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FINAL REPORT

Determination of Drug Resistant Gene Mutations among Rifampicin resistant strains of *M. tuberculosis*

Grant No. RG/ 2007/ BT/03

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Abbreviations

AFB	-	Acid Fast Bacilli
APM	-	Agar Proportion Method
DNA	-	Deoxy Ribonucliec Acid
DSR	-	Drug Susceptibility Testing
ELISA	-	Enzyme Linked Immunoabsorbant Assay
INH	-	Isoniazid
IS	-	Insertion Sequence
L-J	-	Lowenstein-Jensen
MDR-TB	-	Multi Drug Resistant Tuberculosis
MGIT	-	Mycobacteria Growth Indicator Tube
MTB	-	*Mycobacterium tuberculosis*
NPTCCD	-	National Programm for Tuberculosis Control and Chest Diseases
NRA	-	Nitrate Reductase Assay
NTM	-	Non Tuberculosis Mycobacteria
OD	-	Optical Density
PCR	-	Polymerase Chain Reaction
RFLP	-	Restriction Fragment Length Polymorphism
RIF	-	Rifampicin
RRDR	-	Rifampicin Resistant Determining Region
SD	-	Standard Deviation
TB	-	Tuberculosis
US	-	United State
WHO	-	World Health Organization
XDR TB	-	Extensively Drug Resistant Tuberculosis

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Section 1

Information regarding Project/Project Personnel

(A) Grant Number: RG/ 2007/ BT/03

(B) Title of the Project: Determination of Drug (Rifampicin)-Resistant Gene Mutations among Rifampicin Resistant Strains of *Mycobacterium tuberculosis*

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iii) Institute(s) where research was being carried out:
Department of Microbiology,
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i) Date of award: 01/ 02/2008

ii) Date of completion of Project: 01/02/2012

iii) Total allocation of funds (Rs): 4,041,000.00

iv) Total spent (Rs): 3,833,935.39

v) Number of Research Students employed: 1

vi) Post graduate degree completed with dates: In the process of correcting the thesis by supervisors

vii) Number of Technical Assistants and/or labourers employed and period of service: 1

viii) Publications/Communications arising from the project during the reporting period

Please see the Annex I

Section 2

Executive Summary of the Project

This should be limited to 200-250 words and include the scientific background and objectives, methodology and major findings

Background: Tuberculosis (TB) is a disease of poverty that contributes significantly to ill-health in developing countries. Drug resistant TB is a major challenge in controlling and prevention of TB. Therefore, early diagnosis and rapid determination of drug sensitivity is of paramount importance in eradication of TB.

Objectives: The study was divided into 3 main parts. The aim of the first part was to identify the rifampicin resistant gene mutations of *Mycobacterium tuberculosis* (MTB) strains in Sri Lanka (Mainly Western Province). The objective of the second part was to develop a rapid molecular method for determination of rifampicin (RIF) resistance. The aim of the third part was to determine the molecular epidemiology of RIF resistance in Western Province where the highest disease burden in Sri Lanka is reported. Each objective was achieved through several specific objectives.

Methodology: Five hundred and thirty four acid fast bacilli (AFB) positive sputum specimens collected from Chest Hospital- Welisara, Chest Clinic- Colombo and Prison Hospital were used for the study. Lowenstein–Jensen (L-J), Middlebrook7H9 broth and paranitrobenzoic acid incorporated L-J media were inoculated using processed and concentrated sputum. Drug susceptibility testing (DST) was carried out by using the Agar Proportion Method (APM). The Nitrate Reductase Assay (NRA) and the manual Mycobacteria Growth Indicator Tube (MGIT) were evaluated as rapid culture based DST methods. DNA was extracted from isolated *M. tuberculosis* strains. Three fragments in *rpoB* gene were PCR (polymerase chain reaction) amplified and DNA sequenced. PCR based enzyme linked immunoabsorbant assay (PCR-ELISA) was developed and evaluated for detection of RIF resistant *M. tuberculosis* isolates in Sri Lanka. Restriction fragment length polymorphism (RFLP) was carried out to investigate the molecular epidemiology of the RIF resistance.

Findings: Four hundred and one (401) MTB cultures were isolated form 534 AFB positive specimens. The evaluated rapid culture methods, the NRA and the manual MGIT were found to be good alternatives for conventional proportion method that spend more than 28 days for determination of RIF resistance. Two novel mutations (at codon 184 and 626) and 2 prevailing mutations (at codon 526 and 531) were identified in *rpoB* gene of MTB strains collected from Sri Lanka. The PCR-ELISA that was developed as a component of study is simple, rapid, cost effective and an accurate molecular method for determination of RIF resistance. With regard to RFLP analysis of the band pattern of the isolates, there is no clonal relationship of the RIF resistant MTB isolates retrieved from the study.

Section 3

(A) Background

One third of the world's population is estimated to be infected with MTB and the majority of TB cases are concentrated in developing countries (WHO. 2011). Globally, 8.8 million cases of TB were estimated in 2009. Most of the estimated cases occurred in Asia (59%) and Africa (26%) as compared to the Eastern Mediterranean Region (7%), European Region (5%) and the Region of the Americas (3%). Three Asian countries namely India, China and Indonesia, are among the five countries with the largest number of incidents of TB in 2009. India alone contributed 26% of all TB cases worldwide, while India and China together accounted for 38% (WHO. 2010).

Although Sri Lanka is not a high-burden country, TB remains a significant public health issue. The estimated incidence rates of all forms of tuberculosis in 2009 were 66 per 100,000 populations (WHO. The Regional Report 2011) and 9643 new TB cases were notified (NPTCCD annual report. 2009). The overall treatment success rate among smear positive cases was 85% among the new smear positive cases and 70% among re-treatment patients in the year 2008 (WHO. The Regional Report, 2011). A study conducted by the National Reference Laboratory-Sri Lanka during the period 2005 - 2007 revealed that 3.4% of MTB strains were resistant to RIF and 4.5% were resistant to isoniazid (INH) while 2.5% were multi drug resistant (Elwitigala *et al.* 2008).

Rifampicin is the most important drug in the short-course treatment regimen due to its efficient antimicrobial action (Rattan and Ahmad. 1998). Rifampicin interferes with initiation of DNA transcription. It is stated that approximately 90% of world's RIF resistant isolates are also resistant to INH, making RIF resistance a useful marker of MDR-TB that is at least resistant for RIF and INH (Traore *et al.* 2000). Most of RIF resistant MTB strains from different countries appear to harbour specific point mutations in the Rifampin Resistance-Determining Region (RRDR) of *rpoB* gene which comprises an 81bp region containing codons 507–533 (Williams *et al.* 1998; Mani, *et al.* 2001). In addition, studies have also revealed a significant contribution to RIF resistance through mutations in the region outer to RRDR of the *rpoB* gene, suggesting that, the nature and frequency of mutations in the *rpoB* gene of RIF resistant MTB clinical isolates vary considerably, between different geographical regions (Lingala *et al.* 2010; Ozkutuk *et al.* 2007). Further, it has been reported that there is discordance between results of phenotypic methods and genotypic methods that are based on RRDR mutations in the detection of drug resistant TB (Lingala *et al.* 2010).

The WHO recommends to perform DST for at least RIF and INH in resource limited settings, especially in previously treated patients and HIV co-infected patients. Early diagnosis of drug resistant TB and the management of MDR-TB patients are challenging obstacles faced by tuberculosis control programmes all over the world (WHO policy statement .2010). Presently, numerous molecular based identification methods such as INNO-LiPA Rif.TB (INNOGENETICS, Belgium) and Geno Type MTBDR*plus* (HainLifescience, Germany) have been commercialized for identification of rifampicin resistance. However, these methods are based on the detection of commonly reported mutations which are at codon 531, 526 and 516 (WHO, 2010). It is now recognized that there is a need to customize the detection systems according the geographical variation of the mutations (Tan *et al.*2012).

In Sri Lanka, rifampicin resistance is determined by the conventional proportion method that requires more than 28 days for detection of drug resistance. Thus, evolution of the rapid culture methods is very important for early and accurate disease diagnosis. However, drug susceptibility pattern may delay at least 10 days even with the use of rapid culture methods. Thus, molecular methods for determination of drug resistant tuberculosis are vital in controlling and prevention of the disease. Prior to establishing molecular based methods for detection of drug resistance in a particular geographical setting, it is important to characterize the mutations in *rpoB* gene in MTB isolates.

Genotyping of *M. tuberculosis* has been extensively used for investigating epidemics of MDR-TB. There is much debate about the degree of transmissibility as a specific characteristic of drug resistant strains. This controversy focuses on whether MDR strains are more easily transmissible or whether the mutations that confer drug resistance also impair the reproductive function of the organism (Sougakoff. 2011). Application of molecular epidemiological methods was central to the identification and description of all drug resistant outbreaks. The most extensive MDR-TB outbreak reported was from New York, among 267 patients who were infected by Beijing/W genotype (Frieden *et al.*1996). This cluster of cases included drug resistant isolates that were resistant to all first line anti TB drugs. Molecular methods were used to identify, how this initially fully drug susceptible strain expanded to result in a MDR phenotype by sequential acquisition of resistance conferring mutations in several genes (Bifani *et al.* 1996). Beijing/W genotypes can be identified by their characteristic multi-banded *IS*6110 Restriction Fragment-Length Polymorphism (RFLP) patterns and Beijing/W genotypes significantly contribute in drug resistance. However, the studies conducted later suggested that MDR outbreaks are not limited to the Beijing/W genotype (Bifani *et al.* 2002). Thus, studies on molecular epidemiology are very important in a particular geographical area for prevention of drug resistant outbreaks and understanding the transmission pattern of MDR-TB.

(B) Scientific scope of the project (overall and specific objectives)

(1) To study the RIF resistant gene mutations in a selected group of MTB strains in Sri Lanka (part 1).

Specific objectives

(a) To identify the MTB isolates using phenotypic characters of colonies, biochemical test and PCR amplification of specific gene fragment for MTC (part 2).

(b) To determine the rifampicin resistance of MTB isolates using the conventional Agar Proportion Method (part 2).

(c) To evaluate of manual MGIT and NRA for rapid identification of RIF resistance of MTB (part 2).

(d) To identify the rifampicin resistant mutations of the *rpoB* gene of MTB among drug resistant isolates using DNA sequencing (part 3).

(2) To develop a rapid molecular method for determination of RIF resistance

Specific objectives

(a) To optimize PCR-ELISA as a rapid molecular method for detection of rifampicin resistant mutation (part 4)

(b) To validate the PCR-ELISA as a diagnostic method for detection of rifampicin resistance of MTB culture isolates (indirect method) (part 4)

(3) To determine the molecular epidemiology of rifampicin resistance in Colombo district where the highest disease burden reported in Sri Lanka

Specific objectives:

(a) To determine the restriction fragment length polymorphism (RFLP) pattern of 30 rifampicin resistant MTB isolates (part 5)

(b) To determine restriction fragment length polymorphism (RFLP) pattern of 50 rifampicin susceptible MTB isolates (part 5)

(C) Materials and methods (including statistical methods)

Part 1:

Sputum specimens positive for AFB by smear examination were collected from suspected TB patients attending Chest Hospital-Welisaea, Central Chest Clinic - Colombo and Prison Hospital- Colombo. Sputum specimens were processed using the Petroff's method and concentrated by centrifugation at 3500 g in a refrigerated (4 °C) centrifuge for 15 min. Sediment was diluted in 1 ml sterile distilled water. A small portion of the suspension was stained with Ziehl-Neelsen (ZN) stain and examined microscopically for the detection of acid fast bacilli. Two slopes of L-J (Difco, US) (one containing paranitrobenzoic acid to detect Non Tuberculosis Mycobacteria (NTM) species) were inoculated with 100 µl of above bacterial suspension (WHO.1998). The inoculated culture media were incubated at 37 °C in 5% CO_2 until growth was observed or discarded as negative after 8 weeks. Culture isolates were confirmed as MTB, if they were slow growing, non-pigment producing, reduced nitrate and did not grow in the presence of paranitrobenzoic acid. Further, species confirmation was carried out by nitrate reductase test (biochemical test) and PCR amplification of fragment of *IS*6110 insertion element, specific to MTB complex using specific primers, pt18 (5'-GAA CCG TGA GGG CAT CGA GG-3') and INS2 (5'-GCG TAG GCG TCG GTG ACA AA-3') ((1^{st} base -Singapore) (Kolk *et al.* 1998).

