CHAPTER IV

RESULTS

1. Determination of rifampin susceptibility testing by the radiometric method

Out of 108 *M. tuberculosis* isolates, 56 were found to be rifampin resistant and 52 were found to be rifampin susceptible.

2. Amplification of *M. tuberculosis* DNA by PCR

Target DNAs were extracted from sixteen reference and 108 clinical isolates of *M. tuberculosis* by using freeze-boiling method. A 10 µl portion of each target DNA was subsequently subjected to the PCR. The 305-bp DNA fragment was clearly visualized on an agarose gel. An example of an ethidium bromide-stained gel containing amplified DNA from 6 clinical isolates was shown in Fig 1.

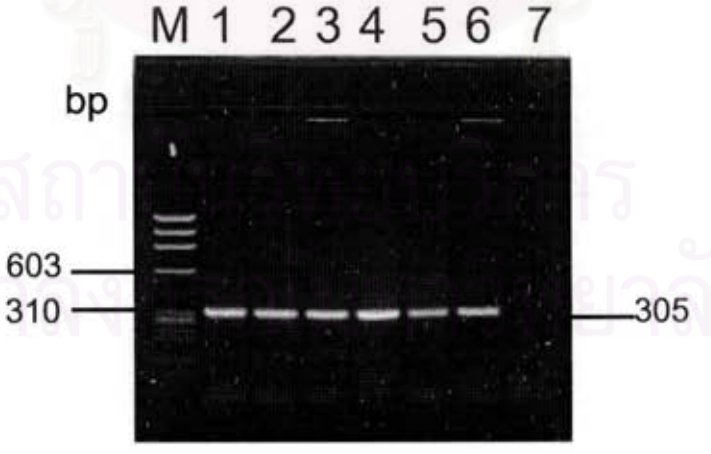

Fig 1. Amplified products (305 bp) by PCR from *M. tuberculosis* clinical isolates. Lane M, *Hae III*-digested phag ϕX174 DNA as molecular size markers; lanes 1-6, *M. tuberculosis*; lane 7, negative control

3. Determination of DNA sequence

Determination of the 305-bp DNA sequences of 16 reference strains of *M. tuberculosis* revealed the correct results as those shown in table 3. Evaluation of 52 *M. tuberculosis* isolates with a rifampin susceptible phenotype revealed a *rpoB* sequence identical to that of the rifampin-susceptible strain *M. tuberculosis* H37Rv. By contrast, thirteen different mutations within a 63-bp region of *rpoB* gene were identified among 56 *M. tuberculosis* isolates with a rifampin-resistant phenotype (Fig 2). Most were point mutations (missense) involving eight codons. Mutation of Ser 531 was present in 28 of the 56 (50%) isolates where a mutation was identified (Fig 3, Table 4). No silent mutations were observed in *rpoB* region examined for any of the *M. tuberculosis* isolates analyzed in this study. This finding reports three new types of mutation (AAC to AAA at codon 519, TCG to CAG at codon 522, and GGG to GAG at codon 523) (Fig 4). One isolate had point mutation in two codons (GGG to GAG at codon 523 and CAC to CTC at codon 526) (Fig 5), and 1 isolate showed double point mutations of two adjacent bases in one codon (TCG to CAG at codon 522) (fig 6). The result showed 100% of the mutations was occured in this DNA region. No isolate was found to contain deletion or insertion mutation.

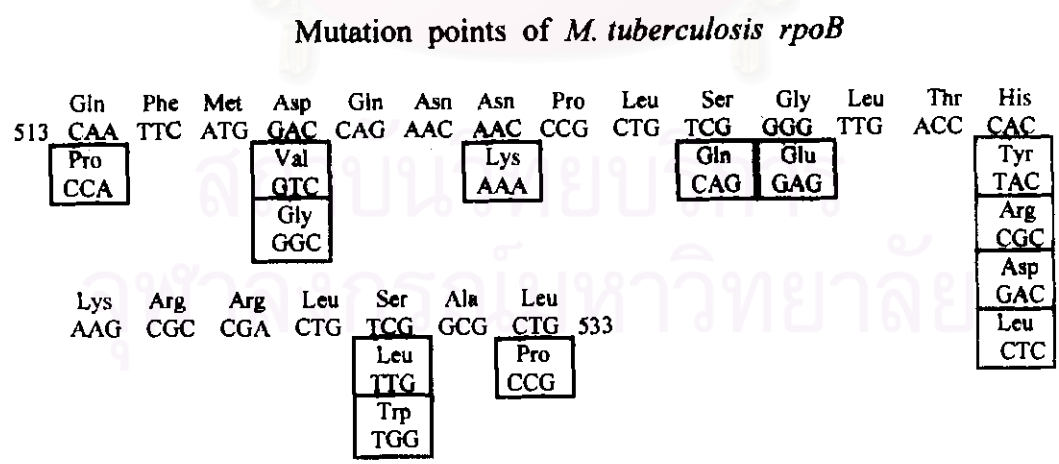

Fig 2. Substituted amino acids in codon 513 through 533 of the β- subunit of RNA polymerase in 56 rifampin resistant *M. tuberculosis* isolates. The position of substituted amino acids due to point mutations are shown in boxes below the line. The numbering system is based on the *E. coli* β-subunit of RNA polymerase (43)

