Philippine Journal of Science
136 (2): 147-153, December 2007
ISSN 0031 - 7683

Characterization of Rifampicin Resistance in Philippine Isolates of *Mycobacterium tuberculosis* by Mutation of the rpoB Gene

Jaime C. Montoya[1,2*], **Maria Sheila Magalonzo-De Jesus**[1], **Gloria Reclusado**[1], **Lydia Sombrero**[1] and **Concepcion F. Ang**[2]

[1]TB Study Group, Research Institute for Tropical Medicine, Muntinlupa City, Philippines
[2]TB Study Group, Section of Infectious Diseases, Department of Medicine, University of the Philippines-Philippine General Hospital, Manila City, Philippines

Rifampicin resistant isolates of *Mycobacterium tuberculosis* in the Philippines were characterized using the rpoB gene (RNA polymerase B gene). A total of 47 isolates were analyzed using polymerase chain reaction and DNA sequencing. Majority of the rifampicin resistant isolates of *M. tuberculosis* in the Philippines showed point mutations in the rpoB gene. Majority of the point mutations were in positions 526 (39.5%) and 531 (34.9%), and most of these involved single nucleotide substitutions. All of the point mutations associated with rifampicin resistance were seen in the isolates from the National Capital Region (NCR), whereas majority of the rifampicin resistant isolates without point mutations were seen in Southern Luzon (Laguna). This information may be used in subsequent studies for determining patterns of drug resistance as well as monitoring changing virulence and drug susceptibility of *M. tuberculosis* that may impact on health policies related to tuberculosis control.

INTRODUCTION

Tuberculosis remains a local and global problem of enormous proportions. Globally, 1.7 billion or approximately 1/3 of the world's population are infected with tuberculosis. In the Philippines, it is the fourth leading cause of death and the fifth leading cause of morbidity. This disease is now not only a problem in the developing world but even in the first world countries as well. Aggravating concerns are the emergence of multi-drug resistant tuberculosis, the pandemic of HIV that is an important co-factor in the development of tuberculosis, and the high immigration rates of populations from highly endemic areas of the world to areas of low endemicity.

An infectious disease of such magnitude, therefore, poses issues and questions regarding treatment. From the public health perspective, treatment is still focused on smear positive cases of tuberculosis that is the reservoir of infection in the community. The recommended treatment is to give at least 3 drugs during the first 2 mo, and at least 2 drugs during the next four months to complete a total of 6 mo (2HRZ/4HR). This is the regimen adopted by the National TB Control Program of the Department of Health. There are, however, mounting concerns regarding the issue of drug resistance. It is also therefore recommended by the TB Consensus Group composed of renowned TB experts in the Philippines to use four drugs for the intensive phase of treatment in cases where the probability of drug resistance is high [2HSRZ/4HR or 2EHRZ/4HR(E)]. This includes patients with history of previous treatment and cavitary cases of tuberculosis. Acquired drug resistance has been shown to be high in patients treated before, while primary drug resistance due to spontaneous mutation is common in high bacillary populations present in cavitary cases.

Multi-Drug Resistant Tuberculosis (MDR-TB) is defined as an isolate of *M. tuberculosis* resistant to

*Corresponding author: mnty_jm@yahoo.com

isoniazid and rifampicin, with or without resistance to the other first line drugs.

Based on a global study to determine the prevalence of drug-resistant TB to the four first line drugs conducted by the World Health Organization -International Union Against Tuberculosis and Lung Disease that was participated in by 35 countries, the problem of MDR-TB is indeed increasing. Overall, there was a 2.2% prevalence of multi-drug resistant tuberculosis. Particularly high prevalence of multi-drug resistant TB were found in the Soviet Union, Asia, the Dominican Republic, and Argentina (Pablos-Mendez et al. 1998). In a community-based surveillance study for drug resistance of *M. tuberculosis* in selected areas in the Philippines, the rate of multi-drug resistance varied from region to region. The rate of MDR-TB in Metro Manila was 6.4% (95% CI 3.9, 9.9), La Union, 9.6% (95% CI 4.6, 17.5), Zamboanga, 4.4% (95% CI 2.4, 7.336), and Leyte 5.2% (95% CI 2.3, 9.98) (Mendoza et al. 2002).

Drug resistance, using microbiologic definition, is present when more than 1 % of the colonies is resistant to a specific drug. This information, however, is not usually available due to the unavailability of culture facilities. Culture requires skilled personnel, expensive culture media, and highly specialized centers.