Part 2:

The MTB isolates retrieved from part one of the study were tested for RIF resistance by APM, the gold standard for culture based drug susceptibility testing of MTB. Agar proportion method was carried out on Middlebrook 7H10 agar (Difco, US) plates as per Clinical and Laboratory Standards Institute (CLSI) guidelines (Forbes *et al.* 2004). Briefly, fourteen days old, fresh MTB cultures grown on L-J medium were suspended in 7H9 broth medium to achieve a turbidity of McFarland No.1 standard. This bacterial suspension was further diluted (two fold and four fold) and used for inoculating rifampicin incorporated (1 µg/ml) agar plates and rifampicin free control agar plates. Colonies on each plate were counted on the 28^{th} and 42^{nd} day of inoculation, and the proportion of organisms growing in the presence of rifampicin was calculated.

The MGIT (BD diagnostics, US) that contains 4ml of modified Middlebrook 7H9 broth medium (figure 1) was used for evaluation of manual MGIT as a rapid culture based drug susceptibility testing. The RIF containing tubes (final rifampicin concentration of 1 µg/ml) were inoculated with 500 µl of a 1:5 dilution of a McFarland No: 0.5 bacterial suspension and rifampicin free control tubes were inoculated with 500 µl of a 1:500 dilution of same bacterial suspension as per manufacturer's guidelines. The emission of fluorescence was measured using the manual MGIT reader (figure 2) from day 2 onwards.

Middlebrook 7H9 broth medium with 0.1% sodium nitrate was used as the medium for NRA. Each RIF containing medium (final RIF concentration of 1 µg/ml) was inoculated with 100 µl of McFarland No: 1 bacterial suspension. The control medium was inoculated with 100 µl of a 1:10 dilution of the same bacterial suspension. Two sets of the drug free and drug containing media were inoculated per isolate. On 6^{th} day of incubation, the first set was examined for a colour change by sequentially adding of Griess reagent (10 µl of 50% HCl, 20 µl of 0.2% sulfanilamide and 20 µl of 0.1% N-napthtylethylene-diamine) to the culture medium. Readings were obtained visually by comparing with the prescribed colour standards (figure 3). Readings between +5 to +3 of the standard colour series were considered positive. If the drug free control medium was negative, the second set was tested at day 12^{th} incubation.

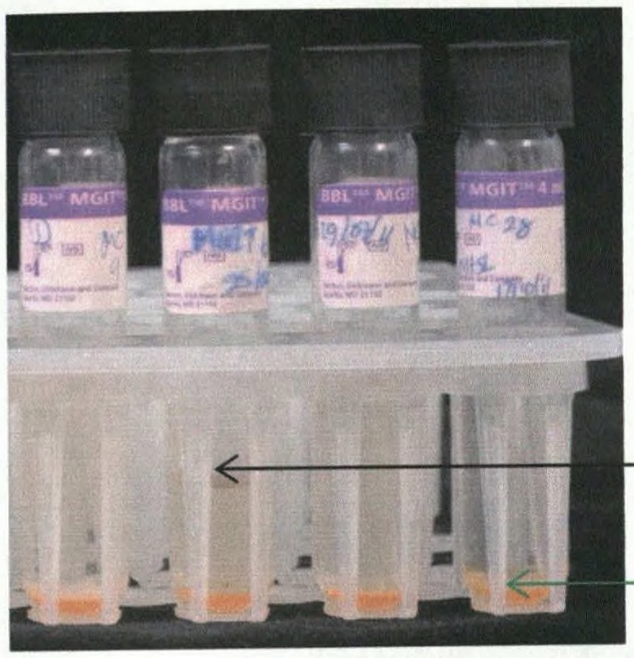

Figure 1: **Mycobacteria growth indicator tube containing 4 ml Middlebrook 7H9 broth medium and fluorescence labeled material on the bottom the tube**

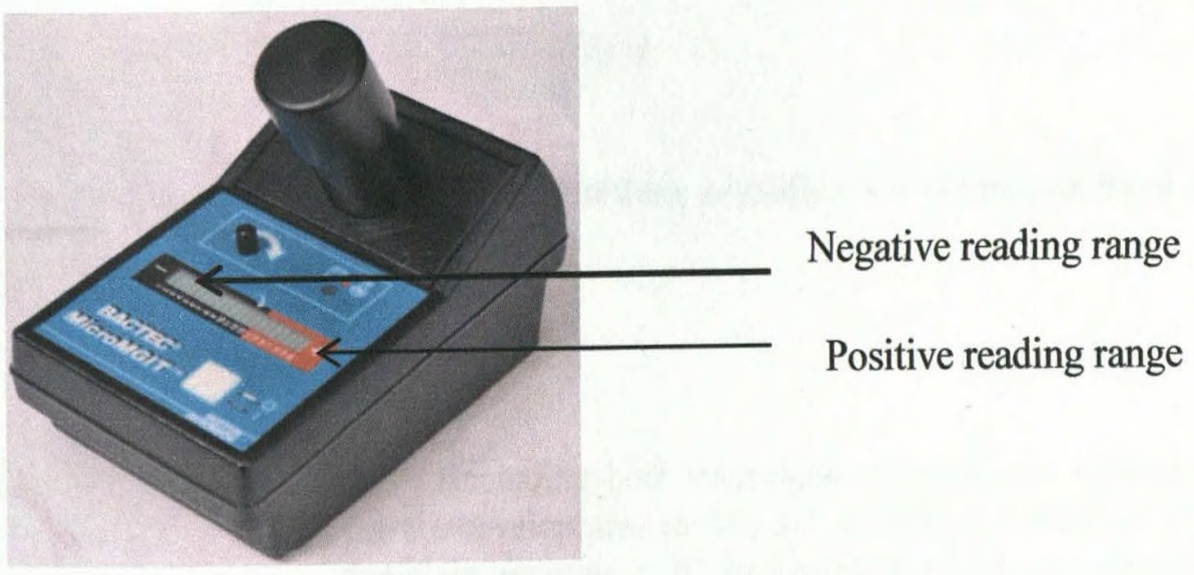

Figure 2: **View of manual MGIT reader**
Reading from 1-14 indicate the absence of bacterial growth in the MGIT.
Readings from 14-20 (red colour region) indicate the growth of bacteria.

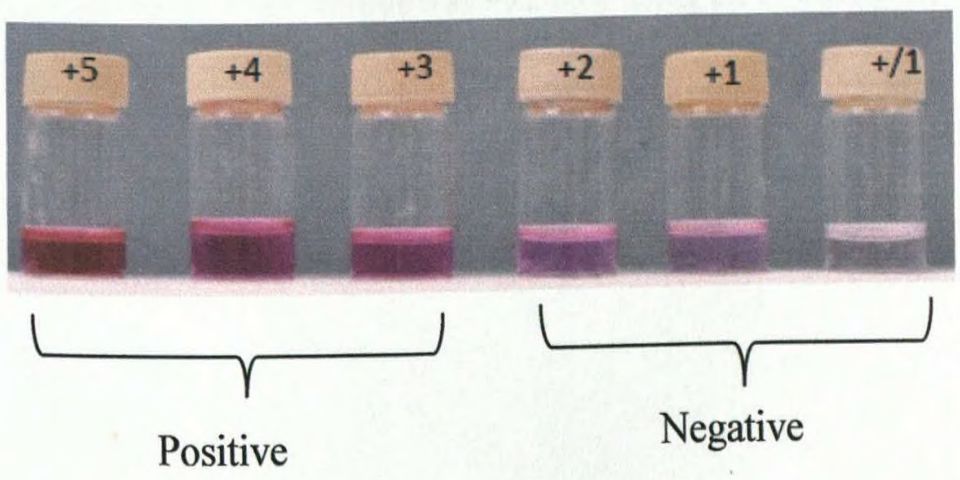

Figure 3: **Standard colour series for NRA**

Part 3

DNA was extracted from identified rifampicin resistant MTB isolates by using phenol chloroform extraction method. Three fragments of the *rpoB* gene (437bp, 872bp and 1395bp) that covers RRDR and spanning RRDR were PCR amplified with specific primers (figure 4). The amplified products were purified and sequenced by Macrogen DNA sequencing service, in Korea.

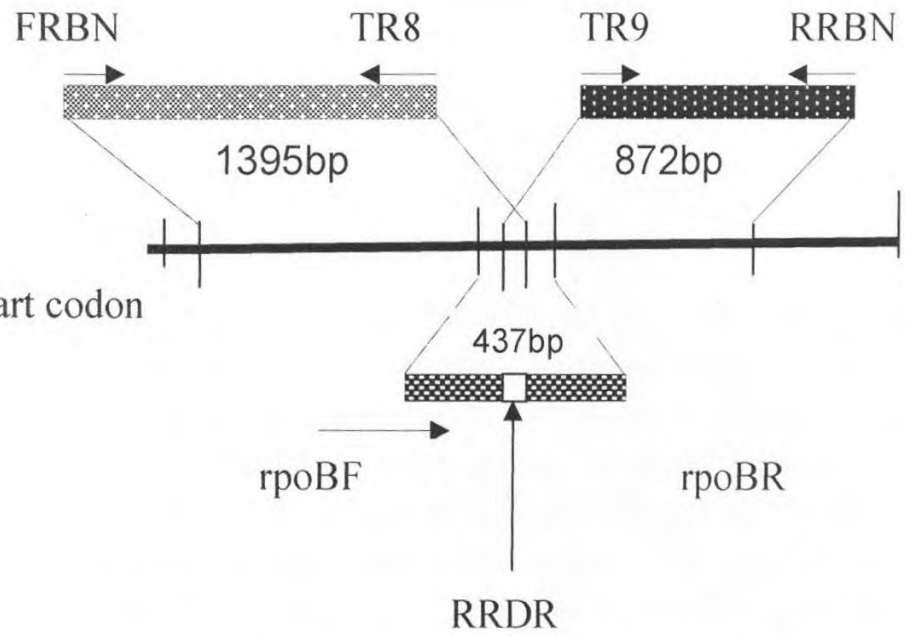

Figure 4: Position of primers used for amplification of selected three regions in *rpoB* gene of MTB

Part 4

Forty two MTB isolates (including both rifampicin resistant and rifampicin susceptible isolates) were used for the development (n=10) and evaluation (n=32) of PCR-ELISA for determination of rifampicin resistance. 5' biotinylated allele specific oligonucleotides corresponding to point mutations at codons 526, 531& 626 (most prevailing mutations of MTB isolates in Sri Lanka as identified in the study-part 3) were custom synthesized. The probes were immobilized on streptavidin coated microtiter plates. Digoxigenin labeled PCR amplified fragments of the *rpoB* gene (437bp and 872bp) were hybridized to immobilized probes. The hybridization was detected using peroxidase conjugated anti-Digoxigenin and the optical density (OD) of colour development was measured at 405 nm (reference wave length was 492 nm) using an ELISA plate reader (Awareness technology, USA). The sensitivity of the PCR-ELISA was measured by a serial dilution of H37Rv DNA and the specificity was determined by using DNA of non-tuberculosis Mycobacteria (NTM).

Part 5

The genomic DNA extracted from RIF resistant isolates (31) and RIF susceptibility (50) isolates were digested by using *PvuII* enzyme. Following electrophoresis of digested DNA mixture on 0.8% agarose gel, southern blotting were carried out in order to transfer the fragments of DNA on to the nylon membrane. After transferring, the membrane was baked and subjected to probe hybridization. The probe was prepared in the standard manner (Soolingen *et al.* 1993). The hybridization was detected by using Amersham ECL^{tm} detection reagents and the RFLP pattern was developed on x-ray film by using standard x-ray development method.

Statistical analysis

The suitability of the manual MGIT and the NRA in comparison with the APM was evaluated in terms of sensitivity (the ability to detect true drug resistance), specificity (the ability to detect true drug susceptibility), positive likelihood ratio and negative likelihood ratio. A positive likelihood ratio above 10 or a negative likelihood ratio below 0.1 was considered to indicate excellent test-performance, and ratios above 5 and below 0.2 were considered to indicate adequate performance. The agreement between the NRA results or the manual MGIT results, and the APM were estimated by kappa statistics. The kappa value(k), a measure of test reliability, was interpreted as follows: <0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; ≥0.81, excellent. The consumable costs per test were calculated for each test method. The suitability of PCR-ELISA as a molecular drug susceptibility testing method was evaluated in same way.

