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SPECIFIC GYRB SEQUENCE OF MYCOBACTERIUM TUBERCULOSIS CLINICAL ISOLATED FROM SPUTUM OF PULMONARY TUBERCULOSIS PATIENTS IN INDONESIA

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Background: Indonesia have many different geographic areas which could be various on the variant strains of *Mycobacterium tuberculosis*. The *gyrB* gene codes GyrB protein as sub unit compound of Gyrase enzyme that functioning in multiplication of bacteria. Detection of *gyrB* gene could be a marker of active multiplication of viable bacteria in the specimen from patients; and some of the DNA sequence regions were conserved and specific in the strain of *Mycobacterium tuberculosis* that would be a marker for identification. This research aims to analyze the sequence of *gyrB* gene of *Mycobacterium tuberculosis* clinical isolates from sputum of pulmonary TB patients in Indonesia, and determine the specific region. **Method**: *Mycobacterium tuberculosis* clinical isolates have been collected from sputum of the patients with pulmonary TB that live in some area in Indonesia. Isolation and identification of *Mycobacterium tuberculosis* clinical isolates using standard culture method; sequence analysis using PCR-direct sequencing of the part bases region of *gyrB*. **Results**: this study revealed that nucleotide sequence on a fragment 764 bases of *gyrB* gene *Mycobacterium tuberculosis* strains among clinical isolates almost identically to a wild type strain *Mycobacterium tuberculosis* H37Rv and subspecies member of *Mycobacterium tuberculosis* complex (MTBC), with a little difference of SNPs; there are many difference nucleotide sequence with MOTT and Gram positive or negative bacteria, except *Corynebacterium diphtheriae* identically with MTBC. **Conclusion**: the *gyrB* sequence in *Mycobacterium tuberculosis* strains among these clinical isolates from sputum of pulmonary TB patients in Indonesia have the conserved specific DNA region that almost identically with wild type strain H37Rv and MTBC.

Keywords: **sequence; *gyrB* gene; Mycobacterium; tuberculosis; sputum.**

INTRODUCTION

Tuberculosis (TB) is a major health problem in Indonesia and in the world. The WHO global TB control report of 2009, mentions in Indonesia noticed 528.063 new TB cases and 102 new sputum smear positive cases per 100.000 population, with the total population 226 million people in 2007 as reported by Indonesian Ministry of Health; the prevalence of TB in Indonesia differs per geographical areas, Java-Bali having the lowest prevalence (67 per 100.000 population), and Eastern Indonesia the highest (198 per 100.000 populations).[1] The other period reported at 2011 in Indonesia there are AFB positive 197.797 cases which the highest cases in Java; on the other hands at 2010 estimated the prevalence of pulmonary TB 725 per 100.000 population with the highest TB prevalence in Papua province 1.441 per 100.000 population, Batan 1.282/ 100.000, north Sulawesi 1.221/ 100.000, and lower in Lampung (270/ 100.000), Bali (306/ 100.000), DI. Yogyakarta (311/ 100.000) (Indonesia MOH, 2012).[2] At 2014, WHO reported the estimates of TB disease problem in Indonesia at 2012 noticed in 246.864.000 population with incidence rate 185 (153 – 220) per 100.000 population (95% CI); prevalence rate 297 (144 – 506); and mortality rate 27 (12 – 48). In South

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East Asia at 2012 estimate TB global incidence 8.300.000 –9.000.000 cases of all forms of TB. In global TB report at 2013 estimated that total TB cases 39 % prevalent in South East Asia region of all TB cases in the world, and mortality estimated 47,6 % from totally in the world, estimated global TB mortality 790.000 – 1.100.000 cases of all form of TB.[3]

The effective control of TB case finding is of utmost importance. Accurate and rapid diagnostic methods are needed for determined TB diagnosis. Until now the culture method is the gold standard for TB diagnosis, on the other hands the culture method constrains with takes the time consuming, more than 3 weeks up till 2 months. Nucleic acid amplification is a rapid diagnostic methods that is sensitive and specific.[4-6] Nucleic acid amplification tests have as the target gene region for identification, i.e. *insertion sequence* IS 6110, 16S rRNA, *hsp*60, *gyrB*, *rpoB*, 16-23S rRNA etc., which have the conserved and specific gene regions of *Mycobacterium tuberculosis.*[7] High copy numberof *gyrB* gene have the important role to distinguish the specific nucleotide sequence with MOTT species or other Gram positive bacteria.[8] Based on the advantages of the role for the specific region identification in *gyrB* gene, as a basis primer design, the sequence of *gyrB* gene of *Mycobacterium tuberculosis* by DNA sequencing must be determine among clinical isolates from TB patients.

This study aims to analyze the sequence of *gyrB* gene *Mycobacterium tuberculosis* clinical isolates from sputum of pulmonary TB patients that live in some area in Indonesia. Some isolates from TB patients in many different geographic areas in Indonesia could be various strains of *M. tuberculosis*, however the *gyrB* gene nucleotide sequence would have the conserved and specific nucleotide sequence in the DNA regions.