Reliable and rapid methods for determining drug susceptibility are not yet available in the Philippines. However, recent studies (Zhang & Young 1994; Musser 1995) have described the molecular basis of resistance in *M. tuberculosis* that offers the genotypic approach for the detection of resistance.

The first study that described the genetic characteristics of *M. tuberculosis* in the Philippines revealed a unique group of isolates distinct from the DNA fingerprint patterns of *M. tuberculosis* isolates in international databases . Forty eight *M. tuberculosis strains* were obtained from patients living in Metropolitan Manila and subjected to three molecular typing methods: IS6110 restriction fragment length polymorphism, spoligotyping, and DNA sequencing of the *oxyR, gyrA* and *katG* loci. Based on the restricted diversity of the isolates , the paper designated the Philippine isolates as the Manila family of *M. tuberculosis* (Douglas et al. 2003).

Rifampicin is considered one of the most potent and bactericidal first line drugs against tuberculosis. The mechanism of resistance to rifampicin is already well understood and amply documented. In most *M. tuberculosis* isolates studied, resistance to rifampicin is attributed to point mutations in a limited region of the gene encoding the B subunit of RNA polymerase (rpoB). Rapid methods for the identification of mutant rpo B gene allow identification of rifampicin resistance, which can be adapted for epidemiological surveillance (Williams et al. 1994; Telenti et al. 1993). Moreover, a molecular epidemiology approach to surveillance of rifampicin resistance will be valuable to the National Tuberculosis Control program.

This study hopes to describe the mutations responsible for rifampicin resistance in Philippine isolates of *M. tuberculosis*.

MATERIALS AND METHODS

M. tuberculosis isolates proven to be resistant to rifampicin by conventional disk diffusion were collected from isolates obtained by the prevalence survey of Tan-Torres and Mendoza in the National Capital Region (Mendoza et al. 2002), and the M. tuberculosis bank at the Research Institute for Tropical Medicine (RITM) (Southern Luzon- Laguna). This included sputum specimens from randomly selected patients diagnosed to have tuberculosis. These isolates were then subjected to DNA sequencing and analysis focusing on the rpoB gene. A total of 47 isolates were included in the analysis.

Determination of Rifampicin MIC for *M. tuberculosis* by the E-test method

Specimen (inoculum) preparation

Forty-seven isolates of *M. tuberculosis* including control strains (ATCC H37Rv and ATCC 35838) were grown in Lowenstein Jensen slants in the P2 Mycobacteriology laboratory of the RITM. Colonies were then scraped from freshly growing (3 to 4 w) LJ slants into 3 mL of Middlebrook 7H9 broth containing glass beads. The colonies were then vortexed to disperse clumps and to homogenize the suspension. The turbidity was adjusted equivalent to 3.0 MacFarland standard using a spectrophotometer.

E-Test susceptibility Testing

The forty-seven rifampicin resistant *M. tuberculosis* isolates were re-grown in Lowenstein-Jensen slants and re-subjected to E-test susceptibility testing. Freshly prepared 7H11 plates with OADC supplement were used. Each culture inoculum was swabbed to the plate and pre-incubated for 24 h at 35° C with 5 to 10% CO_2. Afterwards, the E-test strip containing rifampicin (with concentrations ranging from 0.06 to 0.25 ug/mL) was placed on the agar surface. The plates were then incubated under the same conditions until an inhibition

ellipse was visible (5 to 7 d). The MIC was interpreted as the point at which the ellipse intersects the E-test strip. This procedure confirmed that the 47 isolates were indeed rifampicin resistant.

Characterization of Rifampicin Resistant *M. tuberculosis* isolates

DNA extraction

Twenty-five mL of the suspension of each isolate was spun at 10,000 rpm for 5 min and resuspended in 3 µL of sterile deionized water. To extract the mycobacterial DNA and block PCR inhibitors, 17 µL of the Genereleaser resin (Bioventures, Inc., Murfreesboro) was added to 3 µL of resuspended bacterial pellet, then mineral oil was overlaid. The sample mix was then subjected to lysis procedure according to the manufacturer's instructions.