DNA sequence analysis was done using NCBI data base and Sea View software (version 4.2.12). The RFLP is analysed using SPSS (version15) and gel comparison software.

(D) Results

Part 1

Table 1 details the number of AFB positive sputum specimens received from each of the sampling centers. Four hundred and forty two (442) *Mycobacterium* isolates were yielded from 534 AFB positive sputum specimens in solid and liquid culture media collectively. Of the remaining sputum specimens (92, 17%), 64 did not show any growth on a subsequent culture in any of the media used, while 28 sputum specimens were unable to grow Mycobacterium spp. Among the 92 sputum, 25 (28.7%) isolates were collected from secondary TB patients and reaming 67 (15 %) were from new patients. Among 442 *Mycobacterium* cultures, 401 were *M. tuberculosis* complex and remaining, 41 were NTM (figure 5).

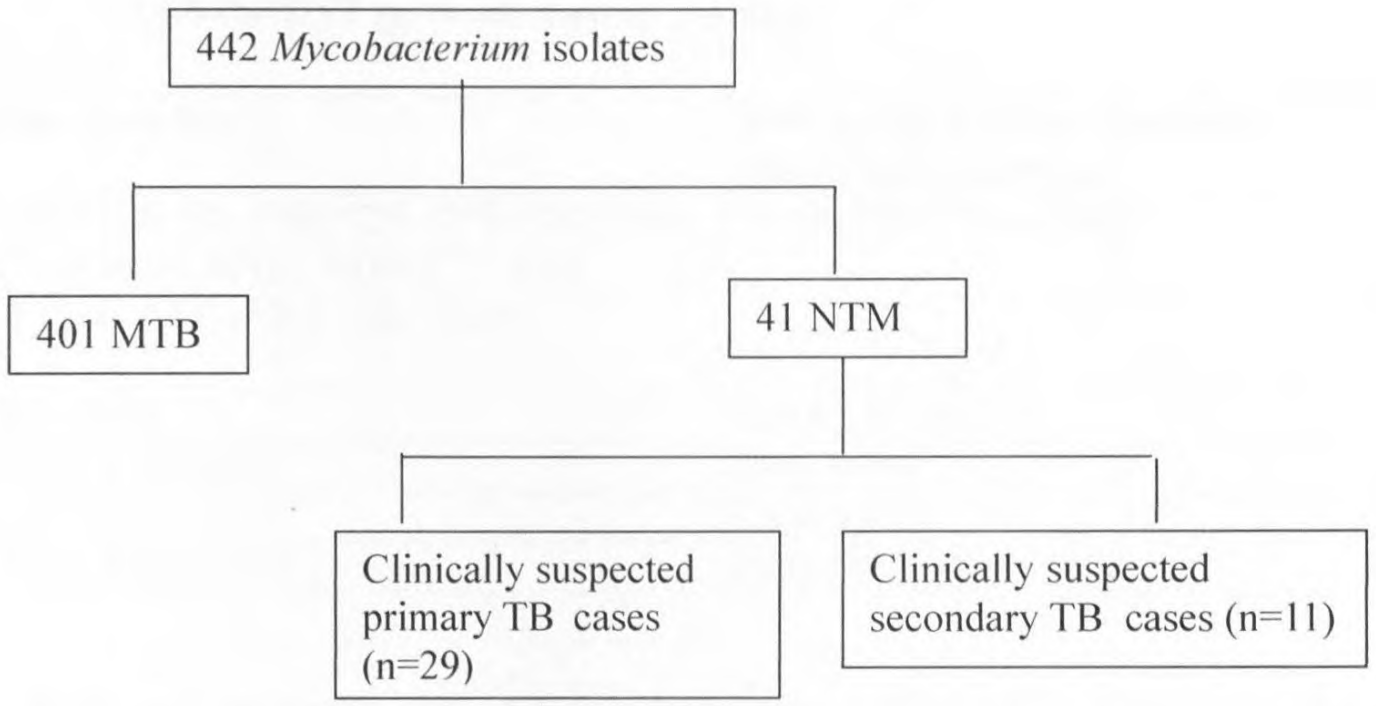

Figure 5: ***Mycobacterium*** **cultures retrieved from collected AFB positive sputum (n=534)**

Table 1: No of AFB positive sputum specimens were received from each sampling centers during March 2008 to October 2011

Collection center	**No. of AFB positive sputum specimen**	**Clinically suspected treatment category of patient**
Chest hospital-Welisara	80	Only secondary TB patients (default, retreatment, treatment failure)
Central chest clinic - Colombo	418	Primary and secondary TB patients
Prison hospital - Colombo	31	Primary and secondary TB patients
Routine laboratory at Dept. of Microbiology	5	Primary and secondary TB patients
Total	534	Primary and secondary TB patients

Part 2

Twenty seven strains were detected as rifampicin resistant by the APM, the currently used gold standard for conventional methods. MGIT and NRA identified 26 isolates each. There were 3 discordant results between the NRA and the APM and one discordant result between the MGIT and the APM in determination of RIF resistance. Three isolates that were detected as rifampicin resistant by NRA were susceptible by the manual MGIT and APM. Furthermore, one isolate that was rifampicin resistant by the manual MGIT was susceptible by APM and NRA (Table 2). Repeat testing of these 4 isolates provided the same results.

Table 2: Pattern of Individual strains (n=31) showing resistance to rifampicin with the DST methods used in the study

Strain no (Lab No.)	DST method/s that confirmed rifampicin resistance
C4, C6, C7, C8, C9, C10, C20, C73, C83, C86, C88, C115, M60, M127, M15, C27, C22 C23,C163, C254 C150, C135, C110 (n=23)	APM, MGIT and NRA
M9, M33 (n=2)	APM and MGIT
PCR 88,PCR 57 (n=2)	Only APM
C120 (n=1)	Only MGIT
M46 , C25, M22 (n=3)	Only NRA

The sensitivity and specificity of the NRA in 7H9 broth medium when compared with the APM were 85 % and 99 % respectively (Table 3). Thus, there was a good agreement between NRA and APM for detection of rifampicin resistance (κ= 0.86). An excellent agreement was also observed between the manual MGIT and APM (κ= 0.94) (Table 4) with 93 % sensitivity and 100 % specificity. The average turnaround time for MGIT and NRA in liquid medium was 08 days (mean) and 10 days (mean) respectively.

Table 3: Sensitivity, specificity, positive and negative likelihood ratio for NRA compared to APM (n=381) in determination of RIF sensitivity

APM	NRA		Sensitivity %	Specificity %	Likelihood ratio	
	No. of resistant isolates	No. of susceptible isolates			Positive	Negative
Resistant(27)	23	4	85	99	101	
Susceptible(354)	3	351	(23/27x100)	(351/354x100)		0.13

Table 4: Sensitivity, specificity, positive and negative likelihood ratio for the MGIT method compared to APM (n=381) in determination of RIF sensitivity

APM	MGIT		Sensitivity %	Specificity %	Likelihood ratio	
	No. of resistant isolates	No. of susceptible isolates			Positive	Negative
Resistant (27)	25	2	93	100	328	
Susceptible(354)	1	353	(25/27)	(353/354)		0.07

Part 3

DNA sequencing of amplified fragments of the *rpoB* gene revealed that mutations in the RRDR region accounted for resistance in 18 (54%) isolates and were confined to two codons, 526 (n=15, 48.4%) and 531 (n=3, 9.7%). The remaining mutations were detected in the regions outer to RRDR and occurred at codons 626 (n=13, 41.9%) and 184 (n=2, 6.4%). Twenty nine isolates showed single point mutations at codon 526 (n=14, 45.2%), 626 (n=11, 35.5%), 531 (n=3, 9.7%) and 184 (n=1, 3.2%). Dual mutations (at codon 526+626 and at codon 184+626) were detected in 2 strains (table 5).

The mutation observed at codon 526 (CAC → TAC) and codon 531 (TCG →TTG) was a C to T transition mutation while the novel mutation at codon 626 (GAC → GAG) was a C to G transversion mutation and at codon 184 (GAC → GAT) a transition mutation. All point mutations in this study revealed only a single mutation pattern and there were no multiple patterns of base changes at a single codon.

NCBI Accession numbers for DNA sequences: HQ377336-HQ377338, HQ377340-HQ377345, HQ377351, and JQ314433-JQ314444

Table 5: **Frequencies and types of mutations in the *rpoB* gene of MTB isolates from Sri Lanka.** The mutated codons with corresponding amino acids are indicated.

*Codons within RRDR, $Codons outer to RRDR, +novel Mutations

Mutated codon	**Specific mutation**	**Primary/secondary TB (No.)**		**Percentage %**
526*	CAC (His) → TAC (Tyr)	Secondary-10 Primary -4		45.2
626$+	GAC(Asp) → GAG (Glu)	Secondary-7 Primary -4		35.5
531*	TCG (Ser) → TTG (Leu)	Secondary-0 Primary-3		9.7
184$+	GAC(Asp) → GAT(Asp)	Secondary-0 Primary-1		3.2
626,184	GAC→GAG,GAC→ GAT	Secondary-0 Primary-1		3.2
526, 626	CAC→TAC, GAC→ GAG	Secondary-1 Primary-0		3.2
Total				100

Part 4

The positive and negative cut off of optical density (OD) value for the PCR-ELISA assay were 1.25±0.37 (Mean OD value ± S.D.) and 0.4±0.12 (Mean D value ± S.D.) respectively. In validation, there was a good agreement between APM and PCR-ELISA with 86% sensitivity and 100% specificity for identification of RIF resistance of MTB. The turnaround time of the assay was 2 days after isolation of primary cultures (table 6).

Table 6: Sensitivity, specificity, positive and negative likelihood ratio for PCR-ELSA compared to phenotypic methods (n=32)

Phenotypic method	PCR-ELISA		Sensitivity %	Specificity %	Likelihood ratio (95%)	
	No. of resistant isolates	No. of susceptible isolates			Positive	Negative
Resistant (22)	22	0	86		Infinite	
Susceptible (10)	0	7		100		0.14

Part 5

The rifampicin resistant strains showed different DNA fingerprinting patterns with 1–15 copies of the *IS*6110. The absence of identical banding patterns demonstrates the presence of epidemiologically nonrelated strains. Thus, it is plausible that rifampicin resistant MTB strains represent epidemiologically different strains. Further analysis of RFLP using computer software is being carrying out currently to arrive at more detailed analysis.

(E) Discussion

The present study on RIF resistance of MTB in from Sri Lanka was split into several components for the purpose of improving clarity of presentation and the articles submitted to journals were based on these different components. Two rapid culture based DST methods were evaluated using the MTB isolates retrieved from AFB positive sputum specimens as an alternative for proportion method. Then, the genetic basis of rifampin resistance of MTB isolates from Sri Lanka was determined by investigation of *rpoB* gene mutations. Then, the PCR-ELISA was developed as a simple, cost effective, rapid and sensitive DST method for determination of RIF resistance. Finally, molecular epidemiology was investigated to determination of transmission of RIF resistant isolates.

Laboratory has a critical role in diagnosing of TB, MDR-TB and monitoring its treatment. The strength of the laboratory network often directs the success of TB control programmes leading to prevention of the disease. Developed countries have sophisticated laboratory infrastructures with high techniques and provide rapid detection, identification and drug susceptibility testing of MTB that combined with good treatment programmes. In contrast, many developing countries, even in high rates of TB burdened regions struggle to provide good diagnosis using microscopy with scarce to non-existent culture and drug susceptibility testing (Drobniewski *et al.* 2003; WHO, 2006).

The AFB positive pulmonary infections should be addressed properly by enhancing the laboratory investigations, due to the emergence of NTM as a regular pathogen. Of 41 NTM isolates, 11 were recovered from suspected secondary TB patients. As NTM are often resistant to first line anti TB medication, AFB will continue to be detected in sputum continuously even after the 6 months of completed anti TB regimen (Vidal *et al.* 1996). Presumably, many of these cases would be considered treatment failures and subsequently treated incurring a high cost, less effective and more toxic second line anti TB drugs (Maiga *et al.* 2012). Most patients treated with second line anti TB drugs experiences at least one side-effect and two thirds of such patients have had at least one drug terminated temporarily or permanently as a result of severe adverse effects such as psychiatric episodes, hepatitis and renal failure (Bloss *et al.* 2010). Thus, improper diagnosis leads to severe complications in patients while making a negative impact on the economy of the country.

