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Detection of Rifampin- and Isoniazid-Resistant Genes in *Mycobacterium tuberculosis* Clinical Isolates

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Abstract

Tuberculosis still remains the leading cause of death worldwide. The morbidity has been reported to decrease, but incident and prevalence of multi-drug resistance Tuberculosis is still on rise. Rifampicin and isoniazid are the first line treatment to TB patients and resistance to these drugs has been linked to mutations in genes such as *rpoB* and k*atG*. In the present study, PCR method was employed to detect three types of restricted genes associated with drug resistance tuberculosis namely *rpoB*, regulatory-*inhA* and *katG*. Sixty-two samples were obtained from different parts of Malaysia hospital which consisted of 35 pulmonary and 27 extra-pulmonary specimens. Twenty-seven specimens showed positive results as detected by duplex PCR method whereas 3 specimens positive as detected by acid fast bacilli and culture method. Out of 27 isolates, 3 isolates from culturable isolates harbored restricted genes that where associated with drug resistance tuberculosis. The mutations involved in *rpoB* genes comprised of acid amino transposition (isolate 148) and frameshift mutations (isolate 624 and 374). This study is clinically important because it focuses in molecular diagnosis and can act as an early warning on the emerging status of multidrug resistance of *M. tuberculosis* in Malaysia.

Keywords: Rifampin, Isoniazid-Resistant, M. tuberculosis, PCR

1. Introduction

Tuberculosis (TB) is an infectious bacterial disease caused by *Mycobacterium tuberculosis* which commonly affects the lungs and respiratory system. The TB morbidity was reported decreasing, but its remains alarming due to increase in incidence and prevalence of Multi Drug Resistance-TB (MDR-TB) [1]. To date, seven antimicrobial agents that have been used in the treatment of resistant TB cases were; - isoniazid, rifampicin, pyraxinamide, enthambutol, streptomycin and fluoroqui-

nolones. MDR phenotype is defined as resistance at least to isoniazid and rifampicin which is the most effective drug recommended by World Health Organization (WHO) and being used as the first line treatment in TB patient. Therefore, resistance towards these two drugs has become major problems in the treatment of TB patients. Resistance to first line anti-TB drugs has been linked to mutations in at least 10 genes; *katG, inhA, ahpC, kasA* and *ndh* for INH resistance; *rpoB* for RIF resistance, *embB* for EMB resistance, *pncA* for PZA resistance and *rpsL* and *rrs* for STR resistance [2]. Nevertheless, nearly 95% of the RIF resistant strains possess a mutation in the *rpoB* gene encoding a DNA-dependent RNA polymerase [3]. In addition, approximately 90% of INH resistant strains have a mutation in the *inhA*, *katG*, and *ahpG* genes encoding enzymes that are related to mycolic acid synthesis of cell wall [3]. Rapid development of drug resistance caused by *M. tuberculosis* has led to measure resistance accurately and easily. This knowledge will certainly help us to understand how to prevent the occurrence of drug resistance as well as identifying genes associated with new drug resistance. Keeping the above facts in mind, the present, this study was carried out to detect resistance-associated mutations gene in *M. tuberculosis* clinical isolates.

2. Materials and methods

2.1. Bacterial isolates and clinical samples

Bacterial samples used in this study were collected from various hospitals in Malaysia. Prior ethical approval was obtained from the institutional ethics committee. The samples consisted of pulmonary (n=35) and non-pulmonary (n=27) specimens. The samples such as sputum, gastric lavage and urine were decontaminated with 4% NaOH for 15 minutes before being used. All samples were investigated for the presence of acid-fast bacilli by Ziehl-Neelsen and cultured on Loewenstein-Jensen medium. *M. tuberculosis* strain ATCC 27294 was grown in Ogawa medium and used as positive control. Whereas, *Bacillus subtilis* (ATCC 26633) was grown on blood agar and used as negative control.

