(Asp)→TAC(Tyr)	1 (1.9)	128
526, 521	CAC(His)→TAC(Tyr), CTG(Leu)→CTA(Leu) [‡]	1 (1.9)	128
526, 529	CAC(His)→CGC(Arg), CGA(Arg)→CAA(Gln) [‡]	1 (1.9)	64
516, 526	GAC(Asp)→GCC(Ala), CAC(His)→GAC(Asp)	1 (1.9)	128
516, 533	GAC(Asp)→AAC(Asn), CTG(Leu)→CCG(Pro) [‡]	1 (1.9)	64
None	–	10	2 (2), 4 (1), 8 (2), 16 (2), 32 (2), 128 (1)
Susceptible			
None	–	12	< 0.25

^{*}Percentages of isolates carrying mutations in the core region are given in parentheses.

[†]Where strains with the same mutation(s) gave different MIC values, the number of strains exhibiting each MIC is given in parentheses.

[‡]Novel pattern of substitution.

511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533
Leu	Ser	Gln	Phe	Met	Asp	Gln	Asn	Asn	Pro	Leu	Ser	Gly	Leu	Thr	His	Lys	Arg	Arg	Leu	Ser	Ala	Leu
CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTC	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
Pro					Val					Leu	Leu				Tyr			Gln		Leu	Pro	
CCG					GTC					CTA	TTG				TAC			CAA		TTG	CCG	
					Tyr										Asp							
					TAC										GAC							
					Gly										Arg							
					GGC										CGC							
					Asn																	
					AAC																	
					Phe																	
					TTC																	
					Ala																	
					GCC																	
					15.1%										18.9%						41.5%	7.5%

Fig. 1. Mutations in a 69-bp region of *rpoB* in Rif^r *M. tuberculosis* isolates. The codon numbering system for *E. coli rpoB* was used. The mutated codons with corresponding amino acids are indicated underneath the original sequence. The frequencies of the four most frequent mutation positions are indicated below.

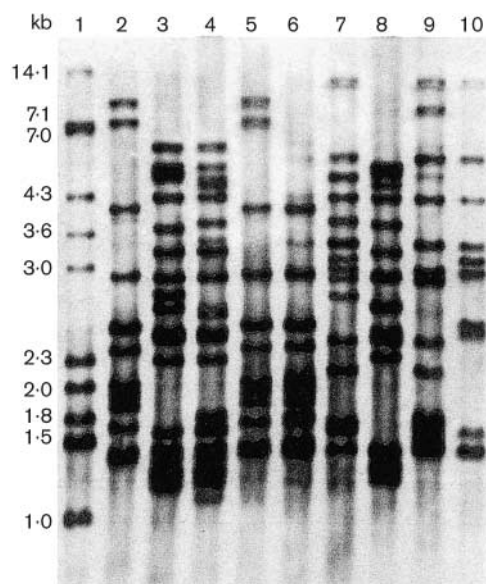


Fig. 2. IS6110-based RFLP patterns of nine Rif^r *M. tuberculosis* isolates from patients. DNA was digested with the restriction enzyme *Pvu*II. The probe was a 245-bp DNA fragment of IS6110. Lanes: 1, reference strain *M. tuberculosis* Mt.14323; 2–10, clinical *M. tuberculosis* isolates.

tuberculosis Mt.14323 (data not shown). These results demonstrated that these 10 Rif^r isolates did not show mutations in the 157-bp or 365-bp fragment of *rpoB*.

In this study, 55 Rif^r *M. tuberculosis* isolates were analysed by the standardized DNA fingerprinting method with IS6110 as a genetic marker. The IS6110 fingerprint patterns generated were highly variable (Fig. 2). The number of copies of IS6110 per isolate varied from two to 19, with sizes ranging from 600 to 16 000 bp. The majority of the 55 isolates (85%), contained six to 16 copies, with a median of 10 bands (Fig. 3). No isolates lacking IS6110 were found.

The similarities of all 55 IS6110 fingerprint patterns were then analysed. A total of 54 of 55 RFLP types were defined at the 90% similarity level (Fig. 4). Two (C14 and C389) of 17 isolates with mutations in codon 531 had 95.5% similarity, with different drug-resistance patterns. Isolate C14 was susceptible to kanamycin and ethambutol, while C389 was resistant to both of these drugs. Close relatedness was not

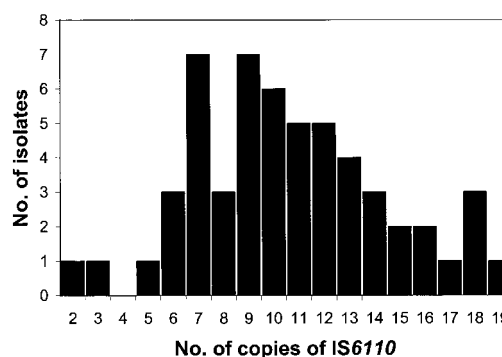


Fig. 3. Number of IS6110 copies in the DNA of 55 Rif^r *M. tuberculosis* isolates.

observed between other resistant isolates carrying mutations in either of the two other highly mutated sites, codons 526 and 516 (data not shown).