Furthermore, out of 401 MTB strains identified during the study, 51 isolates were recovered from suspected secondary TB patients. Thus, their anti TB treatment regimen should have been adjusted with the drug susceptibility pattern of the MTB isolates before continuing the medication with second line anti TB. WHO has recommended DST at least for RIF and INH prior to initiating second line anti TB drugs (WHO. 2010). The use of culture methods or genetic methods for DST is a standard practices in high resource countries. However, most of low-resource countries still struggle to provide culture DST for priority needs such as drug resistance surveillance, extra pulmonary and childhood TB, and MDR-TB (WHO. 2006).

The evaluated NRA in broth medium and manual MGIT are simple cost effective methods for determination of RIF susceptibility that can be easily applied in low resource settings. The drug susceptibility pattern may be available within 8 and 10 days by manual MGIT and NRA respectively. Therefore, these 2 methods are good alternatives for APM in discrimination of RIF resistance. In government sector of Sri Lanka, peripheral microscopic centers have to direct suspected specimen to reference laboratory for DST and

this significantly delays the proper treatment. However, manual MGIT and NRA can be used for culture with minimum infrastructure facilities. The consumable cost per test for APM and NRA is around US$ 4.00 and US$ 3.00 respectively. Comparatively, the manual MGIT is more expensive (~US$ 7.00). Nevertheless, both the NRA and the manual MGIT methods can be initiated with low technical expertise and initial cost. Thus, application of manual MGIT and NRA methods will contribute to decentralize the availability of drug susceptibility testing in Sri Lanka which will improve control and prevention of MDR-TB within the country.

Drug susceptibility results may be delayed at least a week even with use of Rapid culture based methods. Thus, genetic based molecular methods that arrive at precise conclusions on DST of MTB within few days or hours are vital for ensuring early and accurate chemotherapy. In the last century, several genetic based molecular DST methods that based on the mutations of *rpoB* gene were commercialized. However, these methods are costly and are beyond the routine use in developing countries. Additionally, these methods may not be achieve the expected sensitivity in certain geographical areas as mutations of *rpoB* vary geographically (Siu *et al.*2011; Tan *et al.* 2012).

The *rpoB* gene mutations discovered during this study will significantly contribute to expanding the knowledge on the profile of mutations in the *rpoB* gene by revealing 2 novel point mutations at codon 184 and codon 626 that are spanning RRDR. Mutation at codon 626 will significantly contribute (35.5%) to RIF resistance in Sri Lanka. Even though point mutations at codon 531 have been recorded as world prevailing mutation, it contributed to only 9.7% of RIF resistance and the most prevailing mutated codon was 526 (Adikaram *et al.* 2012). The type and frequency of the prevailing mutations that were identified in the present study fairly match with the Sheng *et al* 2008, that reported high mutation frequency at 526 (73.2%) and less mutation rate at cordon 531 (3.5%). Further, absence of mutation at codon 531 of 43 *M. tuberculosis* isolates has been recorded from China (Tan *at al.* 2012). Therefore, the commercialized molecular DST methods will identify around 60% of MDR cases in Sri Lanka (Adikaram *et al.* 2012). Considering all these factors, the PCE-ELISA was established as a simple molecular DST method that can be customized according to user requirement.

Good agreement was shown between PCR-ELISA and APM for identification of RIF resistance with 86% sensitivity and 100% specificity. Further, the sensitivity and specificity of probes were $\leq$ 1 pg and 100% respectively. Ability to design the probes as per user requirement and availability of conclusion of drug susceptibility after 2 days of primary isolation of cultures are added advantages of this method. The sophisticated instruments or infrastructures are not required and these techniques can be made familiar to most of the technicians easily. Technician will be able to carry out the test by qualitative analysis of colour development even in the absence of an ELISA reader. Once, extraction of DNA is completed there is no biohazard risk and people can carry out the test even in a bench where cross-contamination of the PCR amplification can be managed. The initial instrumentation and consumable cost is not behind the settings in Sri Lanka.

In general, the work presented in this thesis has contributed to expand the knowledge on the profile of mutations in the *rpoB* gene and delivered scientific data for increasing the laboratory strength of determination of MDR-TB, a major issue faced by TB control programms.

(F) Conclusion

Sri Lanka is located in a separate geographical area, neighboring India, one of the major contributors for the MDR- TB and XDR- TB in the world. Thus, Sri Lanka is at risk of acquiring XDR-TB in the future, if there are no proper control programs that address the persistent issue of drug resistant tuberculosis in timely manner. The study has focused on a current drawback in controlling and prevention of TB in Sri Lanka, the lack of simple rapid and inexpensive drug susceptibility testing for determination of drug resistance.

The study describes the specific need for definitive laboratory identification following AFB microscopy for the *Mycobacterium* lung diseases in Sri Lanka. The variation of disease outcome generated by this study provided the strong background for the evaluation of the rapid diagnostic tools to determine RIF resistance that is a marker of the MDR TB. Both the NRA in liquid medium and the manual MGIT agreed well with the APM in determination of RIF sensitivity. Further, as need of sophisticated instruments and high technical skills is minimal, the initial establishment cost will be low. Introduction of these methods for low resource settings will make the identification of rifampicin RIF faster and cost effective.

The genetic based molecular DST methods may dramatically reduce the time for the diagnostic of MDT-TB. The two novel point mutations outer to the RRDR and different frequencies of universally prevailing mutations in the RRDR of the *rpoB* gene of MTB isolates in Sri Lanka emphasize the need for expanding the geographical database of the mutations for effective application of the *rpoB*-based diagnosis of MDR-TB in public health settings. It may be necessary to customize the *rpoB*-based molecular diagnostic methods so as to make them suitable to detect locally prevalent mutations.

The PCR-ELISA is a simple, rapid, inexpensive sensitive and specific molecular based DST method that can be customized as per variation of profile of mutation of *rpoB* gene of MTB in a particular geographical region. Application of PCR-ELISA will certainly contribute to the controlling and prevention of MDR-TB in Sri Lanka by early and accurate detection of drug resistance. In future, the PCR-ELISA should be validated for direct detection of RIF resistances from sputum specimens in order to reduce time spend for primary cultures *of* MTB.

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(H) Problems if any, encountered during the implementation of the project

Initially a problem was encountered when patients were recruited from the Central chest Hospital, but managed to circumvent by enrolling additional centres for patient recruitment

(I) Major findings and follow up activities

Major findings

(a) The laboratory differentiation of *Mycobacterium spp* into MTB complex and NTM is essential prior to treatment as well as performing DST, especially in suspected secondary TB cases as NTM are frequently resistant to conventional anti TB drugs.

(b) The evaluated new DST methods, manual MGIT and NRA are rapid and good alternatives for conventional proportion method for determination of MDR-TB.

(c) The presence of a high frequency of new mutations as reported here, compared to those reported earlier, emphasizes the need for expanding the geographical database of mutations for effective application of *rpoB* based diagnosis of MDR TB and customizing rapid molecular based drug susceptibility testing.

(d) The developed PCR–ELISA is a simple, inexpensive, rapid, sensitive and specific molecular based DST method that can be customized as per user requirement. Thus, it is a suitable alternative molecular DST method for determination of RIF resistance of MTB.

(e) The RIF resistant MTB strains in Sri Lanka are not belonged to a single clone and represent epidemiologically different strains.

Follow up activities

(a) The evaluation of PCR-ELISA as a direct method (directly from sputum specimen) for determination of RIF resistance is important as it reduces the time of the assay by eliminating the primary isolation of the MTB strains.

Section 4

Impact of Research results

i) Relevance of results achieved to scientific advancement

The results of the present study contribute to expand the scientific knowledge on RIF resistance of MTB. The new mutations and different frequencies of prevailing mutations that are reported here emphasize the need for expanding the geographical database of the mutations for effective application of the *rpoB*-based diagnosis of MDR-TB in public health settings. It highlighted the necessity of customizing the *rpoB*-based molecular diagnostic methods so as to make them suitable to detect locally prevalent mutations.

The developed PCR-ELISA is a simple molecular drug susceptibility testing method that can be applied even in the resource limited settings. The conventional ELISA that is based on the antigen and antibody has been mimicked by the DNA probe hybridization. This will add data to the scientific knowledge in the field of molecular based microbial drug susceptibility testing. The data on molecular epidemiology of the RIF resistance is more informative with the findings of present study. It fills the gap in the molecular epidemiological evidence of RIF resistance of MTB in different geographical settings.

ii) Relevance of results achieved to national/socio-economic development

In Sri Lanka, patients have to wait for over 2 months to receive their drug susceptibility testing reports after isolation of primary *M. tuberculosis* culture. During this period, empirical therapy is started in order to reduce dispersal of the disease and especially if the patient is seriously ill or the disease is progressing rapidly. However, prolonged inappropriate treatment would cause serious complications in patients as well as spreading of MDR-TB among others is inevitable. The cost of treatment per patient especially in secondary TB is very high and it will negatively impact on the economy of the country. People in productive age are more affected by TB and it will reduce their contribution to the economy of the country. Thus, the delaying of diagnosis of RIF resistance will lead to several complications in society and the economy of the country.

The culture based rapid methods (manual MGIT and NRA) and PCR-ELISA that were addressed by the present study determine the RIF resistance within 10 days and 2 days respectively after primary isolation of MTB cultures. These simple and cost effective methods are suitable for resource poor settings and will enable proper treatment rapidly. Thus, these methods are vital in the control and prevention of the MDR-TB in the country with added advantage to patients and the economy. Therefore, present study is highly relevant in the current context of diagnosing communicable diseases in Sri Lanka.

iii) Dissemination/application of research output

a) The manual MGIT has been applied in routine diagnosis of TB at the department of Microbiology. Faculty of the Medicine, University of Colombo.

b) The elevated rapid culture based manual MGIT and NRA is applicable for determination of RIF resistance in Sri Lanka as well as other settings.

c) The findings of the present study have been applied in customization of the *rpoB* gene based diagnostics methods by developing a PCR-ELISA. The PCR-ELISA may be applied in the health care settings as a rapid molecular diagnostic method for determination of drug resistance of MTB. This is yet to be established.

d) The findings of the present study will be applied in customization of the *rpoB* gene based commercialized diagnostic kits.

Section 5

Miscellaneous

i) List of major equipment acquired during the project period and their functionality

Instrument	**Function**
MGIT reader	Read of MGIT tube

ii) List of publications/communications arising from the project and/or presentations made at seminars, workshops etc. (Please attach copies)

(a) List of publications/communications

Published in a peer reviewed indexed journal

(1) Adikaram CP, Perera J , Wijesundera S. Geographical profile of rpoB gene mutations in rifampicin resistant *Mycobacterium tuberculosis* isolated in Sri Lanka. Journal of Antimicrobial Drug resistance www.ncbi.nlm.nih.gov /pubmed/ 22731859

Manuscript submitted for an indexed peer reviewed journal

1. Adikaram CP, Perera J, Wijesundera S. The Manual Mycobacteria Growth Indicator Tube and the Nitrate Reductase Assay are suitable alternatives for the rapid detection of rifampicin resistant *M. tuberculosis* in low resource settings (2012). Has been submitted to Journal of BMC infectious diseases

Manuscript in preparation for publication

1. Adikaram CP, Perera J, Wijesundera S. Probe based PCR- Enzyme-Linked Immunoabsorbent Assay (PCR –ELISA) as a susceptibility testing method for Mycobacterium tuberculosis (2012)

2. Adikaram CP, Perera J, Wijesundera S. Epidemiology of rifampicin resistance of M. tuberculosis in Sri Lanka (2012)

Research communication

1. **Adikaram CP**, Perera J , Wijesundera S. PCR-Enzyme Linked Immunoabsorbent Assay as a rapid molecular method for detection of rifampicin resistant Mycobacterium tuberculosis in low resource settings. The Ceylon Medical Journal 2012; 57: 31 (Presented at 125th anniversary international medical congress –Sri Lanka Medical Association)

2. **Adikaram CP**, Perera J, Wijesundera SW, Senaratne V. Mutations in the Non Rifampicin Resistance-Determining Region (RRDR) of the rpoB Gene are Important Determinants of *M tuberculosis* (MTB) Rifampicin Resistance (RifR). 51st annual sessions of ICAAC, Chicago, USA 2011; C1: 627.

