POLYMERASE CHAIN REACTION OF *Mycobacterium Tuberculosis* GYRB GENE REGION FOR RAPID SCREENING TEST OF PULMONARY TUBERCULOSIS

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ABSTRAK

*Gen gyr B-Mycobacterium tuberculosis memiliki regio DNA conserved dan spesifik, serta copy number dalam jumlah besar. Primer berdasar regio conserved dan spesifik ini meningkatkan akurasi metode amplifikasi asam nukleat. Penegakan diagnosis tuberkulosis yang akurat dan cepat sangat berguna untuk segera menentukan pemberian pengobatan yang tepat. Tujuan penelitian menentukan sensitivitas dan spesifisitas metode PCR regio gen gyr B untuk deteksi Mycobacterium tuberculosis dibandingkan dengan gold standard metode kultur pada medium Lowenstein Jensen (LJ). Pada 30 sampel sputum dari pasien dengan suspek tuberkulosis paru, dilakukan pemeriksaan kultur metode standar pada medium LJ dan PCR regio gen gyr B. Pada PCR dapat dideteksi gen gyr B Mycobacterium tuberculosis pada 21 sampel sputum sama dengan metode kultur standar, dengan sensitivitas 100% dan spesifisitas 100%. Kesimpulan, PCR regio gen gyr B- Mycobacterium tuberculosis memiliki akurasi yang tinggi, akan berguna untuk uji skrining laboratorium diagnosis TB.**(FMI 2013;49:16-20)***

Kata Kunci: *metode PCR, regio gen gyr B-Mycobacterium tuberculosis.*

ABSTRACT

*gyrB gene of Mycobacterium tuberculosis have conserved and specific DNA region with high copy number. Primer base in conserved and specific DNA region increases the accuracy of nucleic acid amplification method. Determining of tuberculosis diagnosis accurately and rapidly would be useful to appropriately determine the therapy. The objective of this study was to determine sensitivity and specificity of the PCR of gyrB gene region to detect Mycobacterium tuberculosis compared with gold standard method using Lowenstein Jensen (LJ) medium. Thirty sputum samples from pulmonary Tuberculosis suspects were examined using standard culture method in LJ medium and PCR gyrB gene region. Using PCR we positively found gyrB gene region of Mycobacterium tuberculosis in 21 sputum samples, similar to that if we used the gold standard method, with 100% sensitivity and 100% specificity. In conclusion, PCR of Mycobacterium tuberculosis gyrB gene region has a high accuracy, a rapid screening method for TB laboratory diagnosis.**(FMI 2013;49:16-20)***

Keywords : PCR method, gyrB gene region of Mycobactrium tuberculosis

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INTRODUCTION

In WHO report of the year 2009, Indonesian population in 2007 was 231.627.000, with estimated new tuberculosis (TB) cases of 528,063. Estimated TB incidence was 228 per 100,000 population, and the rate of new cases with SS+ sputum was 102 per 100,000 population (WHO 2009). The challenge of global TB problem continues, increases, and becomes more complex, augmented by the emergence of multidrug resistant-TB (MDR-TB) and TB-coinfected HIV, resulting in medication failure, morbidity, and mortality. More challenging problem is marked by the emergence of Extensively Drug Resistant TB (XDR-TB) strain, which is virulent and related to fatal diseases, particularly in TB co-infected HIV (WHO 2009, WHO 2008). The important first step in effective and efficient TB control starts from the improvement of case finding with appropriate method and accurate TB diagnosis establishment. Accurate and rapid TB diagnosis establishment is an important basis for immediate adequate treatment determination, which is expected to increase healing rate and breaking down the transmission chain (Indonesian Ministry of Health 2008).

Early detection and identification of *Mycobacterium tuberculosis* in tuberculosis-suspect patients allows immediate provision of adequate treatment prior to severe and extensive pulmonary tissue damage, and prevent the disease dispersion to other organs (Cho & Brennan 2007, Tortoli & Palomino 2007). Confirmation of tuberculosis diagnosis is not easy, particularly in primary tuberculosis cases, extra-pulmonary TB (with

specimens of pleural fluid, cerebrospinal flid, synovial, pericardial, biopsy tissue etc.), pediatric tuberculosis, TB-co infected HIV, and when there are diagnostic obstacles in increasing prevalence of non-tuberculous Mycobacteria (NTMs) infection (Cho & Brennan 2007). Another problem is slow culture growth of the clinical specimen if we employ standard culture method using solid media. It takes a long time to obtain the result of *Mycobacterium tuberculosis* detection. It may take three weeks up to 2 or even 3 months (Forbes et al 1998). Acid fast bacteria microscopic sputum examination method, which is fast and accurate, is also reported as having limited sensitivity, specificity, and technical constraints, especially on extra-pulmonary TB, pediatric TB, and HIV-TB co-infection (Cho & Brennan 2007).