MATERIALS AND METHOD

Bacterial Strains of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis strains 102 isolated from sputum of 307 suspected pulmonary TB patients that collected in 2010, and 170 from 536 suspected pulmonary TB patients at 2013, patients from some area in Indonesia. Isolation and identification of *Mycobacterium tuberculosis* were carried out using standard culture method -MGIT 960 System (BD) and conventional standard on L-J medium (WHO). All of these were conducted in TB Laboratory in Department of Clinical Microbiology, Dr Soetomo Academic Hospital, Surabaya, Indonesia.

Analysis of DNA sequence of *gyrB* gene of *Mycobacterium tuberculosis* clinical isolates

Molecular study of DNA sequencing conducted in Institute of Tropical Diseases, Airlangga University, Surabaya, Indonesia; eight isolates of TB patients (in 2010) have been done sequencing on a part of the target the 1.020 bases, and three isolates of TB patients (in 2013) with target a part of the almost whole gene sequence. Extraction and purification of DNA from *Mycobacterium tuberculosis* clinical isolates, as is follow, one loop full of fresh colonies of *Mycobacterium tuberculosis* (3 – 4 weeks old on Lowenstein-Jensen medium) was suspended in a buffer solution, extraction and purification of DNA using DNeasy kit (QIAGEN).

PCR to detect and amplify the DNA region of *gyrB* gene *Mycobacterium tuberculosis*, 41 isolates that collected at 2010 were conducted PCR for gene target 1.020-bp of *gyrB.* The suspension of PCR mix Dream Taq "Green PCR Master Mix (Fermentas) was added by primer target 1.020-bp region as is MTUB-f 5'-TCGGACGCGTATGCGATATC-3' and MTUB-r 5'-ACATACAGTTCGGACTTGCG-3' each 1,0 µM, and DNA template 2µl, reaction volume 50 µl. Amplification reaction is 98º C 2 minutes; 96º C 20 seconds; 58º C 20 seconds; 72º C 1 minute; and 72º C 7 minutes, and the reaction 40 cycles.[7,8]

Optimized PCR for target the sequence next to the whole genome of *gyrB* gene were done in 3 isolates from TB patients in 2013 using the five primer settings, Using *Mycobacterium tuberculosis* H37Rv as a positive control and mix reaction suspension without DNA as a negative control.

DNA sequencing and analysis of DNA sequence, PCR product is purified using QIAquick-spin PCR purification kit (QIAGEN). Eight isolates of *Mycobacterium tuberculosis* strains were conducted sequence analysis of 1.020 base region of *gyrB*, and three isolates sequenced of the next to the whole gene using ABI PRISM 310 System Sequencer. Alignment analysis was carried out using Genetix WIN V.10 program.

RESULTS

All of the 41 isolates (100%) *Mycobacterium tuberculosis* could be detect with PCR 41 strains of clinical isolates were positive *gyrB* conserved region at 1.020-bp (Figure 1, with example of five isolates).

Isolates P-1, P-2, P-3, P-4, P-5, P-6, P-7, P-8 from eight TB patients collected in 2010 have been sequenced analysis on alignment and phylogeny tree, revealed that all of these isolates were almost identically, more than 97 % with homolog nucleotide sequence in 764 bases that have been sequenced, with strain *Mycobacterium tuberculosis* H37Rv and other member of MTBC i.e. *Mycobacterium africanum, Mycobacterium bovis, Mycobacterium canettii, Mycobacterium microti*, *Mycobacterium caprae*; on the other hands

also closed related to *Corynebacterium diphtheriae*. These six strains are not identical to *Mycobacterium kansasii* and other MOTT i.e. *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium scrofulaceum, Mycobacterium asiaticum, Mycobacterium smegmatis.*

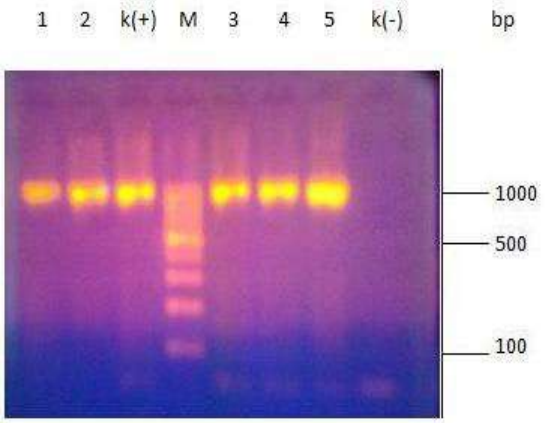


Figure 1
PCR products of *gyrB* gene *Mycobacterium tuberculosis* clinical isolates from sputum of pulmonary TB patients. Amplicon of *gyr* B at 1.020-bp, lane 1, 2, 3, 4, and 5 are amplicon from clinical isolates; M: 100-bp ladder; K+: positive control is amplicon from *Mycobacterium tuberculosis* H37Rv; negative control: suspension of reaction mixture without DNA template.