Amplification by PCR

DNA amplification of the 305-bp fragment of the rpoB gene was carried out in a 25 µL reaction mixture containing 0.25 units of Taq DNA polymerase (Boehringer Manheim) , 1X PCR buffer containing 10 mM Tris HCL, 50 mM KCL, and 10 mM $MgCl_2$ (Boehringer Manheim), 0.1mM dNTPs (Promega), 1pmol of TBRif1 forward primer (5'CAG ACG TTG ATC AAC ATC CG 3'), and 1 pmol of TBRif2 reverse primer (5' TAC GGC GTT TCG ATG AAC 3'). The primers were designed to amplify the 305bp fragment of the rpoB gene where mutations that confer resistance to rifampicin usually occur. The amplification was performed in a thermocycler (Perkin Elmer) with this profile: initial denaturation of 94° C for 3 min then followed by 40 cycles of the following: 94° C for 30 sec, 55 C for 30 sec, and 72° C for 90 sec with final strand elongation at 72° C for 5 min. The PCR products were electrophoresed in a 1% agarose gel containing 0.5 ug/mL of ethidium bromide run in 1X Tris-acetate buffer at 90 volts for 30 min. The bands were visualized using a UV transilluminator and photographed using a polaroid camera. Finally, the PCR products were purified using the Wizard PCR preps purification system (Promega, WI).

DNA sequencing

Purified PCR products were sent to Australian Genome Research Facility (AGRF), University of Queensland, Australia for DNA sequencing with the use of an automated sequencer. The automated sequencing system followed the principle as in Sanger chain termination. However, sequencing reactions were performed using the fluorescent dye labeled reagents. The primer used was the TBRif2 (reverse primer). The sequences were then aligned by comparing with ATCC H37Rv using BLAST 2 sequence alignment program.

RESULTS

A total of 47 isolates were analyzed. All 47 isolates were re-confirmed to be resistant to rifampicin using the E-test. Majority of the isolates (43/47 or 91.49%) showed point mutations with nucleotide substitutions while only 4 (4/47 or 8.51%) showed no point mutation. All isolates with point mutations were from the NCR and all of the isolates with no point mutation were from Laguna. A total of 6 different mutation sites were identified (Table 1) .

Table 1. Point Mutations identified in the isolates of *Mycobacterium tuberculosis*

Point Mutation	No.	%
526	17	39.5
531	15	34.9
516	5	11.6
522	3	7
512	2	4.7
533	1	2.3
Total	43	100

Out of the 43 isolates that showed point mutations, majority showed a point mutation at position 526 (17 out of 43 or 39.5%). In decreasing order, the mutation sites identified were: 526 (n=17); 531 (n=15); 516 (n=5); 522 (n=3); 512 (n=2); 533 (n=1).

Tables (2-4) will show the nucleotide substitutions identified in the predominant point mutations seen.

Table 2. Nucleotide Substitutions Identified as Point Mutation at Position 526

Nucleotide Substitution	No.	%
His-Tyr	11	64.7
His-Arg	3	17.6
His-Asp	1	5.9
His-Leu	1	5.9
His-Gln	1	5.9
Total	17	100

Table 3. Nucleotide Substitutions Identified as Point Mutation at Position 531

Nucleotide Substitution	No.	%
Ser-Leu	14	93.3
Ser-Tryp	1	6.7
Total	15	100

Table 4. Nucleotide Substitutions Identified as Point Mutation at Position 516

Nucleotide Substitution	No.	%
Asp-Val	2	40
Asp-Tyr	1	20
Asp-Gln	1	20
Deletion of Asp	1	20
Total	5	100

DISCUSSION

The World Health Organization recently conducted and published the global surveillance for anti-tuberculosis drug resistance from 1994-1997. The report described the prevalence of resistance to the 4 first-line drugs in the 35 countries that participated. The results of the study showed that among patients with no prior treatment, a median of 9.9% of *M. tuberculosis* strains were resistant to at least 1 drug (range, 2 to 41 %); resistance to isoniazid (7.3%) or streptomycin (6.5%) was more common than resistance to rifampicin (1.8%) or ethambutol (1.0%). The prevalence of primary multi-drug resistance was 1.4% (range 0 to 14.4%). Multi-drug resistance is defined as resistance to isoniazid and rifampicin with or without resistance to the other first line drugs. Among patients with a history of treatment for 1 month or more, the prevalence of resistance to any of the 4 drugs was 36.0% (range 5.3 to 100%) and the prevalence of multi-drug resistance was 13 % (range 0 to 54 %) . The overall prevalences were 12.6% for resistance to any of the 4 drugs (range 2.3 to 42.4%) and 2.2% for multi-drug resistance (range 0 to 22.1%), respectively (Pablos-Mendez et al. 1998). This elucidates the fact that the most important factor that affects the development of drug resistance is the history of previous treatment.