2.2. DNA extraction, PCR amplifications and DNA sequencing analysis

Extraction of DNA from bacterial culture and clinical samples were carried out by using High Pure Viral Nucleic Acid Extraction Kit (Roche Inc. USA) according to manufacturer's recommendation. DNA from all bacterial isolates and clinical samples were subjected to Duplex-PCR using mixture of primers to amplify the IS*6110* gene and p*53* gene as previous report [4] with some modifications. The amplification mixture consisted of 1 µg of template DNA, 1X final concentration of MasterMix (Eppendorf), and primers (0.4 pmole/µl). Amplification was carried out using Master Cycler Gradient Thermocycler (Eppendorf). The cycling parameters were 94 ºC for 3 min followed by 34 cycles of denaturation at 94 ºC for 15 seconds, annealing at 66 ºC for 15 seconds and extension at 72 ºC for 20 seconds. Final extension was then carried out at 72 ºC for 3 min. The PCR product was analysed by a 1.6 % agarose gel. Primers used for detection of *rpo*B, *inh*A and *kat*G resistance genes were design by Vector NTI software. The amplification mixture consisted of 1 µg of template DNA, 1X final concentration of MasterMix (Eppendorf), and primers (0.4 pmole/µl). The amplification was carried out using Master Cycler Gradient Thermocycler (Eppendorf). The cycling parameters were 95 ºC for 3 min followed by 34 cycles of denaturation

at 95 ℃ for 30 seconds, annealing for 30 seconds at 64, 63, and 65 ℃ for *inhA*, *rpoB* and *katG* respectively.The extension cycle were72 ℃ for 90 seconds for *inhA* and *katG* genes whereas 60 seconds for *rpoB* genes. The final extension was carried out at 72 ℃ for 5 min. The PCR product was analysed by a 1.6 % agarose gel. DNA fragments of PCR products were purified using QIA-quick PCR Purification Kit (QIAGEN, Germany) according to manufacturer's recomendation. Purified DNA fragments were sent to the 1st Base Laboratories Sdn. Bhd, Petaling Jaya, Kuala Lumpur for sequencing. DNA sequences were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were conducted using ClustalW (http://www.genome.jp/tools/clustalw/).

3. Result and Discussion

3.1. Detection of M. tuberculosis by PCR and culture method

In total, 27 extra-pulmonary and 35 pulmonary clinical specimens were obtained for AFB smear, culture and PCR analysis. Out of 62 isolates, only 3 isolates namely 148, 374 and 624 were culture positive as detected by AFB. The culture was then confirmed as *M. tuberculosis complex* using GenProbe System performed by Bacteriology Unit, National Public Health Laboratory at Sungai Buloh, Kuala Lumpur. Nevertheless 27 isolates were positive for IS6110 of *M. tuberculosis* as detected by DPCR. Of the 27 isolates, 12 of them were from extra-pulmonary isolates and 15 were from pulmonary isolates (Figure 1). It was interesting to note that the PCR methods were able to detect the AFB negative of the non-pulmonary specimens such as from CSF, bone biopsy, lumbar puncture, rectal biopsy and lymph nodes. A similar result was shown by Suhaila et al. (2008) [4]. Number of positive isolates detected by PCR analysis was higher compared to culture method. Time required for detection of *M. tuberculosis* from clinical specimens via PCR analysis was less than 4 hours whereas more than 8 weeks were required in culture method.