These strains distance correlated with Nocardia, and no correlated to Gram positive bacteria *Staphylococcus aureus* and Gram negative bacteria *Klebsiella pneumonia* and *Shigella sp* were indicated as the outgroup (Figure 2). In these 764 bases sequenced found a single nucleotide polymorphism (SNP) of isolate P4 at base no. 14 A – to – T, no. 169 G – to – T, no. 423 A – to – T, no. 446 G – to – T, no. 449 A – to – T; isolate P5 at base no. 28 C – to – A, no. 32 A – to – C; and isolate P6 no. 480 G – to – C (Figure 2). The 1.020-bp region position in the whole genome, flanked with sequence of the target by next setting primers which target to almost the whole genome.

The strains PS1, PS2, PS3 isolates from patients that collected at 2013 revealed, in around the 1054 bases a part of the target sequenced of almost the whole gene of *gyrB*, the result of alignment analysis and phylogeny tree similar with the strains from TB patients in 2010, were as followed: three strains isolates from patients almost identically with *Mycobacterium tuberculosis* H37Rv and other members of MTBC; distance correlated with MOTT; and no correlated to Gram positive and Gram negative bacteria, except *Corynebacterium diphtheriae* have the same sequence gene region closed correlated to MTBC. These study also found SNPs in sequence *gyrB* gene of isolates PS1 at base no. 192 G – to - C, no. 436 C – to - G, no. 466 C – to - A, no. 866 G – to - C; and PS3 base no. 404 A – to – C, no. 410 A – to - C, no. 852 A – to - G. Isolate PS1 and PS2 also found two bases different with strain H37Rv at no. 417-418 AT – to – CC (Figure 2).

Figure 2 indicates sequence alignment of *gyrB* gene *Mycobacterium tuberculosis* clinical isolates from sputum of pulmonary TB patients in Indonesia at 2010 and 2013 (Genetix WIN V. 10 program), *Mycobacterium tuberculosis* H37Rv, other MTBC, MOTT, *Corynebacterium diphtheriae*, *Staphylo coccus aureus*, and Gram negative bacteria.

Figure 3 Phylogeny tree of *gyrB Mycobacterium tuberculosis* clinical isolates of pulmonary TB patients in Indonesia at 2010 and 2013, *M. tuberculosis* H37Rv, MTBC, MOTT, Gram positive and negative bacteria (Genetix WIN V.10 program).

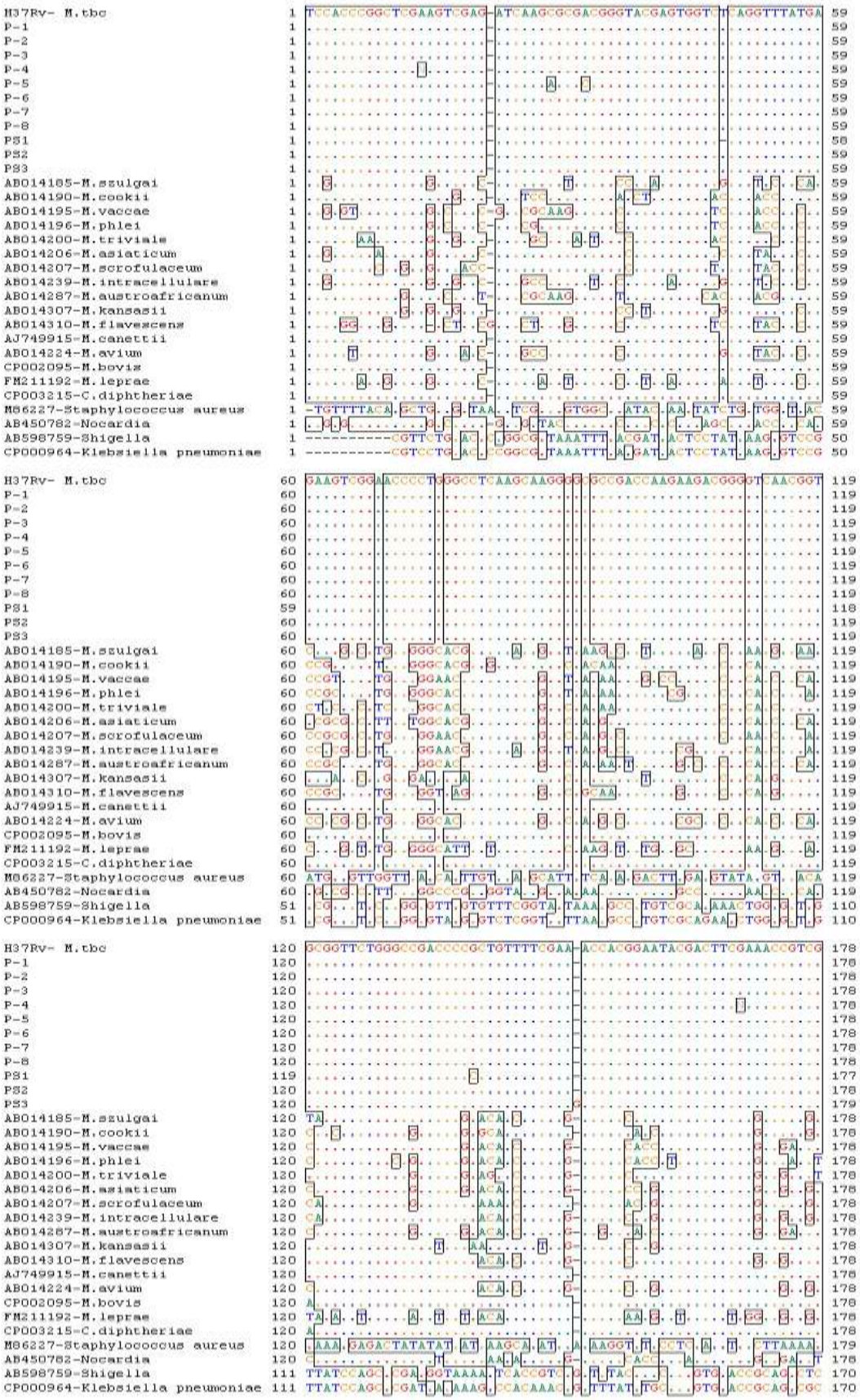
This phylogeny tree of *gyrB Mycobacterium tuberculosis* from clinical isolates have revealed the closed relation among the MTBC strains, on the other hands need more investigate the other DNA region for differentiate to *Corynebacterium spp*.

