Description of polymerase chain reaction and sequencing DNA *Mycobacterium tuberculosis* from specimen sputum of tuberculosis patients in Medan

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Abstract. This study purposed to describe the product Polymerase Chain Reaction (PCR) and sequencing of DNA *Mycobacterium* (M.) *tuberculosis* from sputum of tuberculosis (TB) patients in Medan. Sputum was collected from patients that diagnosed with pulmonary TB by a physician. Specimen processed by PCR method of Li et al. and sequencing at Macrogen Laboratory. All of 12 product PCR were showed brightness bands at 126 base pair (bp). These results indicated similarity to the study of Li et al. Sequencing analysis showed the presence of a mutation and non-mutation groups of M. tuberculosis. The reference and outcome range of the mutation and non-mutation of M. tuberculosis were 56-107, 59-85, 60-120 and 63-94, respectively. The percentage bp difference between the outcome and references for mutation and non-mutation were 3.448-6.569 and 3.278-7.428%, respectively. In conclusion, the successful amplification of PCR products in a 1.5% agarose gel electrophoresis where all 12 sputa contained rpoB-positive M. tuberculosis and 0.644% difference was found between the outcome with reference bp of the mutation and non-mutation M. tuberculosis groups.

1. Introduction

TB, besides is still one of the leading causes clinical impact of mortality and morbidity, also affects the quality of life.[1] The study of [2] highlights the considerable social and economic burdens of Multi-Drug Resistance (MDR)-TB on the patients. Most patients feel isolated intensively because of loss of identity, productive power and family relationships. MDR-TB requires two or more years of treatment and significant treatment costs.[2] MDR-TB has risen as a significant public health problem in the 21st century due to the emergence of resistance to anti-tuberculosis therapy drugs.[1] Delays in TB diagnosis and treatment carry significant risks of worsening disease severity to the patient and increased risk of transmission around. The challenge is to determine how to reduce this duration of the delay, particularly in resource-limited settings where rapid diagnostic techniques may not be available or affordable.[3]

The study of [4] has demonstrated that conventional methods, such as Acid Fast Bacilli and culture, used to detect mycobacterial infections, have led to significant uncertain diagnoses. The direct
consequences of such misdiagnosis are mycobacteria resistance, chronic infections, and poorer outcomes. Species identification is essential to choose the correct therapeutic regimen. Riello et al. suggest that urgent molecular identification of mycobacteria species and resistance tests are incorporated into public health systems in regions with resource-poor settings in order to reduce morbidity by adopting appropriate therapeutic regimens and to prevent the increased burden of antibiotic-resistant mycobacteria.[4]

PCR has revolutionized microbiology by facilitating direct detection and identification of infectious agents in clinical specimens in a short time. This molecular test can reduce to two hours the time it takes to have results from conventional phenotypic sensitivity tests to antimicrobial agents.[5] Faster, more comprehensive diagnostics will enable the earlier use of the most appropriate drug regimen, thus improving patient outcomes and reducing overall healthcare costs. Whole genome sequencing (WGS) has been shown to provide a rapid and comprehensive view of the genotype of the organism, and thus enable reliable prediction of the drug susceptibility phenotype within a clinically relevant timeframe.[6] The present study purpose describes the product of PCR and sequencing DNA M. tuberculosis from specimen sputum of TB patients in Medan.

2. Materials and Methods

Subjects were patients who had diagnosed with pulmonary tuberculosis by a physician. Expectorated sputum samples collected from these patients in Haji Adam Malik General Hospital Medan. Criteria inclusion were adult pulmonary TB with age over 17 years old and had not taken TB drugs. Criteria exclusion were HIV disease and pregnancy. The study was descriptive analysis by comparing the base pair size generated from the mutation and non-mutation groups.