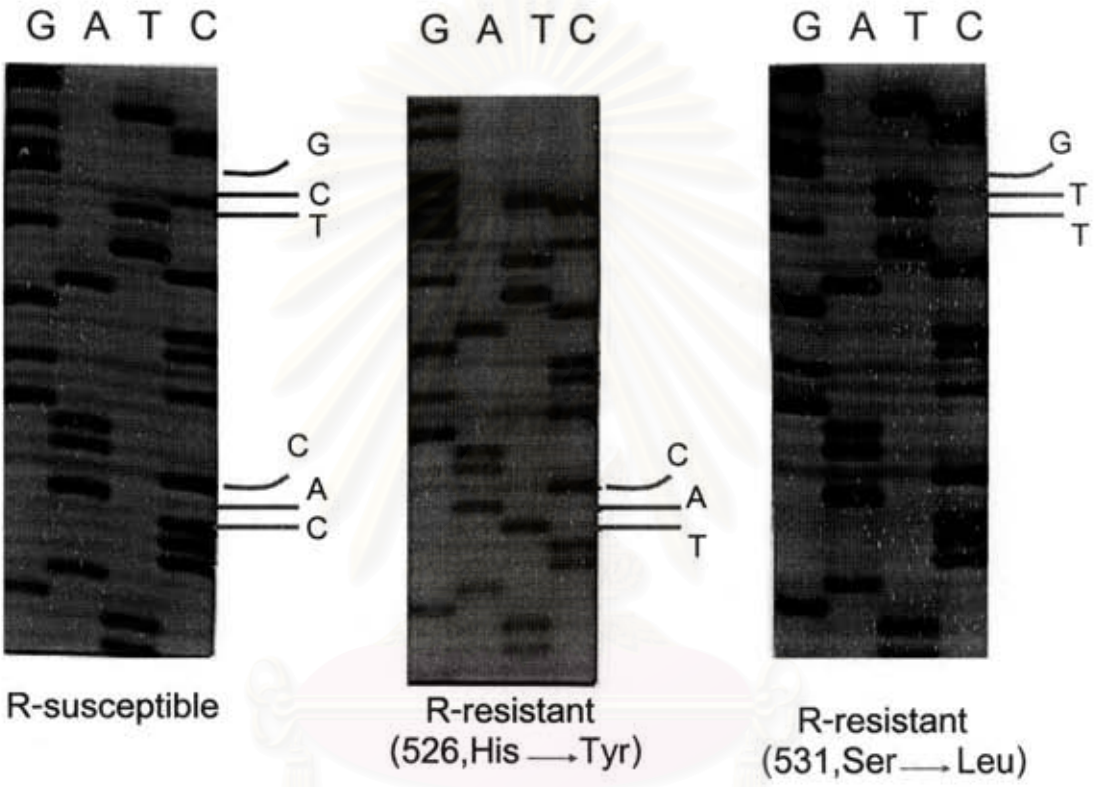

Fig 3. Mutations identified by direct sequencing of PCR products localization is shown of the two most frequent mutations identified in rifampin (R)-resistant *M. tuberculosis*: C → T substitution in position 531 (ser→leu), and a C→T (his→tyr) in position 526

Table 4. Mutation of the *rpoB* gene found in rifampin-resistant *M. tuberculosis* isolates from Thai tuberculosis patients

Mutated *rpoB* codon	Specific mutation	No.(%) of mutated sites
513	CAA(Gln)→CCA(Pro)	1 (1.8%)
516 [a]	GAC(Asp)→GTC(Val)	2 (3.6%)
	→GGC(Gly)	1 (1.8%)
519	AAC(Asn)→AAA(Lys)	1 (1.8%)
522	TCG(Ser)→CAG(Gln) [b]	1 (1.8%)
523	GGG(Gly)→GAG(Glu) [c]	1 (1.8%)
526 [d]	CAC(His)→TAC(Tyr)	9 (16.1%)
	→CGC(Arg)	7 (12.5%)
	→GAC(Asp)	3 (5.4%)
	→CTC(Leu) [c]	1 (1.8%)
531 [e]	TCG(Ser)→TTG(Leu)	26 (46.4%)
	→TGG(Trp)	2 (3.6%)
533	CTG(Leu)→CCG(Pro)	2 (3.6%)

a = subtotal is 3 (5.4%)

b = double point mutations in the one codon

c = one strain contained point mutation in two codons

d = subtotal is 20 (35.7%)

e = subtotal is 28 (50%)