3. **Adikaram CP**, Perera J, Wijesundera S W, Senarathne V. Mutations in the rpoB gene of rifampicin resistant Mycobacterium tuberculosis isolates in Sri Lanka. Association of the Pulmonologist-Sri Lanka 2nd Annual Sessions and Symposium 2010
(This paper awarded 1st place from oral presentation).

4. **Adikaram CP**, Perera J, Wijesundera S W, Weersinghe B. Prevalence of Tuberculosis and Rifampicin resistance in Colombo Prisons. The Ceylon Medical Journal 2010; 55: 48

5. **Adikaram CP**, Perera J, Wijesundera S. Comparison of Mycobacterium Growth Indicator Tube (MGIT) method and Nitrate Reductase Assay (NRA) with agar proportion method (APM) for detection of Rifampicin resistance. Proceedings of the 66th annual sessions of Sri Lanka Association for the Advancement of Science (SLAAS) 2010; 112A :12

6. **Adikaram CP**, Perera J, Wijesundera S. Genus Mycobacterium as an etiological agent of pulmonary infection in Sri Lanka. accepted for oral presentation in annual symposium –University of Colombo, 2012

7. **Adikaram CP**, Perera J, Wijesundera S, Perera GMM, Gamage S. Cord formation in a clinical isolate of *Mycobacterium abscessus.* Has been sent for the annual symposium of Association of Pulmonologist. 2012

(b) Presentations made at seminars, workshops

(1) The findings of the research were presented during the advanced training course on "Molecular Biology, Immunology, Biochemistry and Vaccinology applied to Tuberculosis" at WHO - TDR training centre, University of Lausanne, Switzerland (2010).

(2) The findings of the research were presented during the workshop on "working with pathogen genome" in India (January 2011)

Section 6

Summary Statement of Expenditure (indicate under Personnel, Equipment, Consumables, Travel and Subsistence and Miscellaneous) -

Please see the annex II

Section 7

i) Grantees' signatures

ii) Comments of the Head of the Department/signature

(Same as grantee)

iii) Head of the Institution's signature

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DipWomen's Stu (Col), DipMedEd(Dundee)
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August 6, 2012

To: Director
National Science Foundation
Colombo-07

Thro' Vice Chancellor, University of Colombo
Dean, Faculty of Medicine, Colombo
Supervisors: Professor Jennifer Perera, Dept of Microbiology
Dr Sulochana Wijesundera, Dept. of Biochemistry

Dear Sir

Final report - RG/ 2007/ BT/03

Herewith I am sending the final report alone with the financial report for the research on 'Determination of Drug Resistant Gene Mutations among Rifampicin Resistant Strains of *M. tuberculosis* in Sri Lanka".

Thank you

Yours faithfully

Chamila Adikaram
Research Student

Annex I

Publications/Communications arising from the project during the reporting period

Published in a peer reviewed indexed journal

1. Adikaram CP, Perera J , Wijesundera S. Geographical profile of rpoB gene mutations in rifampicin resistant *Mycobacterium tuberculosis* isolated in Sri Lanka. Journal of Antimicrobial Drug resistance www.ncbi.nlm.nih.gov /pubmed/ 227318 59

Manuscript submitted for an indexed peer reviewed journal

1. Adikaram CP, Perera J , Wijesundera S. The Manual Mycobacteria Growth Indicator Tube and the Nitrate Reductase Assay are suitable alternatives for the rapid detection of rifampicin resistant *M. tuberculosis* in low resource settings (2012). Has been submitted to Journal of BMC infectious diseases

Manuscript in preparation for publication

1. Adikaram CP, Perera J , Wijesundera S. Probe based PCR- Enzyme-Linked Immunoabsorbent Assay (PCR –ELISA) as a susceptibility testing method for Mycobacterium tuberculosis (2012)

2. Adikaram CP, Perera J , Wijesundera S. Epidemiology of rifampicin resistance of M. tuberculosis in Sri Lanka (2012)

Research communication

1. **Adikaram CP**, Perera J , Wijesundera S. PCR-Enzyme Linked Immunoabsorbent Assay as a rapid molecular method for detection of rifampicin resistant Mycobacterium tuberculosis in low resource settings. The Ceylon Medical Journal 2012; 57: 31 (Presented at 125th anniversary international medical congress –Sri Lanka Medical Association)

2. **Adikaram CP**, Perera J, Wijesundera SW, Senaratne V. Mutations in the Non Rifampicin Resistance-Determining Region (RRDR) of the rpoB Gene are Important Determinants of *M tuberculosis* (MTB) Rifampicin Resistance (RifR). 51st annual sessions of ICAAC, Chicago, USA 2011; C1: 627.

3. **Adikaram CP**, Perera J, Wijesundera S W, Senarathne V. Mutations in the rpoB gene of rifampicin resistant Mycobacterium tuberculosis isolates in Sri Lanka. 2nd annual sessions and symposium. Association of the Pulmonologist-Sri Lanka 2010
(This paper awarded 1st place from oral presentation).

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Geographical Profile of *rpoB* Gene Mutations in Rifampicin Resistant *Mycobacterium tuberculosis* Isolates in Sri Lanka

Chamila Priyangani Adikaram,[1] Jennifer Perera,[1] and Sandhya Sulochana Wijesundera[2]

The nature and frequency of mutations in the *rpoB* gene of rifampicin (RIF) resistant *Mycobacterium tuberculosis* clinical isolates varies considerably between different geographical regions. The objective of the present study was the identification of *rpoB* gene mutations responsible for RIF resistance in *M. tuberculosis* isolates in Sri Lanka. Three regions of the *rpoB* gene of *M. tuberculosis*, one corresponding to a 437-bp region, including the rifampicin resistance-determining region (RRDR) and two other regions (1395 bp and 872 bp) spanning the RRDR, were polymerase chain reaction amplified, and were subjected to DNA sequencing. The two mutations found within the RRDR in the 31 RIF resistant strains isolated in this study were at codon 526 ($n=15$, 48.4%) CAC (His)→TAC (Tyr) and codon 531 ($n=3$, 9.7%) TCG (Ser)→TTG (Leu). A significant proportion ($n=15$, 48.3%) showed mutations spanning the RRDR, including two novel mutations at codon 626 ($n=13$, 41.9%) GAC (Asp)→GAG (Glu) and 184 ($n=2$, 6.4%) GAC (Asp)→GAT (Asp), a silent mutation. Two isolates revealed double mutations (codons 626+526 and 626+184). The presence of a high frequency of new mutations, and the different frequencies of the universally prevailing mutations, as reported here, emphasizes the need for expanding the geographical database of mutations for effective application of an *rpoB*-based diagnosis of multidrug resistant tuberculosis.

Introduction

ONE THIRD OF THE WORLD'S POPULATION is estimated to be infected with *Mycobacterium tuberculosis* (MTB), and the majority of tuberculosis (TB) cases are concentrated in developing countries.[34] Globally, 8.8 million incident cases of TB were estimated in 2009. Most of the estimated cases occurred in Asia (59%) and Africa (26%) as compared to the Eastern Mediterranean Region (7%), the European Region (5%), and the Region of the Americas (3%). Three Asian countries, namely India, China, and Indonesia, are among the five countries with the largest number of incidents of TB in 2009. India alone contributed 26% of all TB cases worldwide, while India and China together accounted for 38%.[33]

Multidrug resistant tuberculosis (MDR-TB) is defined as resistance to at least rifampicin (RIF) and isoniazid (INH). The WHO has recognized MDR-TB and XDR-TB, or extensively drug resistant tuberculosis (MDR-TB plus resistance to any fluoroquinolone, and resistance to at least 1 of the 3 injectable drugs: capreomycin, kanamycin, and amikacin) as being a major challenge for controlling TB. Around 440,000 MDR-TB cases are estimated to emerge each year, and 150,000 people die annually from MDR-TB.[32] The four countries that have the largest number of estimated cases of MDR-TB are China, India, the Russian Federation, and South Africa.[34] The WHO progress report of 2011 states that 69 countries had reported at least one case of XDR-TB by the end of 2010, and it is estimated that 25,000 cases of XDR-TB will emerge each year. In an outbreak in South Africa, 52 out of 53 XDR-TB patients died within an average of 3 weeks of being diagnosed.[32]

Although Sri Lanka is not a high-burden country, TB remains a significant public health problem. The estimated incidence rates of all forms of tuberculosis in 2009 were 66 per 100,000 population,[35] and 9643 new TB cases were notified.[15] The overall treatment success rate among smear-positive cases was 85% among the new smear-positive cases and 70% among retreatment patients in the year 2008.[35] A study conducted by the National Reference Laboratory-Sri Lanka during the period 2005–2007 revealed that 3.4% of

Originally presented as poster (abstract) entitled "Mutations in the Non Rifampicin Resistance-Determining Region (RRDR) of the *rpoB* Gene Are Important Determinants of '*M tuberculosis*' (MTB) Rifampicin Resistance (RifR)" at the 51st Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) held in Chicago, Illinois, 2011

Departments of [1]Microbiology and [2]Biochemistry and Molecular Biology, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka.

MTB strains were resistant to RIF and 4.5% were resistant to INH, while 2.5% were multidrug resistant.[6]

RIF is the most important drug in the short-course treatment regimen due to its efficient antimicrobial action.[20] RIF interferes with initiation of DNA transcription. It is stated that approximately 90% of the world's RIF resistant isolates are also resistant to INH, making RIF resistance a useful marker of MDR-TB.[25,27] Most of RIF resistant MTB strains from different countries appear to harbor specific point mutations in the rifampicin resistance-determining region (RRDR) of the *rpoB* gene, which comprises an 81-bp region containing codons 507–533.[11,12,16,30] In addition, studies have also revealed a significant contribution to RIF resistance through mutations in the region outer to RRDR of the *rpoB* gene, suggesting that, the nature and frequency of mutations in the *rpoB* gene of RIF resistant *M. tuberculosis* clinical isolates vary considerably, between different geographical regions.[10,11,17–19] Further, it has been reported that there is discordance between results of phenotypic methods and genotypic methods that are based on RRDR mutations used for the detection of drug resistant TB.[10]

The WHO recommends performing drug susceptibility testing (DST) for at least RIF and INH in resource limited settings, especially in previously treated patients and HIV coinfected patients. An early diagnosis of drug resistant TB and the management of MDR TB patients are challenging obstacles faced by tuberculosis control programmes.[31] Presently numerous molecular-based identification methods such as INNO-LiPA Rif.TB (INNOGENETICS) and GenoType MTBDR*plus* (HainLifescience) have been commercialized for identification of RIF resistance. However, these methods are based on the detection of commonly found mutations at codon 531, 526, and 516.[34]

In Sri Lanka, RIF resistance is identified by the proportion method that requires more than 28 days for detection of drug resistance. Rapid molecular methods have yet to be established for identification of drug resistant tuberculosis. Before establishing molecular-based methods for detection of drug resistance, it is important to characterize the mutations in the *rpoB* gene in MTB isolates in a particular geographical setting. Therefore, the present study was focused on evaluating the nature and the frequency of mutations in the *rpoB* gene that are associated with resistance to RIF in *M. tuberculosis* strains isolated from Sri Lanka. The results of the present study will also contribute to expand the knowledge on the profile of mutations in the *rpoB* gene.

Materials and Methods

Collection of sputum samples

The sample collecting period was from March 2008 to September 2010, and only pulmonary TB cases were considered. The study population represented primary TB patients (patients that had never received treatment for TB or who had received anti-TB treatment for <1 month) as well as secondary TB patients (i.e., relapse, retreatment, treatment failure, and defaulters) (defaulter: A patient who returns to treatment, positive bacteriologically, following interruption of treatment for 2 months or more). Sputum samples were collected from secondary TB patients admitted to the Chest Hospital Welisara (the only chest hospital in Sri Lanka) for supervised treatment and from primary and secondary TB patients presenting to the Central Chest Clinic, Colombo (the location to which secondary TB patients are referred for re-evaluation and treatment from peripheral clinics). In addition, sputum from all TB suspects (cough >3 weeks) were collected from the Prison Hospital Colombo as prisoners are at a high risk of harboring drug resistant TB strains.