Rifampicin is a semi-synthetic derivative of the natural product rifamycin, obtained from culture filtrates of *Amycolatopsis mediterranei*. Rifampicin is not only bactericidal against all 3 populations of tuberculous organisms: actively multiplying, intermittently-dividing, and dormant bacilli but it is also effective against other gram-positive and gram-negative bacteria such as *E. coli, Pseudomonas, Klebsiella, Neisseria meningitidis, and Hemophilus influenzae*.

The mechanism of action of rifampicin is believed to involve interference with transcription and RNA elongation by binding of the drug to the B subunit of RNA polymerase in a locus formed by the appropriate complexing of the different RNA polymerase sub-units (Schlossberg 1987; Konno et al. 1973).

Resistance to rifampicin is increasing rapidly as a result of its widespread use. Rifampicin-resistant tuberculosis, often observed in conjunction with isoniazid resistance, leads to a longer treatment period and significantly poorer chemotherapeutic outcomes. Although newer synthetic derivatives of rifampicin have been developed (rifabutin and rifapentine) (Montaner et al. 1994), they do not appear to be substantially more effective than rifampicin and exhibit cross-resistance with rifampicin (Blanchard 1996).

The rpoB gene has been sequenced and analyzed to understand the mechanism of transcription (Ovchinnikov et al. 1981). This has been further studied in association with rifampicin resistance (Jin & Gross 1998). Results of this study revealed that mutations conferring resistance to rifampicin were mapped to 3 distinct clusters in the middle of the rpoB gene particularly cluster I and II, which led to resistance to high concentrations of rifampicin.

Following the structure function studies of the rpoB gene of *E. coli*, Telenti et al. (Telenti et al. 1993) cloned and sequenced the cognate region of the *M. tuberculosis* gene by using sequence information available from the *M. leprae* rpoB gene. The data were used to design oligonucleotide primers for amplification and sequencing of a 411-bp fragment of rpoB gene from 66 rifampicin-resistant and 56 rifampicin-susceptible isolates recovered from patients from several countries. A total of 15 distinct mutations involving 8 conserved amino acids clustered in a 23 amino acid region (69 bp) were identified in 64 of 66 rifampcin-resistant isolates, but none in 56 of susceptible organisms. Virtually all of the mutations were missense mutations. Amino acid substitutions at 1 of 2 positions (residues 526 and 531) were found in 80% of the resistant isolates. Donnabella et al. (1994) also reported isolation of similar homologous functional rifampicin resistance genes from *M. tuberculosis*. A subsequent study done by Kapur et al. (Kapur et al. 1995) extended knowledge about the spectrum of rpoB mutations, and 5 new mutations were identified. Interestingly, 2 of the previously undescribed mutations occurred outside the 69 bp core region containing nucleotide changes in virtually all rifampicin-resistant *M. tuberculosis* isolates, therefore, expanding the size of the core region to 27 amino acids.

Virtually all isolates resistant to rifampicin and related rifamycins have a mutation that alters the sequence of a 27 amino acid region of the beta subunit of ribonucleic acid (RNA) polymerase (Ramaswamy & Musser 1998).

Based on a review of published researches describing the mutation sites in the rpoB gene of RNA polymerase among isolates of *M. tuberculosis* resistant to rifampicin, several mutation sites were identified. Cooksey et al.

identified 9 distinct rpoB mutations in a collection of 51 rifampicin resistant strains in New York City using a commercial line probe assay kit (Inno-LiPA Rif.TB) (Cooksey et al. 1997). In the United Kingdom, Watterson showed that approximately 95% of rifampin-resistant isolates in the UK showed mutations in a 69 bp region of the rpoB gene, making this a good target for molecular genotypic diagnostic methods (Telenti et al. 1993). In another study in Japan by Hirano et al., 10 distinct single nucleotide substitutions were found among the isolates by automated sequencing (Douglas et al. 2003). In China, 10 distinct rpoB gene mutations were identified among 50 rifampin resistant isolates (Liu et al. 1999). Seven nucleotide substitutions were identified in 21 of 22 rifampin-resistant isolates in another study (Sintchenko et al. 1999).

This local study was able to identify 6 mutation sites in the rpoB gene, majority of which were in the form of single nucleotide substitution. All of these nucleotide substitutions were within the 512-533 bp region. This is consistent with studies done in the region such as Japan and China where several mutation sites were identified.