DISCUSSION

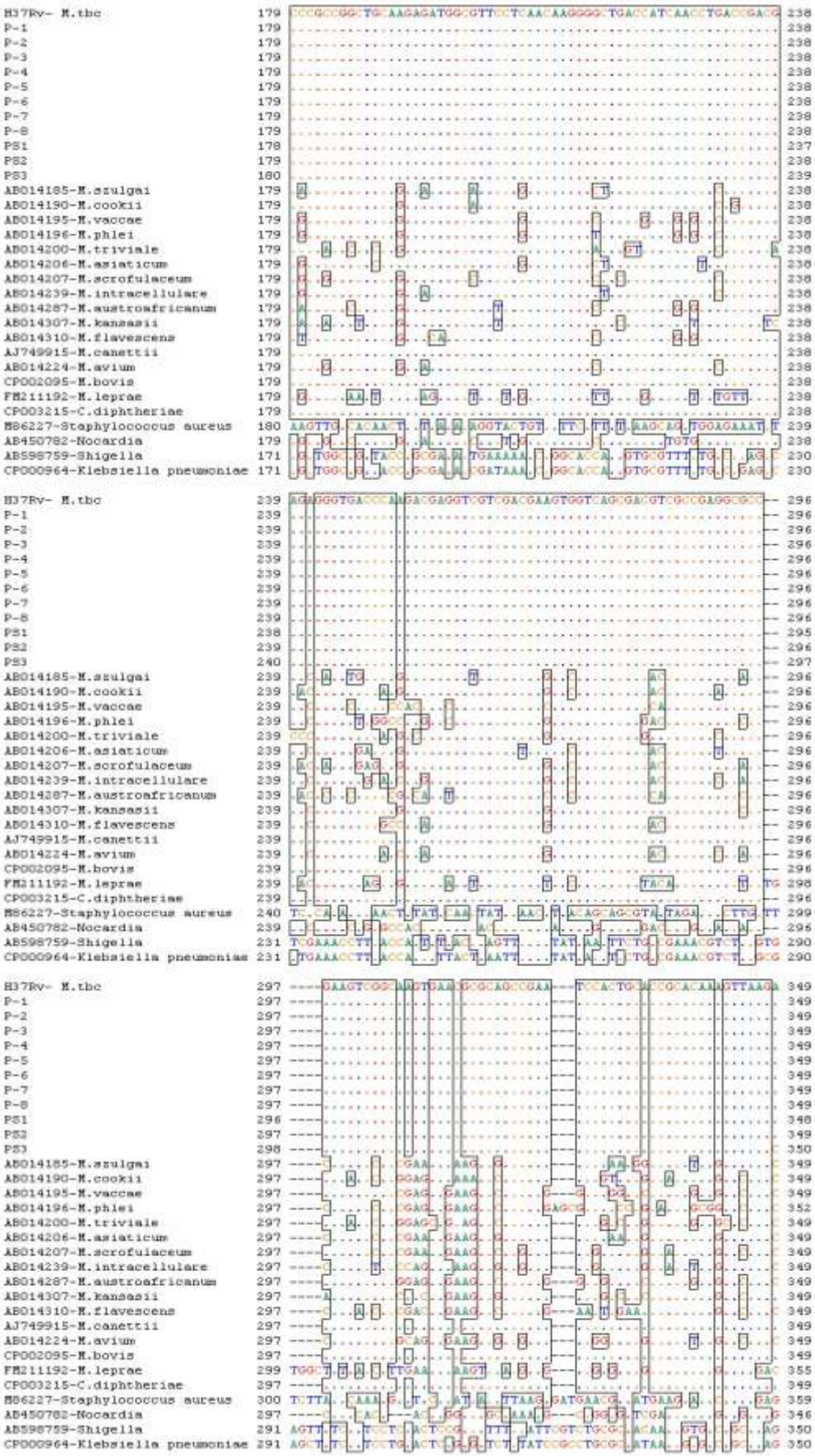
All of the 41 (100%) *Mycobacterium tuberculosis* clinical isolates from pulmonary TB patients in some area in Indonesia were positive for the target gene at 1.020-bp *gyrB*. Primer MTUB-f and MTUB-r were used for amplification of the 1.020-bp fragment of the *gyrB* gene *Mycobacterium tuberculosis* similar to investigation Chimara *et al.*, 2004 and Niemann *et al.*, 2000. The DNA region of *gyrB* gene, 1.020-bp fragments is amplified with species-specific primers MTUBf and MTUBr which do not generate amplicons from other species of mycobacteria.[7,14]