Isolation of M. tuberculosis *strains*

The sputum specimens were processed using the modified Petroff's method. Sodium hydroxide (4%) (Sigma)-treated sputum was centrifuged at 3000 *g* under refrigerated conditions (at 4°C). The centrifuged deposit was diluted in 1ml of sterile distilled water for preparing the bacterial suspension for inoculation. Two slopes of the Lowenstein–Jensen (L-J) medium (Difco), one containing paranitrobenzoic acid (PNB, Sigma), were inoculated with 100 µl of the bacterial suspension. A small portion of the bacterial suspension was examined microscopically, using the Ziehl-Neelsen (ZN) stain, to determine the presence of acid fast bacilli. The inoculated media were incubated at 37°C in a 5% CO_2 incubator up to 8 weeks until growth was observed. Cultures were confirmed as MTB if they were slow growing, non-pigment producing, reduced nitrate, and did not grow in the presence of PNB. The isolates were further confirmed as MTB by detection of the insertion element *IS*6110 by polymerase chain reaction (PCR) as published previously.[14] One Isolate from each patient was used for the study. The H37Rv strain and a known RIF resistant MTB isolate confirmed by the National Tuberculosis Institute, Bangalore, India were used as quality control strains.

DST for M. tuberculosis *strains*

DST was carried out using the agar proportion method,[7] the nitrate reductase assay (NRA),[2] and the manual Mycobacteria Growth Indicator Tube (MGIT).[1] Rifampicin (Sigma) stock solution (10 mg/ml) was prepared using dimethyl sulphoxide (DMSO) (Sigma), and filter sterilized aliquots were stored at −70°C. A working solution (1 mg/ml) was prepared by diluting the stock solution with sterile distilled water. The final concentration of RIF in each drug containing the culture medium used for the DST was 1 µg/ml[7] (Data not shown).

DNA extraction

Genomic DNA was extracted from identified RIF resistant isolates using the phenol chloroform extraction method.[29] Harvested heat-killed bacteria from the L-J slopes were incubated overnight with proteinase K (Sigma). Genomic DNA was extracted using phenol–chloroform (Sigma) and precipitated with absolute ethanol (Sigma) followed by washing with 70% ethanol. The precipitated DNA was resuspended in the TE buffer (pH 8.0). The quality and concentration of the extracted DNA was tested using an agarose gel (1.5%) electrophoresis, and the DNA was stored at −20°C.

PCR amplification and DNA sequencing

Extracted DNA was used for amplification of three different segments (437 bp, 872 bp, and 1395 bp) of the *rpoB* gene (Fig. 1). The amplified segments covered around 60% of

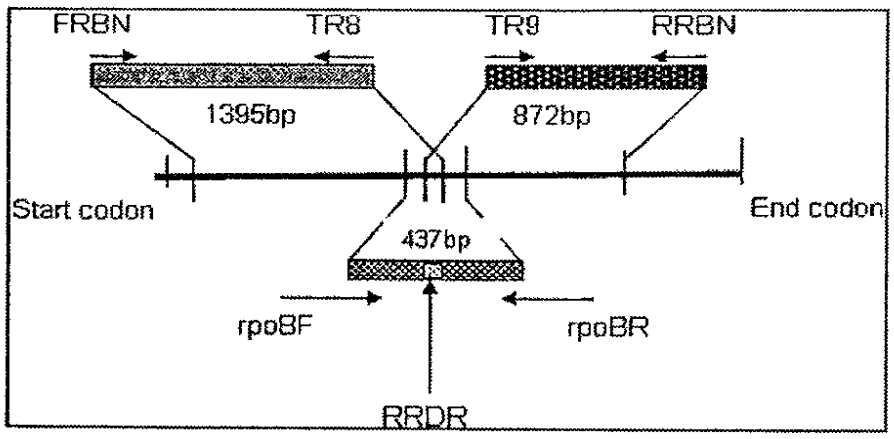

FIG. 1. Position of primers used for amplification of the three regions of the *rpoB* gene in rifampicin resistant *Mycobacterium tuberculosis* isolates.

the total length of the *rpoB* gene. Specific primers were designed using available literature[21,28] and primer-3 software. For amplification of the 437-bp region, including RRDR, rpoBF primer and rpoBR primer were used. The TR9 and RRBN primer pair was used to amplify the 872-bp region downstream of the RRDR region. The 1395-bp fragment upstream of RRDR was amplified using the FRBN and TR8 primer pair (Table 1) (all primers were purchased from Promega).

A 50 µl PCR mixture containing 50 mM KCl, 10 mM Tris (pH 8.0),1.5 mM $MgCl_2$, 200 µM of deoxynucleotides triphosphates (dNTPs) (Promega), 1 U *Taq* polymerase (GenScript), 20 pmol of each primer (Promega), and 2.5 µl of genomic DNA (10 ng) was used for each PCR reaction. The thermo-cycling parameters used for amplification of the three segments of the *rpoB* gene are shown in Table 1. The amplified PCR products were electrophoresed on ethidium bromide stained agarose gels (1.5%) and visualized under a UV illuminator.

Each resistant strain was PCR amplified in duplicate and both amplified products were custom DNA sequenced (Macrogen DNA sequencing service in Korea). A DNA sequence analysis was done using NCBI and Sea View software (version 4.2.12). All resistant isolates were also subjected to *IS6110* restriction fragment length polymorphism typing using the *Pvu*II restriction enzyme.[29]

Results

Three hundred and seventy-three (373) MTB strains were isolated from sputum samples during the period March 2008 to September 2010. The majority of patients were from the Western Province (Colombo, Gampaha, and Kalutara districts) of Sri Lanka, which has a high prevalence of TB.[15] Thirty one RIF resistant isolates were identified among the 373 MTB strains by one or more phenotypic method (APM, NRA, and manual MGIT). Thirteen RIF resistant isolates were from primary cases of TB and the remaining 18 isolates from patients with secondary TB.

DNA sequencing of amplified fragments of the *rpoB* gene revealed that mutations in the RRDR region accounted for resistance in 18 (54%) isolates and were confined to two codons, 526 ($n=15$, 48.4%) and 531 ($n=3$, 9.7%). The remaining mutations were detected in the regions outer to RRDR and occurred at codons 626 ($n=13$, 41.9%) and 184 ($n=2$, 6.4%). Twenty nine isolates showed single-point mutations at codon 526 ($n=14$, 45.2%), 626 ($n=11$, 35.5%), 531 ($n=3$, 9.7%), and 184 ($n=1$, 3.2%). Dual mutations (at codon 526+626 and at codon 184+626) were detected in 2 strains (Table 2).

The mutation observed at codon 526 (CAC→TAC) and codon 531 (TCG→TTG) was a C-to-T transition mutation, while the novel mutation at codon 626 (GAC→GAG) was a C-to-G transversion mutation and at codon 184 (GAC→GAT), a transition mutation. All point mutations in this study revealed only a single mutation pattern, and there were no multiple patterns of base changes at a single codon.

TABLE 1. PRIMER SEQUENCES AND THERMO-CYCLING PARAMETERS USED FOR THE AMPLIFICATION OF RIFAMPICIN RESISTANCE-DETERMINING REGION AND 5′ AND 3′ REGIONS OUTER TO RIFAMPICIN RESISTANCE-DETERMINING REGION OF THE *RPOB* GENE IN RIFAMPICIN RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES

Forward primer (5′-3′)	*Reverse primer (5′-3′)*	*Length of the fragment*	*Thermo-cycling parameters*
rpoBF TGGTCCGCTTGCACGAGGGTCAGA	rpoBR CTCAGGGGTTTCGATCGGGCACAT	437 bp	94°C-10min–denaturing 94°C-1min 57°C-1min 72°C-1min } 40 cycle 72°C-10 min–extension
TR9 TCGCCGCGATCAAGGAGT	RRBN GCGCCATCTCGCCGTCGTCAGTACAG	872 bp	94°C-10min–denaturing 94°C-1min 62°C-1min 72°C-1 min } 40 cycle 72°C-10 min–extension
FRBN GCAAAACAGCCGCTAGTCCTAGTCCGA	TR8 TGCACGTCGCGGACCTCCA	1395 bp	94°C-10min–denaturing 94°C-1min 60°C-1min 72°C-1 min } 40 cycle 72°C-10 min–extension

TABLE 2. FREQUENCIES AND TYPES OF MUTATIONS IN THE *RPOB* GENE OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM SRI LANKA

Mutated codon	*Specific mutation*	*Primary/secondary TB (No.)*	*Percentage%*
526[a]	CAC (His)→TAC (Tyr)	Secondary-10 Primary -4	45.2
626[b,c]	GAC(Asp)→GAG (Glu)	Secondary-7 Primary -4	35.5
531[a]	TCG (Ser)→TTG (Leu)	Secondary-0 Primary-3	9.7
184[b,c]	GAC(Asp)→GAT(Asp)	Secondary-0 Primary-1	3.2
626,184	GAC→GAG, GAC→GAT	Secondary-0 Primary-1	3.2
526, 626	CAC→TAC, GAC→GAG	Secondary-1 Primary-0	3.2
Total			100

The mutated codons with corresponding amino acids are indicated.
[a]Codons within RRDR.
[b]Codons outer to RRDR.
[c]novel mutations.
RRDR, rifampicin resistance-determining region; TB, tuberculosis.

NCBI Accession numbers for DNA sequences: HQ377336-HQ377338, HQ377340- HQ377345, HQ377351, and JQ314433-JQ314444.

Discussion

It is widely reported that MTB expresses a high degree of geographic diversity in mutations to the *rpoB* gene.[9,13,22,26,36,37] Mutations of codon 531, 526, and 516 have been reported as the most frequent mutations in the mutation hotspot region of the *rpoB* gene of MTB worldwide.[3,23,36] In the present study, the majority of mutations occurred at codon 526, and the mutation frequency (48.4%) observed was slightly higher than frequencies reported by China (40%),[36] Japan (35%),[16] and Korea (38%).[9] However, the frequency of occurrence of the mutation at codon 526 in India, the closest neighbor to Sri Lanka, is much higher (93%) as reported by Sharma *et al.* (2003).

Although the majority of published reports have shown CAC to GAC (transversion mutation) as the most prevalent base change at codon 526 (29.7% among Italian isolates[18] and 12% in Greece isolates[12]), our study revealed that the base change was CAC to TAC (transition mutation) in all isolates, similar to reports from India.[24]

The frequency of mutation at codon 531 (9.7%) was comparatively low in MTB isolates from Sri Lanka in relation to mutation frequencies reported by other countries, including India (47%),[10] Australia (52%),[37] Vietnam (31%),[4] and Poland (41%).[23] However, the base change observed at codon 531 was a TCG to TTG (transition mutation) as commonly reported by several Asian countries (Japan,[16] China,[36] Korea,[9] and India[11]) and other countries such as Greece[12] and Italy.[18] Previous studies have reported that a mutation at codon 531 is frequently associated with the codon 526 mutation in certain regions of the world.[22] However, this phenomenon was not observed in our study.

The mutation at codon 626 ($n=13$, 41.9%) GAC (Asp)→GAG (Glu) was the second major mutation found in the present study. Codon 626 is located downstream of RRDR and mutation in this codon has not been previously reported. This novel mutation was present in 13 isolates, and these strains showed different DNA fingerprinting patterns with 1–15 copies of the *IS6110*. The absence of identical banding patterns demonstrates the presence of epidemiologically nonrelated strains. Thus, it is plausible that *M. tuberculosis* strains with mutation at codon 626 represent epidemiologically different strains.

Two isolates showed a silent mutation at codon 184 GAC (Asp)→GAT (Asp) that is located upstream of RRDR. In one isolate, the mutation at codon 184 was associated with a mutation at codon 626. Several mutations in the upstream region of RRDR also have been reported from India.[10]

Mutations at codon 516 have been reported as prominent in several geographical areas such as India, 13%[10] and Iran, 10%[5] and therefore, commercially available RIF resistance diagnosis kits have been optimized to detect mutation at codon 516 among other mutation in the RRDR. However, no mutations were observed at codon 516 in the present study.