In the study done in Japan by Yang et al., they were able to show that point mutations at 515, 521, 533 did not influence susceptibility to rifampicin whereas point mutations at positions 513, 516,526,529 and 531 correlated with resistance (Yang et al 1998). Our study demonstrated that majority of our nucleotide substitutions were at positions 526 and 531, which is more or less consistent with this study. However, the other nucleotide substitutions that were less common like at position 533 (Blanchard 1996) though shown not to be associated with rifampicin resistance in the Yang study was associated with rifampicin resistance in 1 isolate in our study. This means that there may be other mutations involved in this particular isolate outside of the 512-533 bp region that remain to be identified.

It is also interesting to note that all of the rifampicin resistant *M. tuberculosis* isolates that were not associated with any point mutation were seen among patients coming from the Laguna area, whereas all the point mutations in positions 531 and 526 were demonstrated in isolates from patients in the NCR. This may indicate that factors influencing mutations leading to drug resistance may be more rampant in the NCR compared to other areas. Such factors may include inappropriate and inadequate anti-tuberculosis treatment as well as heavy use of drugs that have anti-tuberculous activity such as fluoroquinolones, which are being used for lower respiratory tract infections like pneumonia . Exposure of *M. tuberculosis* to sub-inhibitory concentrations of the drug through incorrect dosing or inadequate treatment may lead to the development of acquired resistance. Further studies have to be done looking at longitudinal prospective studies in the population to identify whether this is the predominant mechanism of development of resistance.

This study demonstrates the common patterns of point mutations seen in local rifampicin-resistant isolates of *M. tuberculosis* that is also seen in similar isolates in other countries in Asia (Taiwan, China, Japan) and in Europe. This valuable information will be vital in future studies for determining patterns of transmission of tuberculosis in the country as well as in identifying changing patterns of mutation that may indicate alterations in virulence, infectivity, and drug susceptibility. All of these will have a major impact on major health policies to be implemented for tuberculosis control in the country.

CONCLUSION

Majority of the rifampicin resistant isolates of *M. tuberculosis* (n=47) studied showed point mutations in the rpoB gene (RNA polymerase B gene). Majority of the point mutations were in positions 526 and 531 and most of these involved single nucleotide substitutions. All of the point mutations associated with rifampicin resistance were seen in the isolates from the NCR, whereas all of the rifampicin resistant isolates without point mutations were seen in the Laguna area. More studies, however, involving a bigger number of rifampicin-resistant isolates should be analyzed to achieve more definite conclusions.

ACKNOWLEDGEMENTS

The authors would like to thank the Essential National Health Research of the Department of Health for providing valuable financial assistance for this project. The authors would also like to thank Dr. Tessa Tan-Torres and Dr. Myrna Mendoza of the TB Research Lab of UP-PGH and Ms Lydia Sombrero of the TB Lab of the Research Institute for Tropical Medicine for providing the Rifampicin-resistant isolates of *M. tuberculosis* analyzed in this study.

REFERENCES

BLANCHARD JS. 1996. Molecular mechanisms of drug resistance in *Mycobacterium tuberculosis*. Annu Rev Biochem 65:215-239.

COOKSEY RC, MORLOCK GP, GLICKMAN S and CRAWFORD JT. 1997.Evaluation of a line probe assay kit for characterization of rpoB mutations in rifampin-resistant *Mycobacterium tuberculosis* isolates from New York City. J Clin Microbiol 35:1281-3.

DONNABELLA V, MARTINIUK F, KINNEY D, BACERDO M, BONK S, HANNA B and ROM W. 1994. Isolation of the gene for the B subunit of RNA polymerase from rifampin-resistant *Mycobacterium tuberculosis* and identification of new mutations. Am J Respir Cell Mol Biol 11:639-643.

DOUGLAS JT, QIAN L, MONTOYA JC, MUSSER JM, VAN EMBDEN JD, SOOLINGEN DV and KREMER K. 2003.Characterization of the Manila Family of *Mycobacterium tuberculosis*. J Clin Microbiol 41:2723-2726.

HIRANO K, ABE C and TAKAHASHI M. 1999. Mutations in the rpo gene of rifampin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay. J Clin Microbiol 37:2663-6.

JIN DJ and GROSS CA. 1988. Mapping and sequencing of mutations in the *Escherichia coli* rpoB gene that lead to rifampin resistance. J Mol Biol . 202:45-58.