In this study have showed the similar result of alignment analysis in the sequence 1.020 fragment and a part of almost the whole genome, that all clinical isolates *Mycobacterium tuberculosis* showed almost identically with H37Rv wild type and members of MTBC, and there are a little number of SNP; however close related to *Corynebacterium diphtheriae*, that could be need to investigate the other region of DNA fragment analyzed in *gyrB* gene regions. The nucleotide sequence of polymorphism in *gyrB* gene represents a unique marker that facilitates the differentiation of the MTBC.[4,8]

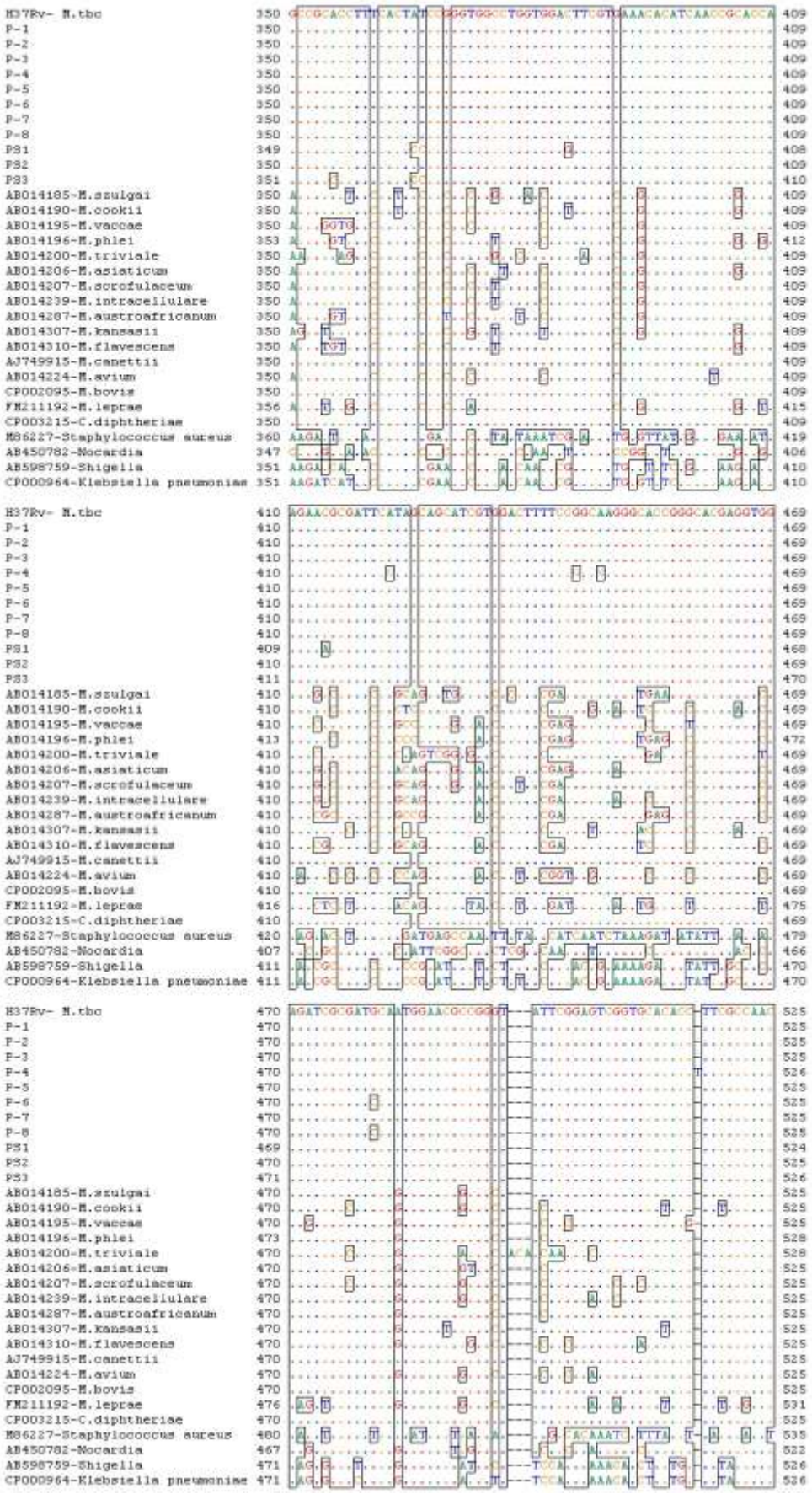
Convenient system for bacterial identification using species-specific *gyrB* gene sequences, because of *gyrB* gene rarely transmitted horizontally, is distributed ubiquitously among bacterial species, its molecular evolution rate is higher than 16S rRNA, and the