Several publications have stated that ~4% of phenotypically resistant isolates did not show any mutations within the RRDR.[4,8] Yue *et al.* (2003) have reported that 10% of phenotypically resistant MTB strains did not show any mutations at RRDR. According to Lingala *et al.* (2010), 21% of phenotypically RIF resistant isolates did not show any mutations when a 255-bp region of RRDR and 350-bp region of non-RRDR of the *rpoB* gene were sequenced. This may be due to mutations occurring in the region outer to RRDR, as was observed in 41.9% of RIF resistant isolates of MTB in the present study. A single discordant result was observed between phenotypic and genotypic results in the present study (phenotypically resistant, but no mutation), although the isolate showed a silent mutation at codon 184. This isolate was confirmed as resistant to RIF by APM and MGIT. It is possible that an additional point mutation is present in this isolate at a codon in the *rpoB* gene not investigated in the present study.

Current molecular diagnostic methods such as INNO-LiPARif are designed to detect common *rpoB* mutations at codon 531, 526, and 516. Therefore, they may not be

applicable universally across various settings, considering the geographical diversity of the mutations.[10,24]

Although the mutations in our strains are limited to a few types, the occurrence of a high percentage of mutations outside the RRDR presents important implications for the use of currently available rapid molecular methods, which target mutations in the RRDR region. Molecular tests used for rapid detection of drug resistance need to be customized to enable detection of novel mutations in different geographical areas. Therefore, identification of drug resistant mutations and their relevant frequencies is an important first step before introducing molecular DST in different geographical settings.

In conclusion, two novel point mutations outer to the RRDR and two universally prevailing mutations in the RRDR of the *rpoB* gene were observed in Sri Lankan RIF resistant MTB isolates. The new mutations and different frequencies of universally prevailing mutations that are reported here emphasize the need for expanding the geographical database of the mutations for effective application of the *rpoB*-based diagnosis of MDR-TB in public health settings. It may be necessary to customize the *rpoB*-based molecular diagnostic methods so as to make them suitable to detect locally prevalent mutations.

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Ethical Approval

The Ethics Review Committee of the Faculty of Medicine, the University of Colombo, Sri Lanka, approved the study (ERC number is EC/06/062).

Disclosure Statement

All authors report no conflicts of interest relevant to this article.

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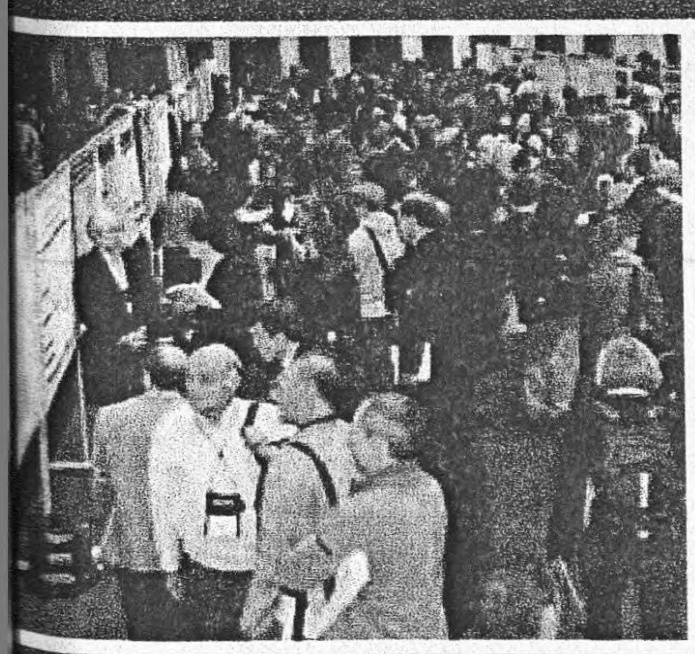

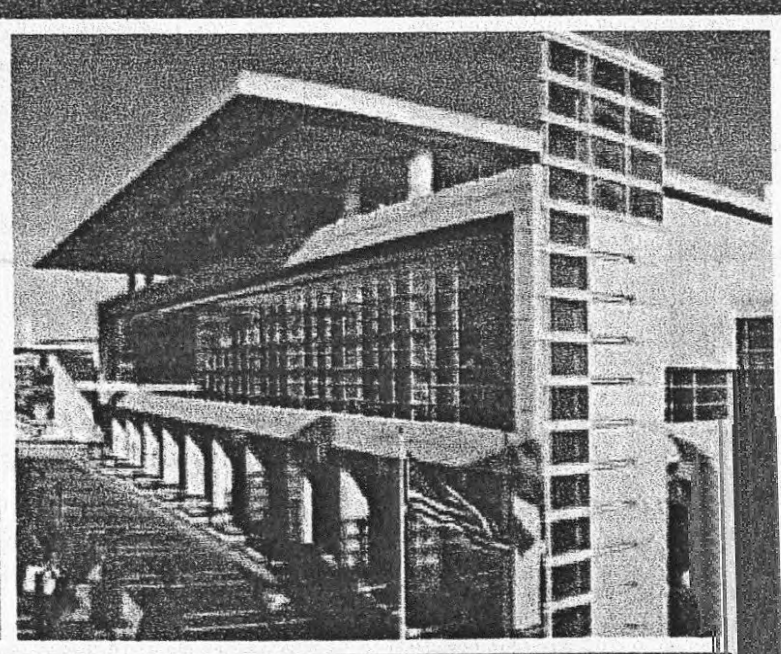

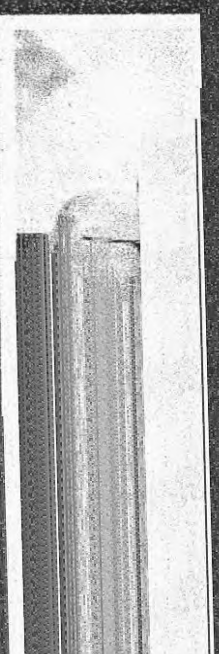

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I. Shukla, M. Ramirez, T. R. Parenteau, M. E. Tolmasky; California State Univ., Fullerton, CA.

C1-622 Identification of an Integrative and Conjugative Element (ICE) Carrying Twelve Resistance Genes in *Pasteurella Multocida*
G. Brenner Michael[1,2], K. Kadlec[1], M. T. Sweeney[3], E. B. Brzuszkiewicz[2], H. Liesegang[2], R. Daniel[2], J. L. Watts[3], S. Schwarz[1]; [1]Inst. of Farm Animal Genetics, Neustadt-Mariensee, Germany, [2]Georg-August-Univ., Göttingen, Germany, [3]Pfizer Animal Health, Kalamazoo, MI.

C1-623 Prevalence of *qnr, aac(6')-Ib-cr* Genes and Mutation of *gyrA* Among Levofloxacin-Resistant Clinical Isolates of *Stenotrophomonas maltophilia* in Brazil
J. Paez, J. Rosa Ferraz, F. Rossi, A. S. Levin, S. F. Costa; Hosp. das Clinicas, São Paulo, Brazil.

C1-624 Mobilization of an IS4 Transposase in a *Pseudomonas aeruginosa* Mutant Following Meropenem Exposure
R. C. Fowler, N. D. Hanson; Creighton Univ., Omaha, NE.

C1-625 The Carboxyl-Terminus of Eremomycin Interacts with the None-D-Ala-D-Ala Segment of Peptidoglycan Pentapeptide Stem for Binding
S. Kim[1], S. S. Solovyeva[2], E. N. Olsufyeva[2], M. N. Preobrazhenskaya[2], J. Schaefer[1]; [1]Washington Univ., St. Louis, MO, [2]Gause Inst. of New Antibiotics, Moscow, Russian Federation.

Late-breaker presentations are listed behind the Late-breaker tab of the Final Program.

088(C1)

Poster Session

Mechanisms of Action and Mechanisms of Resistance in *Mycobacterium* spp. and *Clostridium difficile*

Sunday, 11:15 a.m.–1:15 p.m. . . . . Exhibit Hall F1

Presentations:

C1-626 From XDR to MDR: First Description of Compensatory GyrA Mutations Restoring Fluoroquinolone Susceptibility in a Clinical *M. tuberculosis* Strain
H. Ferrand[1], A. Pantel[1], A. Bouige[1], V. Jarlier[1,2], N. Veziris[1,2], C. Mayer[3,4], A. Aubry[1,2]; [1]UPMC (ER5), Paris, France, [2]NRC for Mycobacteria, Paris, France, [3]Inst. Pasteur, Paris, France, [4]Univ. Paris Diderot, Paris, France.

C1-627 Mutations in the Non-Rifampicin Resistance-Determining Region (RRDR) of the *rpoB* Gene Are Important Determinants of *M. tuberculosis* (MTB) Rifampicin Resistance (RifR)
C. P. Adikaram[1], J. Perera[1], S. S. Wijesundera[1], W. V. Senaratne[2]; [1]Univ. of Colombo, Colombo, Sri Lanka, [2]Chest Hosp., Welisara, Sri Lanka.

C1-628 Mutation Analysis of the *Mycobacterium leprae rpoB* Gene and Rifampicin Resistance
N. Nakata, M. Kai, M. Makino; Leprosy Res. Ctr., Natl. Inst. of Infectious Diseases, Tokyo, Japan.

C1-629 Introduction of Inhibitor Resistance Mutations into BlaC of *Mycobacterium tuberculosis* and Impact on Catalytic Activity
S. G. Kurz[1,2], K. Wolff[3], L. Tremblay[4], C. R. Bethel[5], K. Smith [6], Y. Xu[6], J. Blanchard[4], L. Nguyen[3], R. A. Bonomo[2]; [1]Case Med. Ctr., Cleveland, OH, [2]Louis Stokes Cleveland Dept. of VA Med. Ctr., Cleveland, OH, [3]CWRU, Cleveland, OH, [4]AECOM, New York, NY, [5]Louis Stokes VA Med. Ctr., Cleveland, OH, [6]CSU, Cleveland, OH.

C1-630 The Mycobacterial *ssrA* Promoter: Mapping the Elements Associated with Up-Regulation by Antimicrobial Agents
N. Andini[1,2], K. A. Nash[1,2]; [1]Childrens Hosp., Los Angeles, CA, [2]Univ. of Southern California, Los Angeles, CA.

C1-631 RNA Polymerase Target Modification in *Clostridium difficile* with Reduced Susceptibility to Fidaxomicin
J. Seddon[1], F. Babakhani[1], A. Gomez[1], I. Artsimovitch[2], P. Sears[1]; [1]Optimer Pharmaceuticals Inc., San Diego, CA, [2]Ohio State Univ., Columbus, OH.

C1-632 Fidaxomicin Inhibits Spore Production in *Clostridium difficile*
A. Gomez, P. Sears, L. Nguyen, F. Babakhani; Optimer Pharmaceuticals, Inc., San Diego, CA.

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Presentation Abstract

Session: 088-Mechanisms of Action and Mechanisms of Resistance in Mycobacterium spp. and Clostridium difficile

Sunday, Sep 18, 2011, 11:15 AM - 1:15 PM

Presentation Title: C1-627 - Mutations in the Non Rifampicin Resistance-Determining Region (RRDR) of the *rpoB* Gene are Important Determinants of "*M tuberculosis*" (MTB) Rifampicin Resistance (RifR)

Location: Exhibit Hall F1

Poster Board Number: 93

Presentation Number: C1-627

Pres. Time: Sunday, Sep 18, 2011, 11:15 AM - 1:15 PM

Category: C1

Keywords: tuberculosis ; rifampicin resistant; rpob gene

Author(s): **C. P. Adikaram, Bachelors (BS,Zoology) reading for PhD - *Research Assistant (PhD student)*** [1], J. Perera, MBBS, MD - *Professor of Microbiology* [1], S. S. Wijesundera, PhD - *Senior Lecturer* [1], W. V. Senaratne, MBBS, MD - *Consultant* [2]; [1]Univ. of Colombo, Colombo, Sri Lanka, [2]Chest Hosp., Welisara, Sri Lanka.

Financial Disclosures: **C. P. Adikaram,** None..
J. Perera, None..
S. S. Wijesundera, None..
W. V. Senaratne, None.