To overcome such diagnostic problem, a laboratory method is developed for the diagnosis of tuberculosis in order to improve accuracy. In addition to the development of culture methods, currently a fast method is being developed on the basis of molecular biology. Nucleic acid amplification method based on polymerase chain reaction (PCR) with thermocycler principle has been widely reported to have high accuracy for the detection of DNA region in a gene of organism, which can be applied for diagnosis establishment (Piatek et al 2000). The development of new methods in mycobacteria examination is being studied for its accuracy and practical clinical usefulness (Cho & Brennan 2007, Tortoli & Palomino 2007). The development of rapid diagnostic method based on the principle of nucleic acid amplification is using a detection marker in gene region that are conserved and specific to *Mycobacterium tuberculosis* complex. Conserved region and specific genes of *Mycobacterium tuberculosis* complex was found in gyrB genes have a high copy number.

Primer based on conserved regions of gyrB specific genes increases the accuracy of nucleic acid amplification methods (Piatek et al 2000, Chimara et al 2004). Basic detection of nucleic acids in TB diagnosis, still prevailing as screening test for genotype, is not always expressing the phenotype, while as a screening test it must be combined with phenotypic assay as the gold standard of culture method. The aim of this study was to determine the sensitivity and specificity of PCR nucleic acid amplification method targeting gyrB gene region for the detection of *Mycobacterium tuberculosis* in sputum specimens from patients with suspected tuberculosis for tuberculosis diagnosis, compared with the gold standard culture method using Lowenstein Jensen medium.

MATERIALS AND METHODS

Samples were 30 sputum from patients with suspected pulmonary tuberculosis treated at Pulmonary Clinic and Inpatient Ward, Dr. Soetomo Hospital, Surabaya, from February to May 2010. The research was conducted at Bacteriology Laboratory, Institute of Tropical Disease, Airlangga University. Isolation and identification of *Mycobacterium tuberculosis* from sputum of patients with suspected pulmonary tuberculosis, sputum concentration and decontamination was performed by standard modified Petroff's Method (WHO), and then 100 uL of sediment suspension was subjected to mycobacterial isolation and identification using culture method in Lowenstein Jensen (LJ) medium. In the characteristic colony growth (dry rough, creamy white, cauliflower-like) niacin accumulation and nitrate reduction were tested with BD Strip Test and acid-fast bacterial microscopic examination of Ziehl Neelsen was performed (Forbes et al 1998).

QiAmp DNA Kit (Qiagen) was used for DNA extraction and purification. It was performed to 100 ul sediment suspension resulted from concentration and decontamination, and resulting in DNA-template containing suspension. In 50 ul of PCR reaction mixture Dream Taq “Green PCR Master Mix (Fermentas) we used amplification buffer containing optimized Dream Taq“ Green buffer: Tris-HCl pH 8,3, MgCl2, KCl, enzyme Taq DNA Polymerase, dNTPs consisting of dATP, dCTP, dGTP and dTTP. In this PCR mixture, we added a pair of MTUB-f (5’ TCGGACGCG TATGCGATA TC3’) and MTUB-r5’ACATACAGTTCGGACTTGCG3’) primers in a concentration of 1.0 uM each, and added with template DNA 1 ug (2 ul). Polymerase Chain Reaction (PCR) was performed with Thermocycler (Perkin Elmer, Gene Amp PCR System 2400). PCR reaction comprised 98 degree C, 2 minutes per heating, followed with 40 reaction cycles: denaturation at 96 degree C, 20 seconds; annealing 58 degree C, 20 seconds; and polymeration at 72 degree C, 7 minutes. PCR results were detected with electrophoresis in agarose gel in 2% concentration, containing 0.5 ug/ml ethidium bromide in TBE (Tris 0,089 M, Boric acid 0,089 M, and EDTA 0,002 M) buffer (Piatek et al 2000, Chimara et al 2004). Positive amplificatin revealed *Mycobacterium tuberculosis* gyrB gene region in 1020 bp DNA band in electrophoresis result. It was observed on UV transiluminator and photographed (Chimara et al 2004, Kasai et al 2000). PCR was also done to Amplification Positive control of Mycobaterium tuberculosis H37Rv reference strain (ATCC 27294) and negative control the mixture of PCR reaction without DNA template. Sensitivity and specificity analysis of PCR method of *Mycobacterium tuberculosis* gyrB gene region detection

in sputum of suspected pulmonary TB patients. PCR result of *Mycobacterium tuberculosis* gyrB gene region and the gold standard culture in LJ medium were analyzed with validity test using four fold table.