2A


H37Rv- M.tbc
P-1
P-2
P-3
P-4
P-5
P-6
P-7
P-8
PS1
PS2
PS3
AB014185-M.szulgai
AB014190-M.cookii
AB014195-M.vaccae
AB014196-M.phlei
AB014200-M.triviale
AB014206-M.asiaticum
AB014207-M.scrofulaceum
AB014239-M.intracellulare
AB014287-M.austroafricanum
AB014307-M.kansasii
AB014310-M.flavescens
AJ749915-M.canettii
AB014224-M.avium
CP002095-M.bovis
FM211192-M.leprae
CP003215-C.diphtheriae
M86227-Staphylococcus aureus
AB450782-Nocardia
AB598759-Shigella
CP000964-Klebsiella pneumoniae

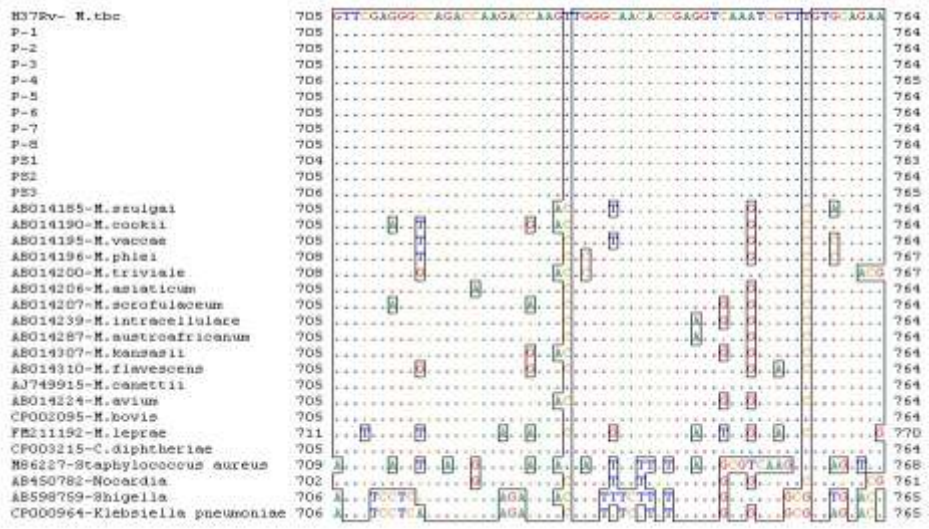
2B


H37Rv- M.tbc
P-1
P-2
P-3
P-4
P-5
P-6
P-7
P-8
PS1
PS2
PS3
AB014185-M.szulgai
AB014190-M.cookii
AB014195-M.vaccae
AB014196-M.phlei
AB014200-M.triviale
AB014206-M.asiaticum
AB014207-M.scrofulaceum
AB014239-M.intracellulare
AB014287-M.austroafricanum
AB014307-M.kansasii
AB014310-M.flavescens
AJ749915-M.canettii
AB014224-M.avium
CP002095-M.bovis
FM211192-M.leprae
CP003215-C.diphtheriae
M86227-Staphylococcus aureus
AB450782-Nocardia
AB598759-Shigella
CP000964-Klebsiella pneumoniae

2C


H37Rv- M.tbc
P-1
P-2
P-3
P-4
P-5
P-6
P-7
P-8
PS1
PS2
PS3
AB014185-M.szulgai
AB014190-M.cookii
AB014195-M.vaccae
AB014196-M.phlei
AB014200-M.triviale
AB014206-M.asiaticum
AB014207-M.scrofulaceum
AB014239-M.intracellulare
AB014287-M.austroafricanum
AB014307-M.kansasii
AB014310-M.flavescens
AJ749915-M.canettii
AB014224-M.avium
CP002095-M.bovis
FM211192-M.leprae
CP003215-C.diphtheriae
M86227-Staphylococcus aureus
AB450782-Nocardia
AB598759-Shigella
CP000964-Klebsiella pneumoniae

2D



2E

Figure 2A,B,C,D,E
Sequence alignment of *gyrB* gene *Mycobacterium tuberculosis* clinical isolates from sputum of pulmonary TB patients in Indonesia at 2010 and 2013 (Genetix WIN V. 10 program), *Mycobacterium tuberculosis* H37Rv, other MTBC, MOTT, *Corynebacterium diphtheriae*, *Staphylococcus aureus*, and Gram negative bacteria.

