MULTIPLE MUTATIONS IN THE *rpoB* GENE OF *MYCOBACTERIUM LEPRAE* STRAINS FROM LEPROSY PATIENTS IN THAILAND

Pongrama Ramasoota[1,4], Waranya Wongwit[1], Prasert Sampunachot[2], Komes Unnarat[3], Maeya Ngamying[3] and Stefan B Svenson[4]

[1]Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand; [2]Prapradaeng Hospital, Samut Prakarn, Thailand; [3]Leprosy Division, Department of Communicable Disease Control, Nonthaburi, Thailand; [4]Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Uppsala, Sweden

Abstract. A new finding is reported of multiple mutations in the *rpoB* gene of 9 *Mycobacterium leprae* strains from leprosy patients in Thailand, who did not respond to therapy even when rifampicin, the main drug in multi-drug therapy was used. By means of sequence analysis of 9 Thai *M. leprae* strains, various mutations in 289 bps of the *rpoB* gene revealed forms of mutation never before described, such as multiple mutations (*ie*, mutation at two, three, six, seven, eight and nine positions in the *rpoB* gene), most of which were point-mutation substitutions (a few of which were silent). and some insertions. This investigation demonstrates that mutation in the *rpoB* gene of *M. leprae* strains from Thailand involves more variety than previously reported for *rpoB* mutation patterns in rifampicin resistance *M. leprae* strains.

INTRODUCTION

Currently recommended by the World Health Organization for the treatment of leprosy (Grosset *et al*, 1989; WHO Study Group, 1982), rifampicin is a key drug in chemotherapeutic regimens for treating both leprosy and tuberculosis, and owing to the exquisite rifampicin susceptibility of *M. leprae*, it is also the main drug for multi-drug therapy. Because of improper use, such as in rifampicin mono-therapy, some mycobacteria are resistant to treatment with rifampicin, threatening its usefulness in treating mycobacterial diseases (Grosset *et al*, 1989; Honore and Cole, 1993).

For monitoring of suspected cases of *M. leprae* rifampicin resistance, previous techniques were the mouse footpad method and the radio respirometric method (BACTEC)(Franzblau, 1989; 1991). Neither the mouse footpad method, which takes up to a year, nor the radio respirometric method, which requires relatively large numbers of metabolically active *M. leprae*, are suitable for biopsy materials. Improvements are needed for a faster and more accurate analysis that requires only small bacillary number materials. DNA diagnostic assays have the potential to provide rapid analysis of rifampicin resistance in mycobacteria because of their high degree of sensitivity and specificity, and because they do not rely on *in vitro* growth for results. Shortening the time between diagnosis and the onset of effective therapy should decrease physical deformities and ocular manifestations (resulting in disabilities and blindness) caused by *M. leprae*. To develop this diagnostic technique requires knowledge of the molecular basis of the rifampicin resistance in pathogenic mycobacteria.

A few years ago, the molecular basis of rifampicin resistance in *M. leprae* was established (Honore and Cole, 1993; Honore *et al*, 1993a) by DNA sequence analysis of the gene encoding the β-subunit of the DNA-dependent RNA polymerase gene (*rpoB* gene). Resistance was shown to be attributed to a limited number of missense (point) mutations in a short region located within this gene (Honore *et al*, 1993a). Other molecular techniques for detecting point mutations and other polymorphisms have been applied, such as single strand conformation polymorphism (SSCP) (Honore *et al*, 1993b) and heteroduplex detection (William *et al*, 1994). In this study, mutations in the *rpoB* genes of 9 *M. leprae* samples isolated from treated lepromatous Thai leprosy patients (who experienced relapse) were analyzed using an automated DNA-sequencer.

Correspondence: Pongrama Ramasoota, Section of Bacteriology, Department of Veterinary Microbiology, Swedish University of Agricultural Sciences, KC Box 7036 S-750 07 Uppsala, Sweden.
Tel: (+46 18) 673194; Fax: (+46-18) 504461; E-mail: pongrama@hotmail.com

MATERIALS AND METHODS

M. leprae strains

Nine 4- to 8-mm skin biopsy samples from Thai lepromatous leprosy patients were obtained. The patients, who appeared to have irregularly taken medication, had not responded to anti-leprosy therapy, including rifampicin; and they had relapsed after some years, and thus were suspected of harboring rifampicin-resistant *M. leprae*. These *M. leprae* strains were acid-fast smear, bacillary count. All samples were kept in 70% ethanol before examination. *M. leprae* Thai 53 and the WHO standard strain were used as reference (control) strains for rifampicin susceptible.