2.1. Decontamination Sputum

The procedure of [7] chose for decontaminated specimens, briefly 2 ml of 4% NaOH solution inserted in the sputum pot, stirred, removed in a2ml falcon tube, then vortex 15s, hold 15 min. Added 2ml phosphate buffer 6.7 mM, pH 7, centrifuged 3000xg for 15 min, discard the supernatant. The precipitate resuspended in buffer up to 2 ml volume, stored in incubator 37°C for 15 min and ready for extraction.

2.2. DNA Extraction

The TIANamp Genomic DNA Kit (TIANGEN, Hilden, Germany) used for DNA extraction according to the instructions of the manufacturer [8], briefly a number of decontaminated sputum inserted into Eppendorf tube, centrifuged 10000rpm 10min, disposed supernatant, resuspension with 200µl buffer GA and 20µl proteinase K, vortex, incubation 56°C 1.5h. Mixed 200µl buffer GB, vortex, incubation 70°C 10min. Added 200µl ethanol absolute, vortex, inserted in a spin column that had been put together with collection tube, centrifuged 12000rpm 30s. The solution in the collection tube was discharged and recombined with a spin. Added 500µl buffer GD, centrifuged 12000rpm 30s, the solution in the collection tube was removed and reassembled with the spin column. Added 600µl buffer PW, centrifuged 12000rpm 30s and liquid in collect tube thrown away, repeated once again. Centrifuge again 12000rpm 2minutes. Collection tube thrown, mixed with new eppendorf tube, added 50µl buffer TE to center of spin column, incubation 2 minutes at room temperature, centrifuged 12000rpm 2min. Removed the spin column. DNA ready for use.

2.3. PCR Amplification

The 81-bp rpoB hot-spotregion (RRDR) that encodes residues 507 to 533 was amplified by PCR using protocols from,[9] Primers forward (5’-CGCCGCGATCAAGGAGTTCT-3’) and reverse (5’-TCACGTCACAGACCCGCGG-3’) used for amplified the 126-bp fragment of the rpoB gene. The volume final of PCR was 50µl. Each PCR mixture contained 2µl of genomic DNA, 25µl of master mix, 2 µl of each primer forward and reversed and 19 µl of deionized water. The assays run on a Thermal Cycler (Applied Biosystems). The cycling conditions for rpoB gene PCR were briefly, a
cycle of 94°C for 2 min, 35 cycles of 94°C for 45s, 55°C for 45s, 72°C for the 30s, and a cycle of 72°C for 7 min.

2.4. Quality control
The appropriate negative (no-DNA) and positive (H37Rv DNA) controls used in each step to identify possible contamination or inhibition.

2.5. Electrophoresis
A 5μl aliquot of PCR product was analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide. The amplified products visualized and photographed using a UV-transilluminator.

2.6. Sequencing of genomic rpoB-RRDR
PCR product purified then sequences determined by MacrogenLaboratory in Korea.

2.7. Sequence analysis
DNA sequencing analyzer by Basic Local Alignment Search Tool (BLAST) algorithm.

3. Results and Discussions

3.1. Results
3.1.1. PCR Results. Standard M. tuberculosis H37Rv and normal human sputum used as a positive and negative control, respectively. The brightness of the PCR bands was determined by the concentration of M. tuberculosis.[9] PCR fragment of the rpoB gene from 12 samples were amplified and performed by gel agarose electrophoresis. All of 12 sputa showed positive DNA at 126 bp (Figure 1).

![Figure 1. Electrophoresis results from PCR product samples no. 1-12 M=DNA Marker, -= no DNA, +=H37Rv.](image)

3.1.2. Sequencing Results. The results of DNA sequencing (Tables 1) of 12rpoB-positive PCR products, showed respectively that 6 specimens displayed mutation and non-mutation in rpoB gene. The range bp of reference mutation M. tuberculosis are from 56 to 107 bp and non-mutation between 59 and 85. The present study showed outcome bp of mutation and non-mutation M. tuberculosis respectively 60-120 and 63-94. The percentage difference between outcome and reference are 3.448-6.569% and 3.278-7.428% for mutation and non-mutation, respectively. The mutation and non-
mutation \textit{M. tuberculosis} with the sequencing and percentage difference for all 12 samples summarized in Table 1.