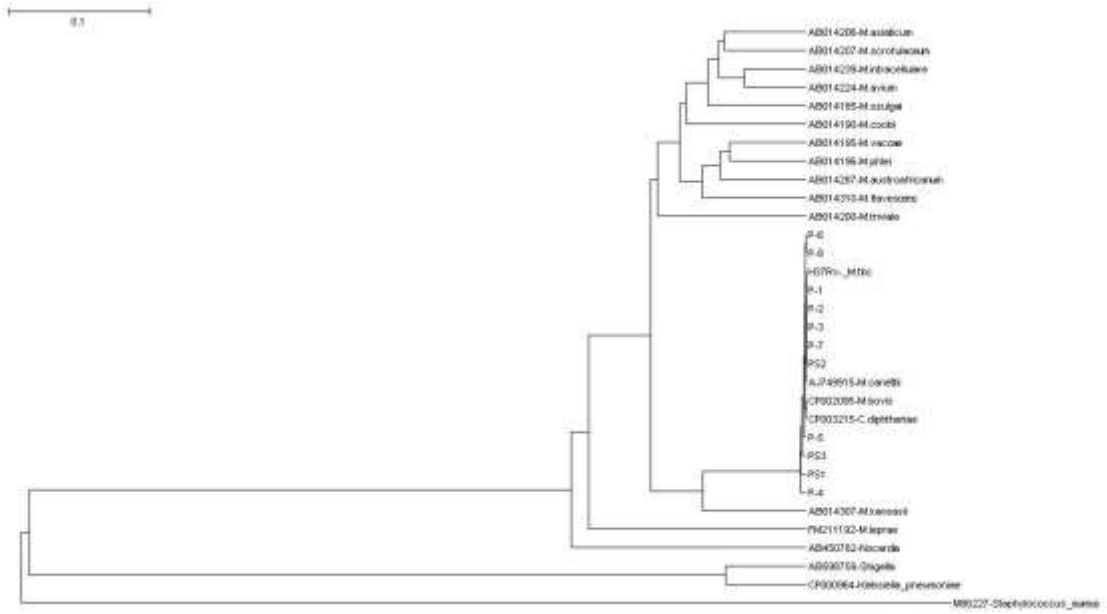


Figure 3
Phylogeny tree of *gyrB Mycobacterium tuberculosis* clinical isolates of pulmonary TB patients in Indonesia at 2010 and 2013, *M. tuberculosis* H37Rv, MTBC, MOTT, Gram positive and negative bacteria (Genetix WIN V.10 program).

essential roles of the *gyrB* product in DNA replication and transcription functions against the introduction of foreign *gyrB*. The *gyrB* sequences have the high correlation between the phylogenetic distance and the total genome homology analyzed by DNA-DNA hybridization.[17,21] DNA topoisomerase are essential for DNA replication, transcription, recombination, repair and control the level of supercoiling by cleaving and resealing the phosphordiester bond of DNA. They are classified into type I and type II according to enzymatic properties. The bacterial DNA gyrase is a type II topoisomerase that is capable of introducing negative supercoilling into a relaxed closed circular DNA molecule. DNA gyrase consists of two subunit protein-active form enzymes in the hetero-quaternary

complex structure of A2B2, A protein (GyrA) and B protein (Gyr B) that coding by *gyrA* and *gyrB* gene, only in prokaryotes.[16,17,19] In *M. tuberculosis* presence only single topoisomerase I and DNA gyrase as a type II DNA topoisomerase.[19,21,23] Expression of virulence genes depend on topological status of the genome, the topoisomerase as sensor of supercoils influence the specific gene expression.[19] The high validity of *gyrB* sequences as taxonomic marker was evaluated mainly: the rate of their base substitutions and the consistency of the results of *gyrB*-based analysis. Protein-coding genes evolve faster than rRNA genes because synonymous substitutions mainly at the third positions of codons in the protein-coding genes are permitted without causing any changes the amino acid sequences. The average base-substitution rate of 16S rRNA genes was 1% per 50 million years, while *gyrB* was estimated 0.7 – 0.8% per one million years, therefore some species with identical 16S rDNA sequences can be differentiated using their *gyrB* sequences.[17] The two genes encoding DNA gyrase in *Mycobacterium tuberculosis* are present next to each other in the genome, with *gyrB* upstream of *gyrA*, primary transcript is dicistronic with multiple promoters that appear to fine-tune transcription, the *gyr* genes in *Mycobacterium tuberculosis* as in other species are subject to autoregulation, albeit with slower kinetics, probably reflecting the slower metabolism of organism.[16,19,21,24] The most probable translational start codon for *M. tuberculosis* GyrB is GTG (Val) which results in translation of a protein of 674 amino acids (74 kDa) at position 5240-5242 in *M. tuberculosis* genome sequence.[16]

Mycobacterium tuberculosis complex (MTBC) are the causative agents of tuberculosis in humans and animals, genetic close relationship, but differ in epidemiology pathogenicity, geographic range, host preferences, and in importance for tuberculosis disease in human. MTBC includes *Mycobacterium tuberculosis, M. bovis subsp.bovis, M. bovis subsp. caprae, M. bovis BCG vaccine strain, M. africanum (subtype I and II), M. microti, M. canettii, M. pinnipedii*.[6,7,9,26] Routine differentiation is still base on phenotypic characteristics are time-consuming. Species belonging to MTBC cannot be differenteated by small subunit rRNA (16S rRNA) or internal trancribed spacer (ITS) 16-23S rRNA sequencing.[5] Niemann *et al*. 2000 showed the result of DNA sequencing on gene region 1.020 bases of *gyrB* gene *Mycobacterium tuberculosis* and *Mycobacterium africanum* subtype II have an identical *gyrB* sequence that facilities discrimination from the other species.[8] All members of MTBC are identical sequence gene, closely related gene with *gyrB* discriminatory regions in 1.020 bases. Base on these revealed, DNA sequence of species-specific region on 1.020-bp of *gyrB* gene *Mycobacterium tuberculosis* could be sole as the primer design for nucleic acid amplification that purpose to detect and identificate MTBC.