Sample preparation for PCR

Biopsy samples were resuspend with 1 x TE buffer and minced by sterile scissors until homogenized, then frozen at -80ºC for 30 minutes and thawed at 95ºC for 5 minutes three times before being resuspended in 100 µl of lysis buffer (protienase K 10 mg/ml and 0.5% Tween 20 in 1M Tris-HCl) and incubated overnight at 58ºC. Proteinase K was then inactivated by heat treatment at 94ºC for 10 minutes. The mixture was centrifuged at 13,000 rpm for 5 minutes in an Eppendorf centrifuge 5042. The DNA from the supernatant was purified by phenol: chloroform extraction and ethanol precipitation, a method described by Sambrook *et al* (1989). Afterwards, the resulting purified DNA pellet was dissolved in 100 µl sterile distilled water in order to determine its purity and concentration, spectrophotometrically, using Gene Quant RNA/DNA calculator (Pharmacia Biotech, Sweden).

PCR procedures

The aliquots of purified *M. leprae* DNA were added to PCR reagents and primers having the following sequences: Rpo-F, 5′GGT GGT CGC CGC TAT CAA G 3′ and Rpo-R, 5′TTT GCG GTA CGG TGT TTC G 3′. These primers were prepared to completely match the *M. leprae rpoB* gene sequence, giving a product of 289 bps of the gene. The reactants were put in a thermal cycler with touch down program:

94ºC for 5 minutes, 1 cycle;
45 seconds at 94ºC, 45 seconds at 65ºC, 90 seconds at 72ºC, 1 cycle;
45 seconds at 94ºC, 45 seconds at 64ºC, 90 seconds at 72ºC, 1 cycle;
45 seconds at 94ºC, 45 seconds at 63ºC, 90 seconds at 72ºC, 1 cycle;
45 seconds at 94ºC, 45 seconds at 62ºC, 90 seconds at 72ºC, 1 cycle;
45 seconds at 94ºC, 45 seconds at 61ºC, 90 seconds at 72ºC, 1 cycle;
45 seconds at 94ºC, 45 seconds at 60ºC, 90 seconds at 72ºC, 1 cycle;
45 seconds at 94ºC, 45 seconds at 59ºC, 90 seconds at 72ºC, 30 cycle; and
72ºC for 7 minutes, 1 cycle.

DNA sequencing

The PCR products for direct DNA sequencing were purified to separate the unincorporated nucleic acid and primers from amplified DNA by filtration with the QIA quick Purification Kit (Qiagen, USA). Sequencing reactions followed, carried out by means of a DNA sequencing kit (Dye terminator Cycle sequencing ready reaction; Applied Biosystem Inc, Foster City, CA, USA) with 5 µl of purified PCR product and 3.2 pmol of the Rpo-F-for primer. Centrisep spin columns (Applied Biosystems Inc) were then used to separate the unincorporated dye terminators and primers from the extension products. The products were dried in a vacuum centrifuge, resuspended in a loading buffer, heat denatured for 2 minutes at 90ºC, and immediately loaded onto a 4% acrylamide gel in an Applied Biosystems Prism 377 automated DNA sequencer (Perkin Elmer Corp).

RESULTS

Using the PCR procedure described earlier to amplify the designated region within the *rpoB* gene, all 11 strains, comprised of the nine experimental strains and the two reference strains, produced a strong single 289-bp PCR product that was used as a template for DNA sequencing. This PCR product was purified and then further sequenced using an automated DNA sequencer. DNA sequences obtained from the Thai 53 *M. leprae* reference strain and the *M. leprae* WHO standard reference strain were identical to those of previously reported sequences of the *rpoB* region in other *M. leprae* rifampicin-susceptible strains (Honore and Cole, 1993; Honore *et al*, 1993a).

DNA sequence analysis of the 9 experimental *M. leprae* strains suspected of being rifampicin-resistant (RIF^r) revealed 36 different mutation positions within the 289-bp region of the *rpoB* gene (Table 1), and revealed that all nine strains had multiple mutations. Two of the nine had mutations at 2 positions (codons); three, at 3 positions; one,

Table 1
List of all 27 codon positions at which 36 *rpoB* mutations (from codon Gln-517 to Glu-565) occurred for the 9 *M. leprae* strains from Thailand. The last column gives the number and percentage of mutations for each position.