RESULTS

Detection and identification of *Mycobacterium tuberculosis* culture in Lowenstein Jensen medium reveals colonies with creamy white, dry rough, and cauliflower-like characteristics (Figure 1) with positive nicacin accumulation test, positive nitrate reduction test, and positive acid-fast bacteria. Sputum culture examination from 30 samples of pulmonary-tuberculosis suspected patients, using standard culture method in LJ medium (Table 1), revealed 21 with positive *Mycobacterium tuberculosis* isolates. Mean detection time of characteristic colony and *Mycobacterium tuberculosis* identification requires 3 weeks 4 days, and 7 isolates (23.33%) were detected after 4 to 8 weeks.

Table 1. Results of PCR test on *Mycobacterium tuberculosis* gyrB from suspected pulmonary TB patients' sputum, compared to gold standard culture method in LJ medium.

PCR gyrB. M.tbc Test Method	Gold standard, culture in LJ medium		
	+	-	Total
+	21	0	21
-	0	9	9
Total	21	9	30

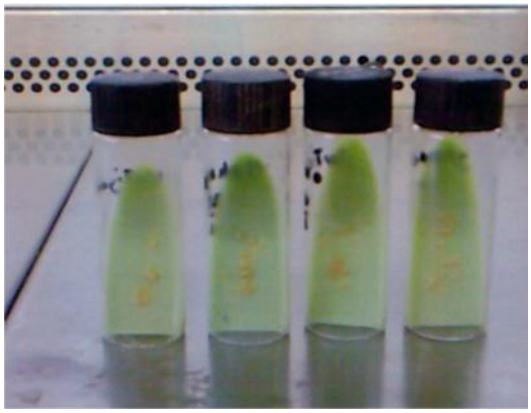

Figure 1. Culture of colony growth from sputum specimen of suspected pulmonary tuberculosis patients in Lowenstein Jensen medium.

PCR result showed that *Mycobacterium tuberculosis* gyrB gene region amplification can be detected in electrophoresis as DNA band at 1020 bp (Figure 2). PCR results were positive in 21 out of 30 sputum samples of pulmonary tuberculosis suspected patients, and the result of validity test analysis showed that sensitivity and specificity level of 1020 bp region of *Mycobacterium tuberculosis* gyrB gene was 100%, and the conformity of positive and negative results between this PCR method and standard culture method in LJ medium was also 100% (Table 1).

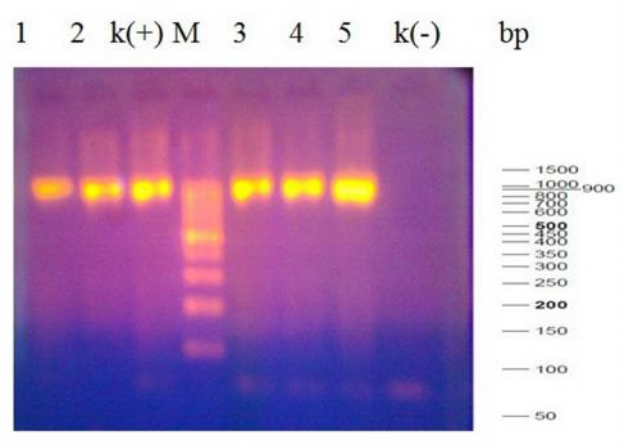

Figure 2. PCR results, *Mycobacterium tuberculosis* gyrB gene region amplification.

Amplification of *Mycobacterium tuberculosis* gyrB gene region in 1020 bp DNA band (1, 2, 3, 4, 5 positive PCR results); M : marker 50-bp ladder; k(+) was positive control (suspension of *Mycobacterium tuberculosis* strain reference H 37Rv); k(-) was negative control, mixture of PCR reaction without DNA template. *Mycobacterium tuberculosis* gyrB PCR laboratory examination requires averagely 6 hours to produce results.

DISCUSSION

Culture method in LJ medium is the gold standard in pulmonary tubeculosis diagnosis. However, it is constrained by the length of time to obtain the result. In this study, mean detection of characteristic colony growth after more than 3 weeks incubation with identification procedure required averagely 4 weeks, and 7 isolates were detected after 4 to 8 weeks. In addition to the length of diagnosis, there are also difficulties in taking sputum from suspected pediatric tuberculosis patients and suspected tuberculosis co-infected HIV patients, so that the specimen is frequently presenting as saliva or gastric or blood aspirate, which,

by using culture, negative results are often reported (Cho & Brennan 2007). Therefore, we need a detection method employing a principle of nucleic acid amplification, in order to improve detection sensitivity and specificity, by using conserved gene region marker and specific *Mycobacterium tuberculosis* (Chimara et al 2004, Kasai et al 2000, Niemann et al 2000).