The *gyrB* could be useful for differentiation of MTBC and MOTT. The *gyrB*-based phylogeny was better than ITS-based phylogeny. The *gyrB* sequence of MTBC *M. tuberculosis, M. bovis, M.africanum, M. microti* found substitution at four sites, at 675 in *M. microti* , *M. bovis* at 756, at 1410 of *M. bovis*, *M. tuberculosis* at 1450, these substitutions can be regarded as the result of naturally occurring divergent evolution. The quantitation analysis *gyrB*-based method more useful than 16S rDNA-based method because the copy number of *gyrB* is single, while that of 16S rDNA is variable.[9,17]

Based on sequenced genome of MTBC and outside the MTBC (*M. leprae, M. ulcerans, M. avium, M. paratuberculosis, M. marinum,* and fast-growing *M. smegmatis,* the most distant of the sequenced mycobacterial genomes are minimally related by 60% DNA/DNA homology, and comparative genomic analysis has shown that gene loss is a significant part of the ongoing evolution of the slow-growing mycobacterial pathogens. Single nucleotide polymorphisms (SNPs) can result a silent amino acid substitution in which the protein sequence remains unchanged, synonymous or can alter the protein coding sequence (non synonymous) and act as a substrate for evolutionary selection. The identification of SNP markers for study of evolution, pathogenesis, and epidemiology in clinical *M. tuberculosis* and *M. bovis*, the ratio of SNPs type within a genome can act as a molecular clock, the high ratio of non synonymous to synonymous mutations across coding sequences suggests a recent divergence of *M. tuberculosis* and *M. bovis.* The prominent role of genomic deletion relative to *M. tuberculosis* i.e. *M. bovis* contains 66.037 bp less than *M. tuberculosis* H37Rv, genomic deletion can be used to reconstruct phylogeny trees. Genomic deletion behave as unidirectional event polymorphism which represent one time events in the evolution, can serve as robust markers of clonal organisms for determining phylogeny classification. MTBC present relatively little genomic diversity, based on SNPs similar with large-sequence polymorphism, genomic flexibility exist within MTBC for specific host adaptation. Genomic deletions seem to several regions of difference (RD) as a genetic instability at a locus.[20] Namouchi at 2012 reported the nucleotide substitution is a major mechanism for the

emergence of *M. tuberculosis* pathogenesis, MTBC genome exhibit significant regional variation in the density of SNPs, the region of high SNPs may harbor rapidly evolving genes which positively selected for adaptation to human environment, the extremely conserved genes essential for survival.[26] The other advantages of *gyrB* as a marker differentiation, there were only a little evidence showed mutations that are related to quinolone resistance.[10,12,13,22,25]

Gutierrez *et al*., 2005, The highly successful human pathogen *M. tuberculosis* has the extremely low level of genetic variation, incongruence among gene phylogenies as mosaic gene sequences, despite its apparent homogeneity its genome appears to be a composite assembly resulting from horizontal gene transfer events predating clonal expansion, with synonymous nucleotide variation in housekeeping genes.[15] Members of MTBC are the most successful human pathogens, *M. tuberculosis, M. bovis, M. africanum, M. microti, M. pinnipedii,* and species *M. caprae,* these members display different phenotypic characteristics and specific mammalian host ranges, they represent one of the most extreme genetic homogeneity with about 0.01%-0.03% synonymous nucleotide variation, and no significant trace of genetic exchange among them, it is believed the members are clonal progeny of a single successful ancestor that occurred 20.000 to 35.000 y ago. The six housekeeping genes, *katG, gyrB, gyrA, rpoB, hsp65, sodA,* and sequence of 16S rRNA revealed a smooth strain *M. canettii* and other members of MTBC form a single species defined by a compact phylogenetic clade.

Goh KS *et al.,* 2006, MTBC has been expanded additional members such as *M.canettii, M. pinnipedii, M. caprae,* dassie bacillus. *M. africanum, M. canettii* infect only human, *M. tuberculosis* infect human may also occasionally infect domestic and wild animals upon exposure. Host range *M. bovis* and *M. caprae* is very broad, causing disease among a wide range of feral, domestic mammals, and human. Analysis of *gyrB* and *hsp65* targets could be used for differentiation of the species within MTBC.[14]

CONCLUSION

The nucleotide sequence of *gyrB* gene *Mycobacterium tuberculosis* clinical isolates from sputum of TB patients in Indonesia that collected at 2010 and 2013 revealed almost identically to *Mycobacterium tuberculosis* H37Rv and MTBC; all of 41/ 41 (100%) clinical isolates positive PCR with target 1.020-bp as the specific and conserved gene region.

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