Codon position no.[a]	Nucleotide mutation	Type of mutation	Amino acid changes	No. and (%) of mutations
Gln-517	CAG→CGG	Substitution	Gln→Arg	1(2.8)
Asn-518	AAC→AAT	Silent	Asn→Asn	1(2.8)
Asn-518	AAC→AGC	Substitution	Asn→Ser	1(2.8)
Pro-520	CCT→GGT	Substitution	Pro→Gly	1(2.8)
Pro-520	CCT→GCT	Substitution	Pro→Ala	1(2.8)
Leu-521	CTG→GTG	Substitution	Leu→Val	1(2.8)
Ser-522	TCG→TCA	Silent	Ser→Ser	1(2.8)
Lys-527	AAG→ATG	Substitution	Lys→Met	1(2.8)
Arg-528	CGC→TTC	Substitution	Arg→Phe	1(2.8)
Ser-531	TCG→TTG	Substitution	Ser→Leu	1(2.8)
Ala-532	GCG→GCT	Silent	Ala→Ala	1(2.8)
Gly-534	GGC→GCC	Substitution	Gly→Ala	1(2.8)
Gly-536	GGT→GCT	Substitution	Gly→Ala	1(2.8)
Ser-539	TCG→TCA	Silent	Ser→Ser	1(2.8)
Ser-539	TCG→TGG	Substitution	Ser→Trp	1(2.8)
Gly-540	GGT→CTGT	Insertion	Gly→	1(2.8)
Glu-541	GAG→CAG	Substitution	Glu→Gln	1(2.8)
Pro-543	GCC→CCC	Silent	Pro→Pro	1(2.8)
Gly-544	GGG→CTG	Substitution	Gly→Leu	1(2.8)
Gly-544	GGG→CCG	Substitution	Gly→Pro	1(2.8)
Leu-545	CTA→ATA	Substitution	Leu→Ile	1(2.8)
Glu-546	GAG→AAG	Substitution	Glu→Lys	3(8.3)
Val-547	GTC→GTG	Silent	Val→Val	1(2.8)
Arg-548	CGT→CGA	Silent	Arg→Arg	1(2.8)
Arg-548	CGT→GGT	Substitution	Arg→Gly	1(2.8)
Val-550	GTG→ATG	Substitution	Val→Met	2(5.5)
His-554	CAC→AAC	Substitution	His→Asn	3(8.3)
Gly-556	GGC→CGC	Substitution	Gly→Arg	2(5.5)
Gly-556	GGC→CGT	Substitution	Gly→Arg	1(2.8)
Gly-556	GGC→GTGC	Insertion	Gly→	1(2.8)
Arg-557	CGG→ACGG	Insertion	Arg→	1(2.8)
Glu-562	GAG→GAA	Silent	Glu→Glu	1(2.8)
Glu-562	GAG→AAG	Substitution	Glu→Lys	1(2.8)
Pro-564	CCG→CTG	Substitution	Pro→Leu	1(2.8)
Glu-565	GAG→CAG	Substitution	Glu→Gln	1(2.8)
Glu-565	GAG→TAG	Substitution	Glu→STOP	1(2.8)

[a] Based on *E. coli* numbering system for β-subunit of the RNA polymerase (Ovchinnikov *et al*, 1981).

at 6 positions; one, at 7 positions; one, at 8 positions; and one, at 9 positions. The details for each are given in Table 2.

DISCUSSION

Rifampicin, the main drug in multi-drug therapy (MDT) which has had an immense impact on leprosy control in Thailand has helped to decrease the level of cases there to less than 1 per 10,000 population (the level in 1997 was 0.4 per 10,000 population), which meets the criteria of the World Health Organization for eliminating leprosy as a public-health problem (Ramasoota and Intaratip, 1995). However, in Thailand, because available methods for detecting drug resistance to *M. leprae*

Table 2
The origin and *rpoB* mutation of 10 *M. leprae* strains from Thailand, which includes the nine experimental strains and the Thai 53 *M. leprae* reference strain. Most of the mutatations are point mutations, some of which are silent; and a few are insertions, indicated by a change from a three base sequence to a four base sequence; and one is a change to a stop codon.

rpoB mutation position[a]	Number of strains/(%)	Patients / origin (province)
Glu-546(Lys), Gly-556(Arg)(CGC)	1 (10)	Relapse / Samut Prakarn
Pro-564(Leu), Glu-565(Gln)	1 (10)	Relapse / Samut Prakarn
Gln-517(Arg), Gly-540(CTGT), Gly-556(Arg)	1 (10)	Relapse / Samut Prakarn
Asn-518(Gln), Pro-520(Ala), Ser-522 (Silent)	1 (10)	Relapse / Samut Prakarn
Gly-556(GTGC), Glu-562(Lys), Glu-565(stop codon)	1 (10)	Relapse / Samut Prakarn
Ser-531(Leu), Ala-532(Silent), Ser-539(Trp), Glu-541(Gln), Pro-543(Silent), Gly-544(Pro)	1 (10)	Relapse / Samut Prakarn
Leu-545(Ile), Glu-546(Lys), Val-547(Silent), Arg-548(Gly), His-554(Asn), Gly-556(Arg)(CGT), Arg-557(ACGG)	1 (10)	Relapse / Nakhon Rachasima
Lys-527(Met), Arg-528(Phe), Ser-539(Ser), Gly-544(Leu), Glu-546(Lys), Val-550(Met), His-554(Asn), Gly-556(Arg)(CGC)	1 (10)	Relapse / Nakhon Rachasima
Asn-518(Ser), Pro-520(Ala), Leu-521(Val), Gly-534(Ala), Gly-536(Ala), Arg-548(Silent), Val-550(Met), His-554(Asn), Glu-562(Silent)	1 (10)	Relapse / Nakhon Rachasima
No mutation	1 (10)	Thai 53 *M. leprae* reference strain