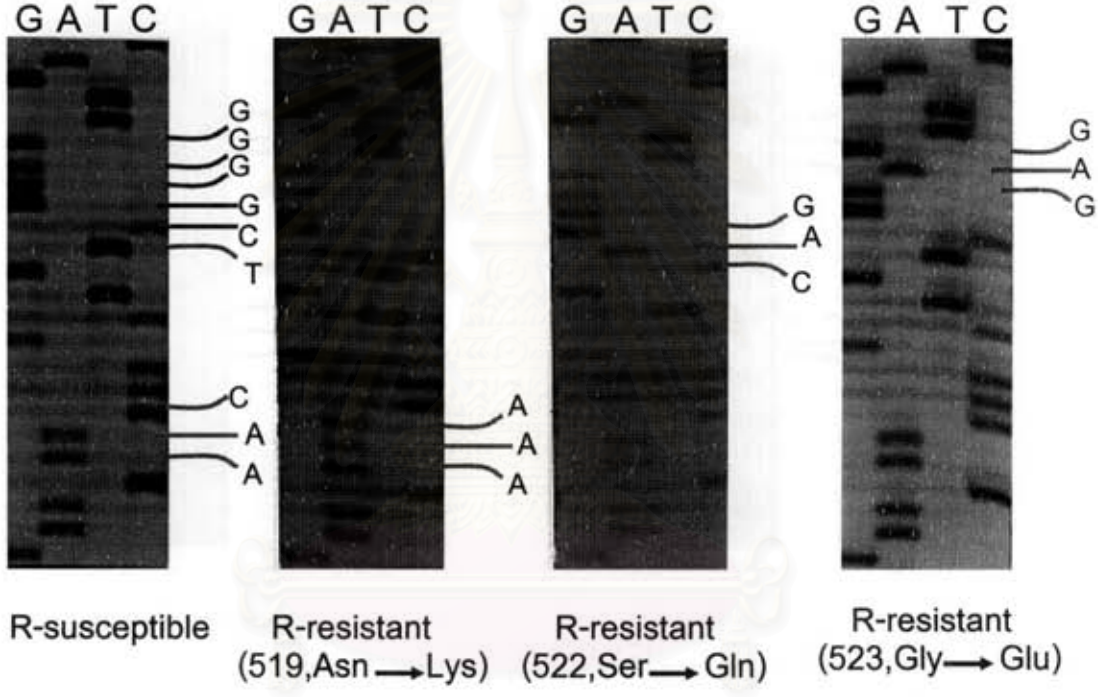

Fig 4. Mutations identified by direct sequencing of PCR products demonstrated three new types of mutation.