Mean time to obtain the result of PCR in this study was 6 hours, starting from decontamination to sputum concentration. *Mycobacterium tuberculosis* gyrB gene region, which is highly conserved and specific at species level, becomes the basis of primary design (MTUB-f and MTUB-r) for nucleic acid amplification procedure, and it can be used as a method for detecting *Mycobacterium tuberculosis* for an accurate tuberculosis diagnosis establishment (Kasai et al 2000).

DNA gyrase is an important topoisomerase enzyme that has a function in DNA replification process in organisms cells, including in *Mycobacterium tuberculosis*, with a high copy number, particularly during the active multiplication or growth. The gyrase can be employed as viability marker of the cells and active division process. DNA gyrase enzyme is signaled by gyrB and gyr A genes. In each bacterial species, DNA sequence and gyrB and gyr A organizations have a region with homologue sequence and can be used to differentiate from other species (Nagaraja 2004, Unniraman et al 2002). Nakajima et al (2010) reported the that specificity of laboratory diagnosis confirmation for *Mycobacterium tuberculosis* identification was 100% by detecting 1020 bp fragment of *Mycobacterium tuberculosis* gyrB gene among all 350 *Mycobacterium tuberculosis* clinical isolates from tuberculosis patients in India. *Mycobacterium tuberculosis* species confirmation between the members of *Mycobacterium tuberculosis* complex group is vital for proper treatment, because first line anti-TB drugs are suitable for *Mycobacterium tuberculosis*, while Mycobacterium bovis has natural resistance against pyrazine amide (Nakajima et al 2010). In addition, another advantage of gyrB gene target in PCR is that the gene has region with stable DNA sequence, which may increase both specificity and sensitivity (Nakajima et al 2010).

Similarly, the report of Chimara et al (2004) stated that *Mycobacterium tuberculosis* identification between 311 *Mycobacterium tuberculosis* complex from clinical isolate of TB patients in Sao Paulo, using gyrB – RFLP PCR method, detected 1020 bp fragment of *Mycobacterium tuberculosis* gyrB gene in all (100%) of 311 TB clinical isolates, and not detected 1020 bp amplicon in other non-*Mycobacterium tuberculosis* (NTM) species (Chimara et al 2004). Specific and conserved DNA sequence in *Mycobacterium tuberculosis* gyrB gene is located at 1020 bp DNA region, which is different from that in other species, and only identical with that in Mycobacterium africanum subtype II (Niemann et al 2000, Nagaraja 2004). This identical condition has clinical similarity as the cause of infection in human, where there are similar pathogenesis, clinical manifestations, and treatment procedure (Niemann et al 2000).

Nucleic acid amplification method is highly useful for clinical application in *Mycobacterium tuberculosis* specific DNA detection, particularly in pediatric TB and TB in patients with HIV/AIDS (Cho & Brennan 2007). In children, it is rather difficult to obtain sputum specimen and the extra-pulmonary proportion, such as in meningitis and lymphadenitis, is high, with low number of bacille within cerebrospinal fluid specimen, aspirate, and biopsy. In TB-coinfected HIV many extrapulmonary TB cases are also found, and is often along with other opportunistic infections, so that the bacille was also found in very low number, whose detection can be improved by the amplification of its nucleic acid (Cho & Brennan 2007). Another advantage of *Mycobacterium tuberculosis* gyrB gene PCR is that it provides fast result, averagely in 6 hours. Thus, nucleic acid amplification method has an important role in the development of Tuberculosis diagnosis establishment with a strict application of quality control and quality assurance in each molecular laboratory examination setting (Cho & Brennan 2007).

CONCLUSION

PCR method of *Mycobacterium tuberculosis* gyrB gene region has 100% sensitivity and 100% specificity. It is suggested that PCR method gyrB gene region should be subjected to further clinical trial for use as screening test.

ACKNOWLEDGMENT

We would like to thank to DP3M Ditjen Dikti, Ministry of Education and Culture, the Rector of Airlangga University, LPPM of Airlangga University, Dean of Faculty of Medicine, Airlangga University, LPT Airlangga University, and Dr. Soetomo Hospital for all kinds of support for the implementation of this research.

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