[a]Based on *E. coli* numbering system for β-subunit of the RNA polymerase (Ovchinnikov *et al*, 1981).

are unsuitable (as stated earlier), such as mouse footpad inoculation and BACTEC, research on early detection and confirmation of drug-resistant *M. leprae* from MDT is still needed (Pirayawaraporn, 1996).

In our study, we used a touch down PCR program (TD-PCR), which starts with a high annealing temperature for the first primer-annealing step, followed by a reduced annealing temperature of 1ºC in each later cycle. This program, which yielded a strong single band of 289 bp PCR product, helps reduce nonspecific amplification, improves specificity and product yield, and yet reduces the time for optimized PCR reaction (Hecker and Roux, 1996).

The DNA sequences obtained from *M. leprae* rifampicin-susceptible strains (Thai 53 strain) and the WHO standard strain were identical to those of previously published sequences for the same region of the *rpoB* gene in other *M. leprae* rifampicin-susceptible strains (Honore and Cole, 1993, Honore *et al*, 1993a). The sequence analysis of 9 Thai *M. leprae* strains revealed new forms of mutation in the 289 bp region of the *rpoB* gene, which have never been described previously, such as mutations at two, three, six, seven, eight and nine positions in that region of the *rpoB* gene (Table 2).

This is the first report of multiple mutations (with more than 2 positions mutated) in the *rpoB* gene of *M. leprae* strains, which when traced back to patient clinical data, were found to have come from relapse patients experiencing chronic infection for many years, and most of these patients had non-compliance treatment records (data not shown). In our study, the possible explanation for the presence of the multiple mutations in the *rpoB* gene of *M. leprae* is as follows. After the uncompleted treatment, the likelihood of *M. leprae* survival may be enhanced by selection of mutant strains that balance their high levels of antibiotic resistance with levels of deleterious metabolic effects that are minimal for their own survival. To precisely explain this evolutionary phenomenon, we need more detailed understanding of the structure-function relationships of the RNA polymerase β-sub-unit as determined by X-ray crystallographic and other

types of analyses.

Regarding mutation frequencies for codon positions in our nine-strain sample of *M. leprae* from Thailand, point mutations at the Glu-546 (Lys) and His-554 (Asn) positions predominated. Mutations at both of these positions occurred 3 times (8.3%). For two other positions, Val-550 (Met) and Gly-556 (Arg), mutations occurred two times (5.5%); and for the remaining codon positions, only once (Table 1).

This finding demonstrates that mutation in the *rpoB* gene of *M. leprae* strains from Thailand have more variety than previously reported for *rpoB* mutation patterns in RIF^r *M. leprae* strains. Most of the *rpoB* mutations from the previous studies were single point mutations causing a single amino acid substitution to occur at positions Ser-531 (Leu) (Williams *et al*, 1994) and Ser-425 (Leu, Phe, Met) (Honore and Cole, 1993). In contrast, for our study, all strains had more than one mutated position in the *rpo*B gene, and the frequencies and positions with point mutations were also different, as presented in Tables 1 and 2. These results were unexpected insofar as the *M. leprae* chromosome was believed to have low diversity for DNA sequences (Williams *et al*, 1990).

However, further investigation of RIF^r *M. leprae* with larger sample sizes from different geographic regions is needed, along with evaluation of the significance of these mutations in mechanisms of rifampicin resistance, and also along with drug-susceptibility tests using nude-mouse footpad inoculation followed by daily provision of rifampicin to the mice. An ongoing study is underway to propagate these multiple *rpoB* mutant strains of *M. leprae* in mice, and then test for drug-susceptibility, investigate thoroughly their *rpoB* genes, carry out DNA fingerprinting for epidemiological study, and describe their phenotype and genotype characteristics using clinical data for the patients infected with these strains. This study will be useful, not only from the epidemiological or public health point of view, but also for clarifying and understanding the molecular evolution aspect of *M. leprae*.

ACKNOWLEDGEMENTS

We thank Drs Thomas P Gillis and Diana L William from GWL Hansen's Disease Center, Louisiana State University, USA, for providing the *M. leprae* WHO standard strain, and for technical assistance. This work was partly supported by a grant from National Research Council of Thailand, Faculty of Tropical Medicine, Mahidol University, Thailand, and Dr SB Svenson from Swedish University of Agricultural Sciences, Uppsala, Sweden.

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