Abstract: **Background:** During 2005 - 2007 the national reference laboratory reported drug resistance in 17.9% MTB isolates with multi drug resistance rate of 2.5%. The objective of study was to identify mutations in the *rpoB* gene of resistant isolates. This is the first report on drug resistant gene mutations in *rpoB* gene of MTB isolates from Sri Lanka. **Methods:** DNA was extracted using phenol chloroform

method from 31 RifR MTB isolates confirmed as resistant by agar proportion method. A 437bp fragment including RRDR and other 2 fragments cover large part of *rpoB* gene (872bp and 1395bp) were amplified by PCR. DNA sequences of amplified products were analyzed using NCBI, EMBL and Sea View (version 4.2.12). **Results:** DNA sequencing confirmed 2 point mutations in the RRDR region for 18 (58.1%) isolates, at codons 526 (n=15, 48.4%) CAC (His) to TAC (Tyr) and 531(n=3, 9.7%) TCG (Ser) to TTG (Leu). The balance 15 isolates had mutations in the region outer to RRDR at codons 626 (n=13, 41.9%) GAC (Asp) to GAG (Glu) and 184(n=2, 6.4%) GAC (Asp) to GAT (Asp) and these have not been reported previously. Twenty nine isolates showed single point mutations and were at codons 526(n=14, 45.2%), 626 (n=11, 35.5%), 531(n=3, 9.7%) and 184 (n=1, 3.2%). Double mutations were detected in 2 strains (526+626 & 184+626). A single discordant result was observed between phenotypic and genotypic results due to the silent mutation at codon 184. **Conclusions:** Two novel point mutations outer to RRDR and some universally prevailing mutations in the RRDR of *rpoB* gene were observed in Sri Lankan RifR MTB isolates. Although codon 531 is reported as the most common mutation site worldwide, the majority of RifR strains in the present study showed mutations at codon 526. The second commonest mutation seen at codon 626 has not been previously reported. Although the mutations in our strains are limited to few types, the new mutations and their high frequency emphasizes the need for raising the geographical profile of the mutations for effective application of *rpoB* gene based molecular diagnosis of MDR.

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112/A

Comparison of Mycobacterium Growth Indicator Tube (MGIT) method and Nitrate Reductase Assay (NRA) with agar proportion method (APM) for detection of Rifampicin resistance

C Adikaram [1], J Perera [1] and S Wijesundera [2]

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[2]Department of Microbiology, Faculty of Medicine, University of Colomb

Testing of drug susceptibility prior to initiating treatment is vital for controlling and managing drug resistant TB. Drug susceptibility testing by the nitrate reductase assay (NRA) and manual Mycobacterium Growth Indicator Tube (MGIT) method has been used extensively for detection of growth. The objective of the study was to compare Mycobacterium Growth Indicator Tube (MGIT) method and Nitrate Reductase Assay (NRA) with agar proportion method (APM) for detection of rifampicin resistance.

Three hundred and seventy three clinical isolates of Mycobacterium tuberculosis were collected from Chest Clinic Colombo, Chest Hospital, Welisara and Prisons of Colombo from March 2008 to May 2010. Rifampicin susceptibly tests were carried out by APM (gold standard), MGIT method and NRA with 14 days old fresh cultures. The final rifampicin concentration used was 1.0µg/ml in all three methods.

Thirty one rifampicin resistant isolates were detected from among 373 Mycobacterium tuberculosis isolates by APM, NRA and MGIT methods. Twenty seven isolates out of 31 were identified as rifampicin resistant by the APM. MGIT and NRA methods were able to identify 28 rifampicin resistant isolates each. One resistant isolate was identified only by MGIT method and another 3 isolates by NRA only. The sensitivity and specificity of the NRA method was 93% and 99% respectively and there was very good agreement between NRA and APM. There was complete agreement between APM and manual MGIT methods with 100% sensitivity and 99.7% specificity.

Rapid identification of drug resistance is a prerequisite for initiating effective anti- TB treatment. Newer methods (NRA and MGIT) showed a high level of agreement with the gold standard, APM. The NRA and MGIT methods are rapid and easy to perform and results could be used for timely management of patients.

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Mutations in the rpoB gene of rifampicin resistant Mycobacterium tuberculosis isolates in Sri Lanka

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Objectives:
To identify the mutations in the rpoB gene of rifampicin resistant Mycobacterium tuberculosis isolates in Sri Lanka.

Design, setting and method :
Two hundred ninety eight Mycobacterium tuberculosis isolates were collected from chest clinic Colombo, Chest Hospital Welisara and Prisons of Colombo from March 2008 to May 2010. Decontaminated sputum was cultured on LJ, 7H10 and 7H9 medium. Drug susceptibility testing was carried out with agar proportion method, Nitrate Reductase assay and Mycobacterium Growth Indicator Tube method. DNA of resistant isolates was extracted by phenol chloroform extraction method and the 437bp fragment of rpoB gene was amplified by polymerase chain reaction with specific primers. The DNA sequencing was carried out and resulted sequences were analyzed with NCBI, CLUSTAL W (1.83) and DNAman software.

Results:
From 298 Mycobacterium tuberculosis isolates, 20 isolates were rifampicin resistant. DNA sequence analysis showed that there is one point mutation at codon 526 CAC(His) TAC (Tyr) and one point mutation at codon 531TCG(Ser) TTG(Leu) within mutation hotspot region of rpoB gene .

Conclusion:
Mutations in the rpoB gene indicate resistance to rifampicin and it is associated with resistance to other classes of drugs, most notably isoniazid. The point mutation at the codon 526 and 531 has reported as the most frequent mutation of rpoB gene worldwide and the frequency and place of mutation can vary geotropically. The identification of mutations is vital to develop a rapid molecular drug susceptibility testing method which is suitable for Sri Lanka.

OP 35: Utility of quantitative polymerase chain reaction in leptospirosis diagnosis - association between level of leptospiraemia and clinical manifestations in Sri Lanka

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Introduction and Aims: Quantitative polymerase chain reaction (qPCR), has the potential to provide accurate and timely diagnosis for leptospirosis at the point-of-care in endemic areas. We studied optimal sample types for qPCR, timing of sampling, and clinical manifestations in relation to quantitative leptospiraemia.
Methods: A new qPCR assay using pathogenic leptospira-specific 16S ribosomal RNA(rRNA) gene taqman primers and an optimised temperature stepdown protocol was used to analyse patient blood samples. Serum was compared with whole blood as sample source. Quantitative leptospiraemia was compared with clinical manifestations of leptospirosis and outcome.
Results: The diagnostic sensitivity of qPCR of whole blood and serum was 18.4% (95%CI: 9.97%–31.4%) and 51.0% (95%CI:37.5%–64.4%) respectively. The qPCR on suspected cases confirmed infection in 58 of 381 cases (15.2%). Of these, 6 cases confirmed by nested PCR and sequencing were serologically negative using a standard but not regionally optimised microscopic agglutination test panel. The bacterial load in serum/blood ranged from 102 to 106 leptospira/ml. Median leptospiral load for uncomplicated, renal failure, myocarditis, and multi-organ failure patients were 8616, 11 007, 36 100, and 15 882 Leptospira/mL respectively. The qPCR window of positivity ranged from day 2 to day 15; sensitivity of qPCR was not affected by the length of the interval between the onset of symptoms and sample collection (p=.328).
Conclusions: Quantitative PCR shows potential as a valid diagnostic test with a wider window of positivity than previously thought. Quantitative leptospiraemia in serum/whole blood samples did not directly correlate with clinical manifestations of outcome in this patient population.

OP 36: PCR-enzyme linked immunoabsorbent assay as a rapid molecular method for detection of rifampicin resistant *Mycobacterium tuberculosis* in low resource settings

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Introduction: Rifampicin resistance (RR) of *M.tuberculosis* (MTB) is due to point mutations in the *rpoB* gene. Early detection of drug resistance is important for prevention and control of tuberculosis (TB). Commercially available molecular based methods cannot be adopted in low resource settings due to high cost.
Aims: The study was focused on developing a PCR-ELISA, for rapid detection of RR, a surrogate marker of multi drug resistance.
Methods: Thirty RR- MTB strains with mutations at codon 526, 531 or 626 of *rpoB* gene, confirmed by DNA sequencing were selected for the study. 5' biotinylated allele specific oligonucleotides corresponding to point mutations at codons 526, 531& 626 were designed as probes and were immobilized on streptavidin coated microtiter plates. Digoxigenin labeled PCR amplified fragments of the *rpoB* gene were hybridized with the immobilized probes and detected using peroxidase conjugated anti-Digoxigenin. The sensitivity of the PCR-ELISA was measured by a serial dilution of H37Rv DNA and the specificity was determined by using DNA of non tuberculosis Mycobacteria (NTM).
Results: The PCR–ELISA was able to detected mutations in all 30 RR-MTB strains. The sensitivity of detection was 1pg of DNA for mutation at codon 531 and 626 and 100 fg for mutation at codon 526. Specificity of the PCR-ELISA was 100% against tested NTM.
Conclusions: The PCR–ELISA for the detection of RR was in total agreement with DNA sequencing results (Kappa=1). Hence the developed PCR–ELISA is a rapid, sensitive and specific assay for detection of RR, suitable for low resource settings.

OP 58: Characteristics of *Staphylococcus aureus* isolates from patients with atopic dermatitis and healthy controls

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[4]*Asha Central Hospital, Colombo*
[5] *MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK*

Objectives: Staphylococcus aureus (SA) shows a wide range of antibiotic resistance and therefore, it would be useful to know the general patterns of antibiotic sensitivity of this organism in patients with atopic dermatitis (AD).
Methods: Skin and nasal swabs were collected from 100 patients with AD and 100 healthy individuals. All swabs were cultured and antibacterial susceptibility testing was performed when indicated.
Results: SA was isolated from 59% of patients with AD and 16 % of healthy individuals. Of the SA strains isolated from patients, 8 (10.7%) were MRSA and only 10% showed susceptibility to penicillin and 37% to erythromycin. Susceptibility was much higher to gentamicin (95%), co-trimoxazole (91%) and fusidic acid (98%). The 8 MRSA strains were consistent with those of community acquired MRSA according to Centre for Disease Control criteria. Although 98% of isolates from patients were sensitive to clindamycin, 47% showed erythromycin induced resistance. Antibiotic resistance was not seen in SA isolates from healthy controls. Out of the 75 isolates from both groups 67 isolates were β lactamase producers.
Conclusions: Patients with AD are more likely to be colonized with SA strains resistant to conventional antibiotics. Emergence of community acquired MRSA appears to be a significant problem in Sri Lanka and could worsen with the indiscriminate use of antimicrobials.

OP 59: Prevalence of tuberculosis and rifampicin resistance in prisons of Colombo

Adikaram C[1], *Perera J*[1], *Wijesundera S*[2], *Weersinghe B*[3]
Departments of [1]*Microbiology and* [2]*Biochemistry, Faculty of Medicine, University of Colombo*
[3]*Department of Prisons, Colombo*

Introduction and Objectives: Prevalence of tuberculosis (TB) in prisons is reported to be up to fifty times higher than in the general population and is considered a public health problem globally. Prisoners are more likely to die from TB and default treatment than non-incarcerated populations and once released from the prison are infectious hazards to the community. This study was done to determine prevalence of TB and rifampicin resistance of *M. tuberculosis* isolates from prisons in Colombo which houses 9000 prisoners with a daily turnover rate of 250.
Methods: During the study period October 2008 – July 2009, sputum samples were collected and examined by smear and culture for TB from all prisoners in Colombo prisons who had fever and cough. Rifampicin susceptibility was tested by Agar proportion method (conventional method), Mycobacterium growth indicator tube (MGIT) and Nitrate reductase assay. H37Rv and rifampicin resistant reference strains were used as control strains.
Results: Eligible participants (605) comprised 592 (97.25%) males and 13 (2.15%) females. 28 (4.6%) (27 male; 1 female) were smear positive. All smear positives, except one grew *Mycobacteria* on culture. 96.3% (26/27) isolates were confirmed as *Mycobacterium tuberculosis* (Mtb) by PCR and biochemical tests while 3.7% (1/27) were non-tuberculosis Mycobacteriae. 7.69 % (2/26) were Rifampicin resistant.
Conclusion: The prevalence of Mtb (0.31%) and Rifampicin resistance (7.69%) is higher in the Colombo prisons compared to figures in the general population (0.079% and 3.14% respectively).

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