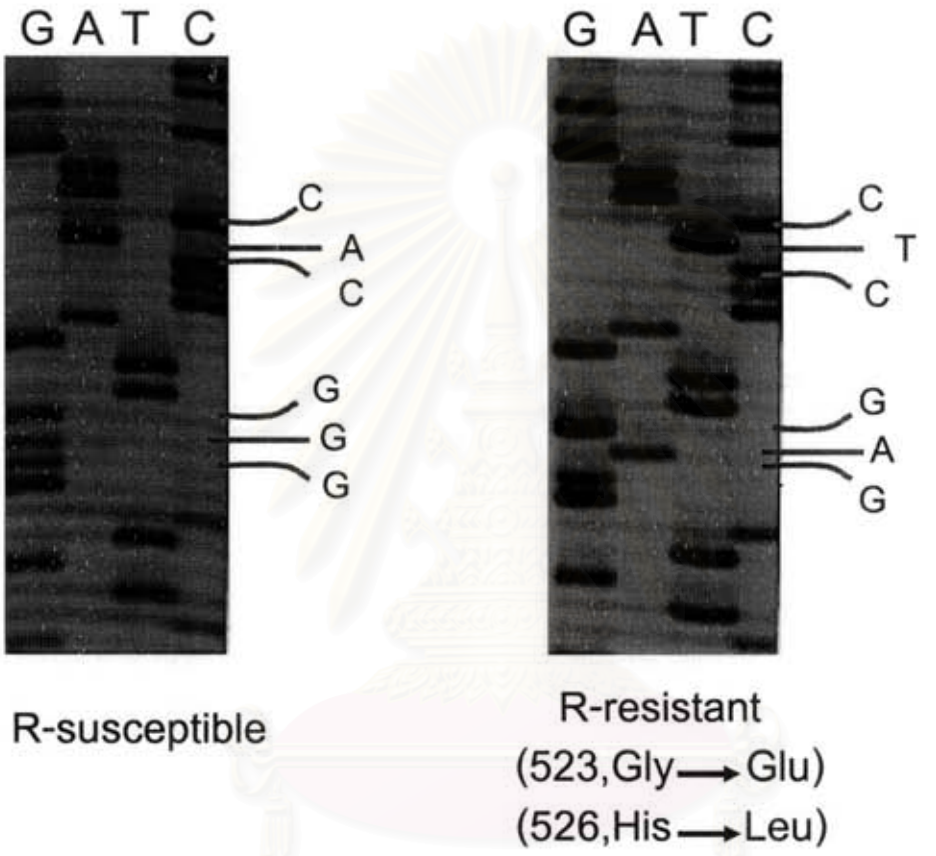

Fig 5. Mutations identified by direct sequencing of PCR products demonstrated that 1 isolate had point mutation in two codons.

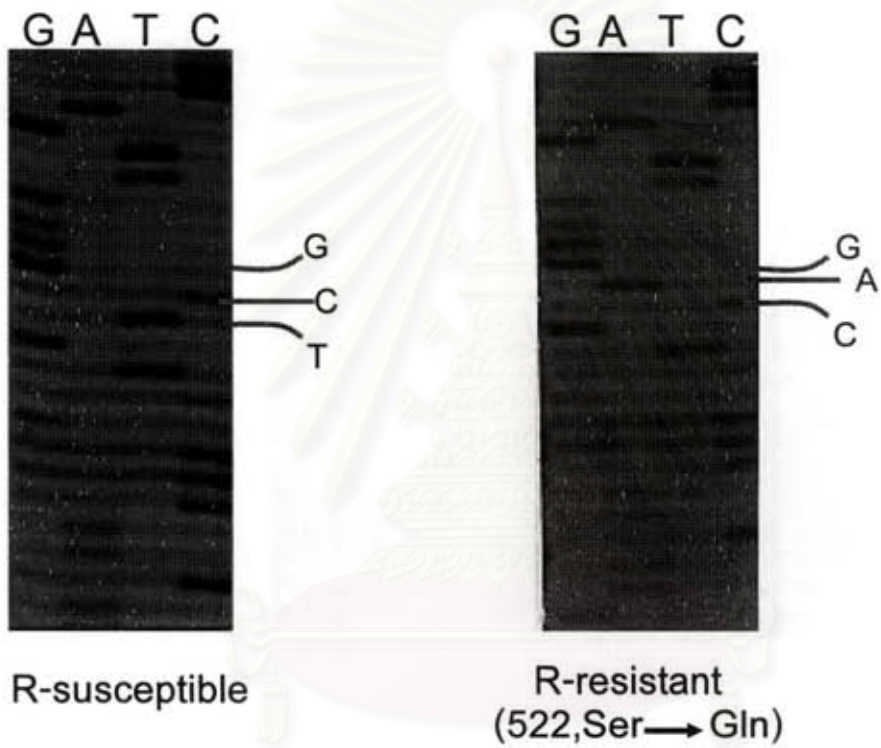

Fig 6. Mutation identified by direct sequencing of PCR products demonstrated that 1 isolate had double point mutation in two adjacent bases of one codon.

4. PCR-HDF analysis of rifampin-resistant *M. tuberculosis* isolates

To test the feasibility of using the PCR-HDF technique for the rapid detection of point mutations in *rpoB* gene in rifampin-resistant isolates, PCR-HDF analysis was performed with selected rifampin-resistant clinical isolates. The results indicated that rapid identification of mutations in the *rpoB* gene was unsuccessful in this study. Analysis of 31 random rifampin-resistant and rifampin-susceptible *M. tuberculosis* isolates did not show more than one band in nondenaturing polyacrylamide gel. However, reference strain (YE12) which contains 3-bp ttc insertion in codon 514-515 showed two bands in HDF pattern (lane 4,5 Fig 7).

lanes 1,2 = rifampin-susceptible *M. tuberculosis*
lanes 3,6 = rifampin-resistant *M. tuberculosis*
lanes 4,5 = rifampin-resistant *M. tuberculosis*
lane 7 = H37Rv

Fig 7. Mutations identified by PCR-HDF analysis