DISCUSSION

It was observed that the majority (75.5%) of Rif^r *M. tuberculosis* isolates contained missense mutations that led to substitutions of amino acids at Ser-531 (41.5%), His-526 (18.9%) or Asp-516 (15.1%) in the core region of *rpoB*. While codon 531 is the most common site of nucleotide substitutions worldwide, mutations at the other two prevalent sites (codons 526 and 516) in our strains occur at different frequencies in strains from other geographical regions (Qian *et al.*, 2002; Pozzi *et al.*, 1999; Taniguchi *et al.*, 1996). Distinctly, about 8% of Rif^r *M. tuberculosis* isolates displayed point mutations in codon 533 in this study. However, this missense mutation in codon 533 only occurred at low frequency (< 3.3%) or no mutations at all were reported in other geographical regions (Mani *et al.*, 2001; Pozzi *et al.*, 1999; Bartfai *et al.*, 2001; Valim *et al.*, 2000). These differences may be attributed to geographical genetic differences in Rif^r *M. tuberculosis* strains and the transmission of these strains among patients in different countries.

Unexpectedly, 10 (15.9%) of the 63 Rif^r clinical isolates in this study showed no mutations in the sequenced 157-bp region of *rpoB*, despite the fact that these isolates were resistant to rifampicin. A comparable high frequency (20%; 4/20) of Rif^r isolates with no mutations in this core region was also reported recently from northern Taiwan (Qian *et al.*,

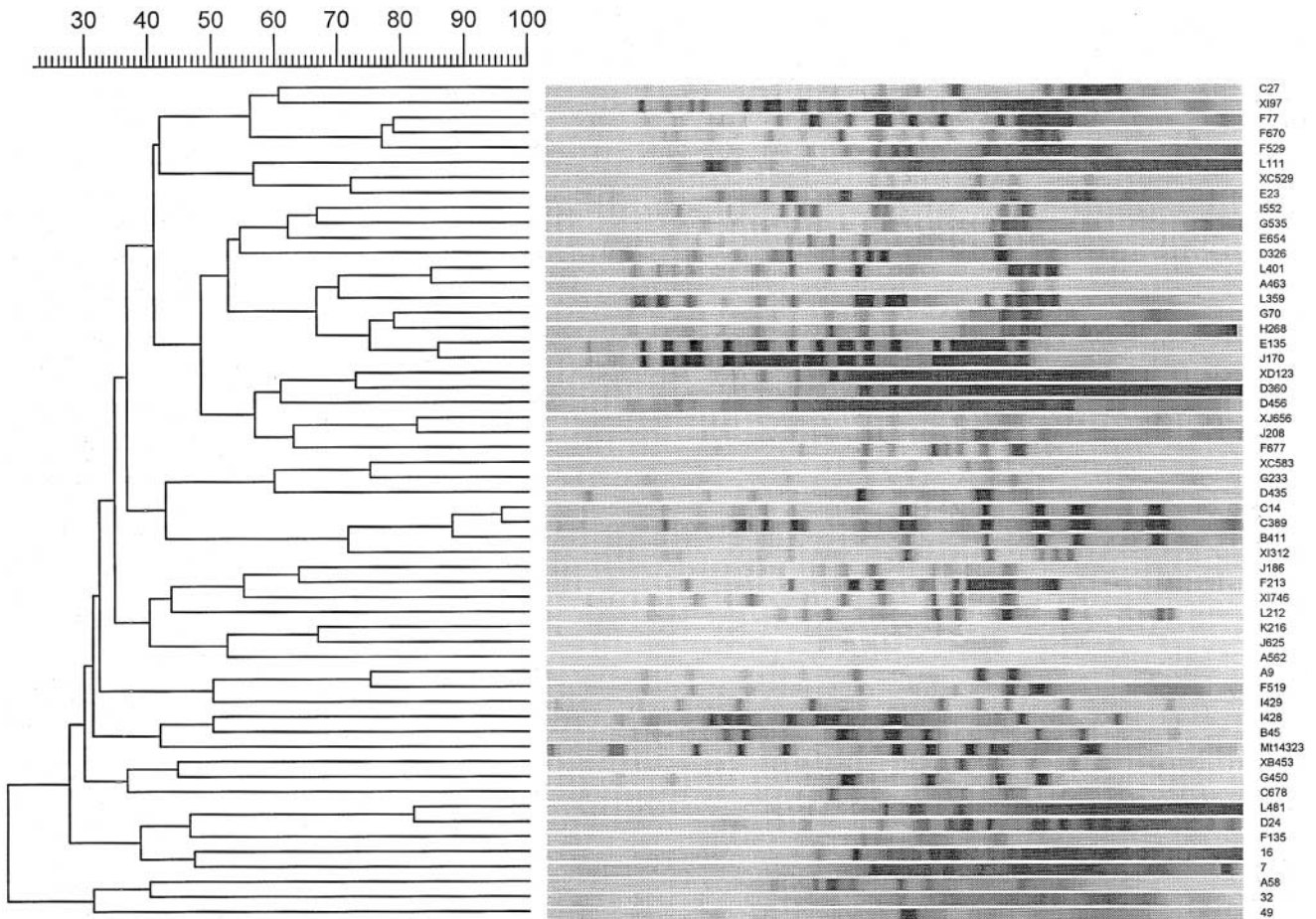


Fig. 4. Dendrogram of *M. tuberculosis* isolates ($n = 55$) by RFLP typing.

