Abstract

The thesis comprise studies on rifampicin resistance of *Mycobacterium tuberculosis* in Sri Lanka by sequentially addressing the isolation of *Mycobacterium tuberculosis*, drug susceptibility testing methods, characterisation of *rpoB* gene mutations and transmission pattern of rifampicin resistance.

Mycobacterium cultures (n=442) were isolated from acid fast bacilli (AFB) positive sputum specimens collected from clinically suspected tuberculosis patients in Sri Lanka. Four hundred and one (401) isolates were identified as belonging to *M. tuberculosis* complex while the remaining 41 were recognised as belonging to non-tuberculosis mycobacteria group. The prevalence of non-tuberculosis mycobacteria (9.7%) in an acid fast bacilli positive sputum cohort in Sri Lanka indicates the necessity for species identification of *Mycobacterium* isolates prior to treatment as non-tuberculosis mycobacteria are frequently resistant to conventionally used anti tuberculosis drugs.

In Sri Lanka, drug susceptibility testing of *M. tuberculosis* still depends on time consuming, conventional proportion method. Thus, the nitrate reductase assay (NRA) in broth medium and the manual mycobacteria growth indicator tube (MGIT) were evaluated as rapid culture based drug susceptibility testing methods for determination of rifampicin resistance. The nitrate reductase assay and the manual mycobacteria growth indicator tube demonstrated excellent agreement (κ= 0.86 and κ= 0.94 respectively) with the agar proportion method (APM). With the manual mycobacteria growth indicator tube and the nitrate reductase assay, it was possible to determine the rifampicin susceptibility within 8 and 10 days respectively from primary

M. tuberculosis isolates with high sensitivity (93% and 85% respectively) and specificity (100% and 99% respectively).

Rifampicin resistance has immerged due to point mutations in the *rpoB* gene and majority of world's prevailing mutations are restricted to the rifampicin resistance determining region (RRDR) of *rpoB* gene. However, the presence of mutations varies geographically and is not restricted to the rifampicin resistance determining region. Therefore, selected fragments (437bp, 872bp and 1395bp that cover RRDR and regions spanning the RRDR) of *rpoB* gene of the 31 rifampicin resistant *M. tuberculosis* strains isolated during the study were subjected to PCR amplification and DNA sequencing. The DNA sequences revealed 2 point mutations within the rifampicin resistance determining region 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 two novel mutations in the region spanning the RRDR at codon 626 (n=13, 41.9%) GAC (Asp) →GAG (Glu) and codon 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 new mutations with a high frequency 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 *rpoB* based diagnosis of drug resistant tuberculosis.

The commercialized molecular drug susceptibility testing methods are based on world's prevalent mutation in rifampicin resistance determining region of *rpoB* gene (codon, 531,526 and 516). Thus, available molecular drug susceptibility testing methods may not achieve the required sensitivity in Sri Lanka as they will only be able to identify 58% of drug resistant TB cases. Additionally, these methods are not accessible in developing countries due to the high cost per

test. Therefore, the polymerase chain reaction linked immunoabsorbent assay (PCR-ELISA) was developed using rifampicin resistant and susceptible *M. tuberculosis* isolates identified by agar proportion method. The dig labelled PCR amplified fragments of *rpoB* gene were hybridized with 5' biotinylated allele specific oligonucleotide probes corresponding to point mutations at codons 526, 531 & 626. The hybridization was determined by colour development. There was a good agreement between agar proportion method and PCR-ELISA with 86% sensitivity and 100% specificity for identification of rifampicin resistance of *M. tuberculosis*. The turnaround time of the assay was 2 days after isolation of primary cultures. Thus, PCR-ELISA is a rapid, sensitive and specific drug susceptibility testing method that can be customized as per user requirement.

DNA fingerprinting of IS6110 insertion element of rifampicin resistant and susceptible *M. tuberculosis* isolates revealed that none of the rifampicin resistant isolates have similar fingerprinting patterns to that of susceptible isolates. This observation indicated the absence of the acquisition of rifampicin resistance following infection of a rifampicin susceptible strain. Thus, the rifampicin resistance of *M. tuberculosis* in Sri Lanka is due to the transmission of rifampicin resistant strains (primary drug resistance) based on the IS6110 DNA fingerprinting.