3.2. Detection of resistance genes

Out of 27 isolates positive for *M. tuberculosis* genes, only 3 culturable isolates showed amplification of all resistance genes tested (Figure 2). Low quantity of *M. tuberculosis* DNA in the clinical sample and the presence of inhibitor [5] might be the contributed factors. It was interesting to note that all the positive samples bearing the Zielhl Neelson staining scores more that 10/3L directly showed the high bacteria numbers. Approximately 104 organisms/ml are required for reliable detection with Ziehl-Neelsen stains [6] In addition, the properties of the genes of interest also affect the success of amplification. Only one specific site of *katG*, *rpoB* and regulatory-*inhA* genes presence in the genome of *M. tuberculosis* and may explain the limited excess to the genes. Hence, the amplification cannot be made. The primer used for detection of resistance genes have been successfully design by Vector NTI software. Amplification of resistance genes from clinical specimen have been succeeding in three isolates namely 148, 374 and 624. Amplified genes for *rpoB*, *katG* and *inhA* were shown in Figure 2a, 2b and 2c respectively. The product size for *rpoB*, *katG* and *inhA* were 442bp, 2206bp and 442bp, respectively as confirmed by sequencing analysis.
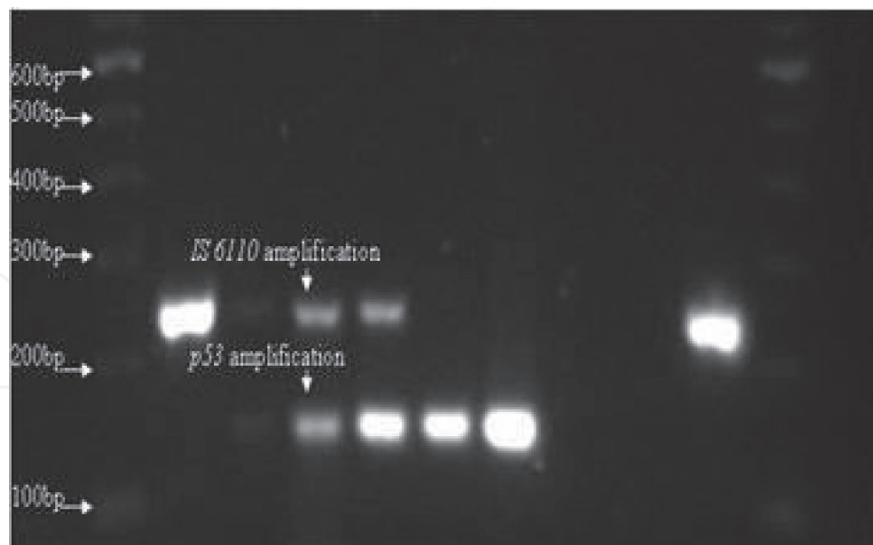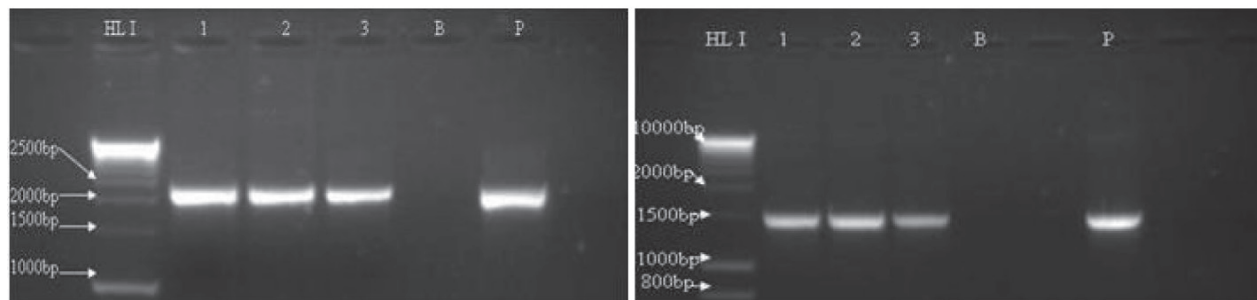
Figure 1. Agarose gel showing amplification of p53 and ISP6110. Amplifications were performed using marker 100 bp (M) and DNA from M. tuberculosis ATCC 27294 (P), various isolate (1-5), negative control, B. subtilis (N), positive control (PP).

a

b

c

Figure 2. Amplification of rpoB gene (a), katG gene (b) and inhA gene (c) From left; Lane HL: Hyperladder maker, Lane 1, isolate 148; Lane 2, isolate 374; Lane 3, isolate 624; Lane B, negative control, Lane P, positive controlMutation analysis

PCR products amplified from regulatory-*inhA, rpoB* and *katG* genes of *M. tuberculosis* were sequenced with the same primer used in amplification of the genes. The sequences obtained were compared to the sequences available in Gen Bank Data using BlastN analysis. The DNA nucleotide sequences were translated to amino acid sequences through BlastX software available in http://www.ncbi.nlm.nih.gov. The nucleotide and amino acid changes of each isolates for respective genes were compared using ClustalW (Vector NTI). As for mutation analysis of *rpoB* genes,

isolate 148 showed minor mutation where the additional, changes and transposition of certain base of amino acid Whereas, isolate 374 showed showed frameshift mutation in which total loss of A base that subsequently affects the rest of amino acid coding. It is known as.. Nevertheless, isolate 624 showed both minor and major mutation at different base position in which all amino acid code was totally changed.

4. Conclusion

Two isolates of 624 and 374 showed frameshift mutations in which all the amino acid codes were totally changed. the quantity of DNA and the present of inhibitor may affect the successful of amplification. In addition, only one specific site of *katG*, *rpoB* and *inhA* sequence in whole genome of *M. tuberculosis*.. As for conclusion, the identification of *M. tuberculosis* through amplification of *IS6110* was successfully achieved whereas amplification of restricted genes associated with drug resistance was achieved in culturable clinical specimens.

5. Acknowledgment

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