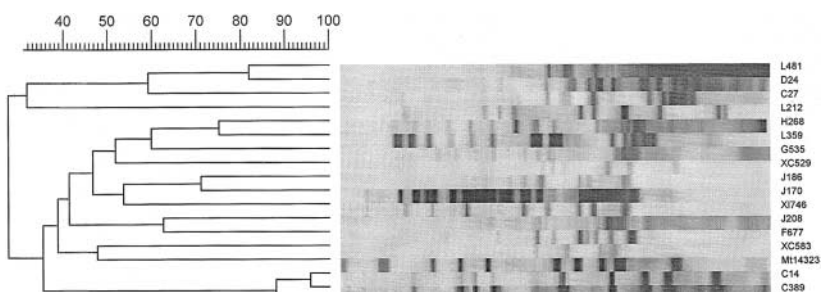


Fig. 5. Dendrogram of *M. tuberculosis* isolates with mutations in codon 531 ($n=17$).

2002). The frequency is comparatively higher than those that have been reported for Rif^r isolates from other geographical areas. More than 90 % of Rif^r strains from other regions had mutations located in the 69-bp core region (Telenti *et al.*, 1993; Mani *et al.*, 2001; Matsiota-Bernard *et al.*, 1998; Ohno *et al.*, 1996; Williams *et al.*, 1994). DNA sequencing of the early part of *rpoB* in these 10 Rif^r isolates showed no mutations in a 365-bp region. This indicated the possible occurrence of an alteration outside the two regions of *rpoB* examined, such as mutations in V176F (Heep *et al.*, 2001) and codon 381 (Taniguchi *et al.*, 1996). Among other

explanations, several additional genes may be involved in rifampicin resistance. A change in the antibiotic permeability of a membrane or in metabolism could also give rise to the rifampicin-resistant phenotype (Hui *et al.*, 1977; Konno *et al.*, 1973).

In general, amino acid substitution in the 69-bp core region led to higher levels of resistance to rifampicin than those isolates without mutations in this region ($P < 0.05$, Table 1). Some investigators have even demonstrated that different levels of resistance are associated with different mutational

sites in this region (Ohno *et al.*, 1996, 1997; Taniguchi *et al.*, 1996). Thus, strains with mutations in either codon 531 or 526 were usually highly rifampicin-resistant, as revealed in this report and others (Ohno *et al.*, 1996; Williams *et al.*, 1998; Taniguchi *et al.*, 1996). However, in contrast to previous findings on mutations in codon 533 (Ohno *et al.*, 1996), our four isolates with amino acid substitution in this codon (Leu→Pro) did not show a consistently low level of resistance (MIC < 2 µg ml⁻¹). Likewise, high MICs (>128 µg ml⁻¹) for isolates with mutations in codon 516 or 533 were also observed in India (Mani *et al.*, 2001). Thus, the association between particular mutational sites on *rpoB* and the drug susceptibility of multidrug-resistant *M. tuberculosis* strains is not apparent in some areas, including Taiwan. Noticeably, the same mutation in the same codon in the 69-bp region did not reflect the same level of drug resistance in these resistant isolates (Table 1; Ohno *et al.*, 1996). Some other additional factor(s) might contribute to the variation in drug resistance (Hui *et al.*, 1977; Konno *et al.*, 1973). Thus, sequencing of *rpoB* is not completely able to replace traditional methods of susceptibility testing to detect the level of rifampicin resistance of *M. tuberculosis*.

The results showed that Rif^r *M. tuberculosis* strains from this area were highly polymorphic. The majority of the TB cases were therefore presumed to be the result of re-activation of previously contracted *M. tuberculosis* infections. Furthermore, our results showed that most isolates, even with the same mutated codon, did not have similar patterns or locations of IS6110 copies (Fig. 5). This suggests that acquisition of rifampicin resistance followed the infection of a rifampicin-susceptible strain in this work. In contrast to a report from Cape Town, where most drug-resistant TB cases represented new infections (van Rie *et al.*, 2000), our results did not reveal an outbreak of Rif^r *M. tuberculosis* strains in this area.

In conclusion, five novel alleles, as well as some universal prevailing mutations, in the core region of *rpoB* were observed in our Rif^r *M. tuberculosis* isolates. The mutated codon might affect the MIC for rifampicin, although other additional factors might contribute to variation in drug resistance. Analysis of RFLP patterns of our Rif^r *M. tuberculosis* isolates suggested that latent re-activation rather than active transmission accounted for most TB cases. Nevertheless, we consider that the establishment of a DNA fingerprinting bank will be extremely helpful for tracing recent sources of infection, for the control of a possible spread of multidrug-resistant organisms and for the surveillance of TB in general.

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