\textbf{Table 1. Outcome base pair (bp) of sample sequencing.}

| No. | Significant alignments sequences | Reference (bp) | Outcome (bp) | Difference bp (%) |
|-----|---------------------------------|----------------|--------------|-------------------|
| 1   | Non-mutation of \textit{M. tuberculosis} | 59             | 63           | 3.278             |
| 2   | Non-mutation of \textit{M. tuberculosis} | 81             | 94           | 7.428             |
| 3   | Non-mutation of \textit{M. tuberculosis} | 79             | 88           | 5.389             |
| 4   | Non-mutation of \textit{M. tuberculosis} | 64             | 73           | 6.569             |
| 5   | Non-mutation of \textit{M. tuberculosis} | 85             | 94           | 5.027             |
| 6   | Non-mutation of \textit{M. tuberculosis} | 77             | 88           | 6.667             |
| 7   | Mutation of \textit{M. tuberculosis} | 56             | 60           | 3.448             |
| 8   | Mutation of \textit{M. tuberculosis} | 58             | 63           | 4.132             |
| 9   | Mutation of \textit{M. tuberculosis} | 72             | 82           | 6.493             |
| 10  | Mutation of \textit{M. tuberculosis} | 93             | 101          | 4.123             |
| 11  | Mutation of \textit{M. tuberculosis} | 107            | 120          | 5.726             |
| 12  | Mutation of \textit{M. tuberculosis} | 64             | 73           | 6.569             |

3.2. Discussion

The presence of a 126-bp band on the agarose gel from all of 12 sputa in this study indicated successful amplification. The PCR results (Figure 1) showed that 100% (12/12) of sputum specimens were positive for the \textit{rpoB} gene \textit{M. tuberculosis}. This result indicates the similarity study of\cite{9} showed that 93.3% (84/90) of sputum specimens were positive for the \textit{rpoB} gene. PCR-based diagnostic systems, such as Xpert, cannot distinguish between dead or live bacilli and only focuses on \textit{rpoB} (Rifampicin). Currently, MDR \textit{M. tuberculosis} can be detected rapidly on the Xpert MTB/RIF system, but unavailable for extensively drug-resistant (XDR) cases.\cite{10}

WGS becomes the gold standard of detecting polymorphisms in the continuing genomes of \textit{M. tuberculosis} bacteria during infection, treatment and the acquisition of drug resistance so as to identify mutations in the specific loci present in the proportion of the ordered bacteria.\cite{11} WGS of bacteria has been shown to provide comprehensive data on antimicrobial resistance which could be used to inform treatment. The use of diagnostic WGS appropriate for \textit{M. tuberculosis} because \textit{M. tuberculosis} has a well-characterized clonal nature, with relatively low levels of sequence variation, and does not undergo recombination or horizontal transfer. Thus, a stable set of oligonucleotide baits can be created, and sequence data can be mapped to a reference genome.\cite{10} The results of DNA sequencing in the study \cite{9} showed that 65 specimens displayed mutations in the \textit{rpoB} gene from 84 (77.4\%) \textit{rpoB}-positive PCR products. The present study (table 1) found that 6 from 12 (50\%) \textit{rpoB}-positive PCR products showed a mutation in \textit{rpoB} gene. Average percentage outcome bp of significant alignments sequences mutation and non-mutation of \textit{M. tuberculosis} were 5.082\% and 5.726\%, respectively, the difference from reference sequences.

Sequencing directly from the clinical sample may reduce any possible biases. The quality of sequence data allowed us to accurately call mutations that are known to be associated with resistance to first- and second-line drugs.\cite{10}

4. Conclusion

The presence of a 126-bp band on the agarose gel from all of 12 sputa in this study indicated successful amplification product PCR of \textit{rpoB}-positive \textit{M. tuberculosis}. There was a slight difference of 0.644\% between outcome to reference bp mutation and non-mutation \textit{M. tuberculosis} in this study.

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