KAPUR V, LI L, HAMRICK R, PLIKAYTIS B, SHINNICK T, TELENTI A, JACOBS W, BANERGEE A, COLE S, YUEN K, CLARIDGE III J, KREISWIRTH BN and MUSSER JM. 1995. Rapid Mycobacterium species assignment and unambiguous identification of mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis* by automated DNA sequencing. Arch Pathol Lab Med 119:131-138.

KONNO K, OIZUMI K, ARIJI F, YAMAGUCHI J and OKA S. 1973. Mode of action of rifampin on Mycobacteria. Am Rev Respir Dis 107:1002-1012.

LIU YC, HUANG TS and HUANG WK. 1999. Line probe assay for rapid detection of mutations in the rpoB gene of *Mycobacterium tuberculosis*. J Formos Med Assoc 98: 582-5.

MENDOZA MT, TAN-TORRES T, ANG CF, ARCIAGA R, ELONA F, RETUTA M, CRUZ N and MANTALA M. 2002. Community-Based Surveillance for Drug Resistance of *Mycobacterium tuberculosis* in Selected Areas un the Philippines. Philipp J Microbiol Infect Dis. 31:169-175.

MONTANER LJ, NATAL S and YONGCHAIYUD P. 1994. Rifabutin for the treatment of newly-diagnosed pulmonary tuberculosis;a multinational, randomized, comparative study versus rifampicin. Tuber Lung Dis 75:341-347.

MUSSER J. 1995. Antimicrobial agent resistance in Mycobacteria:molecular genetic insights. Clin Microbiol Rev 8:496-514.

OVCHINNIKOV Y, MONASTYRSKAYA G, GUBANOV V, GURYI S, CIRTOV O, MODYANOV V, GRINKEVICIH V, MAKAROVA IA, MACHENKO LV, POLOVNIKOVA IN, LIPKIN VM and SVERDLOV ED. 1981. The primary structure of Escherichia coli RNA polymerase. Eur J Biochem 116:621-629.

PABLOS-MENDEZ, A, RAVIGLIONE MC, LASZLO A, BINKIN N, RIEDER HL, BUSTREO F, COHN DL, LAMBREGTS-VAN WEEZENBEEK CS, KIM SJ, CHAULET P and NUNN P. 1998. Global surveillance for anti-tuberculosis drug resistance, 1994-1997. World Health Organization-International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance. N Engl J Med .338 :1641-9.

RAMASWAMY S and MUSSER JM. 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis 79:3-29.

SCHLOSSBERG D. 1987 Tuberculosis. In: Tuberculosis, 2nd ed. Schlossberg D. (ed.). Philadelphia: Springer-Verlag p. 39-53,

SINTCHENKO V, CHEW WK, JELFS PJ and GILBERT GL. 1999. Mutations in rpoB gene and rifabutin susceptibility of multidrug-resistant *Mycobacterium tuberculosis* strains isolated in Australia. Pathology. 31:257-6.

TELENTI A, IMBODEN P, MARCHESI F, LOWRIE D, COLE ST, COLSTON MJ, MATTER L, SCHOPFER K and BODMER T. 1993. Detection of rifampin resistance mutations in *Mycobacterium tuberculosis*. Lancet 341:647-650.

WATTERSON SA, WILSON SM, YATES MD and DROBNIEWSKI FA. 1998. Comparison of three molecular assays for rapiddetection of rifampin resistance in *Mycobacterium tuberculosis*. J Clin Microbiol 36:1969-73.

WILLIAMS D, WAGNESPACK C, EISENACH K, CRAWFORD J, PORTAELS F, SALFINGER M, NOLAN CM, ABE C and GILLIS T. 1994. Characterization of rifampin resistance in pathogenic Mycobacteria. Antimicrob Agents Chemother 38:2380-2386.

YANG B, KOGA H, OHNO H, OGAWA K, M FUKUDA , HIRAKATA Y, MAESAKI S, TOMONO K, TASHIRO T and KOHNO S. 1998. Relationship between antinycobacterial activities of rifampicin, rifabutin and KRM-1648 and rpoB mutations of Mycobacterium tuberculosis. J Antimicrob Chemother 42:621-8.

Zhang, Y., and D. Young. 1994. Molecular genetics of drug resistance in Mycobacterium tuberculosis. J Antimicrob Chemother 34:313-319.