NUCLEIC ACID AMPLIFICATION OF THE rpoB REGION OF Mycobacterium tuberculosis IN PULMONARY TUBERCULOSIS DIAGNOSIS

Yudita Wulandari, Nurul Wiqoyah, Ni Made Mertaniasih
Department of Medical Microbiology, Faculty of Medicine, Airlangga University

ABSTRACT

Tuberculosis (TB) is one of the major public health concerns worldwide. The detection of the pathogen Mycobacterium tuberculosis complex (MTBC) as early as possible has a great impact on the effective control of the spread of the disease. It is difficult to diagnose Mycobacterium tuberculosis infection due to a lack of rapid, sensitive and specific test. Newer methods, which are easy and reliable, are required to diagnose TB. This research aim is to evaluate the accuracy polymerase chain reaction (PCR) technique, using primers the rpoB gene region compare to culture method in Lowenstein-Jensen medium as a gold standard for the detection of Mycobacterium tuberculosis in the sputum samples. Sputum samples from TB suspected patients are examined by culture and PCR, using rpoB target gene. Specimens are digested and decontaminated by the modified Petroff method (WHO). Approximately from 1.0 ml of resuspended sediment, each 100 ul is used to inoculate Lowenstein-Jensen slants in duplo and 100 ul resuspended sediment is processes for PCR. Mycobacterium tuberculosis is identified using a specific pair of primers designed to amplify 541 bp sequences of rpoB gene. Conclusion: PCR have the high accuracy, sensitivity 100% and specificity 100% for pulmonary TB diagnosis. The performance of a rpoB Mycobacterium tuberculosis PCR assay have value in the rapid diagnosis of pulmonary tuberculosis.

Keywords: rpoB gene, Mycobacterium tuberculosis, diagnosis

Correspondence: Yudita Wulandari, Department of Medical Microbiology, Faculty of Medicine, Airlangga University

INTRODUCTION

Tuberculosis (TB) is still a public health problem that is important to immediately controlled globally around the world. Furthermore, in line with the emergence of HIV / AIDS, it is estimated that there is an increase in the incidence of tuberculosis. One of the important TB control strategy starts from suspected tuberculosis case finding effective and efficient with the proper diagnosis of TB. Proper diagnosis and rapid TB can determine an adequate TB treatment that could eventually break the chain of transmission. Early diagnosis of tuberculosis and accurately is critical to the success of treatment of TB.
DNA-dependent RNA polymerase. In some previous studies reported that some *Mycobacterium tuberculosis* rpoB gene sequence can be used for identification of Mycobacteria to more easily, quickly and accurately than with conventional molecular biology methods that use polymorphism of 16S rRNA (of BJ Kim, 1999). DNA Structure rpoB gene region are "conserved" and specific to *Mycobacterium tuberculosis* may become the target nucleic acid amplification by PCR for identification of the species of interest. Based on the background of the study aims to determine the accuracy (sensitivity, specificity, positive predictive value, negative predictive value) method of nucleic acid amplification primer PCR method with a target gene "rpoB" for detection and identification of *Mycobacterium tuberculosis* in sputum specimens of patients with suspected tuberculosis lung and pulmonary tuberculosis diagnosis.

**MATERIAL AND METHOD**

The design of this study is the diagnostic test. The study was conducted from January-March 2011, sputum sampling conducted in Surabaya BP4 and research conducted at the Tropical Disease Center (TDC) Airlangga University. As the subjects were patients with suspected pulmonary tuberculosis clinically. Samples were taken at consecutive sampling that every patient who met the study criteria included in the study until the number of subjects met. The sample size formula based diagnostic tests obtained as many as 11 samples. All samples were examined by smear, culture and PCR. Gold standard culture used Lowenstein-Jensen media while using the target gene PCR with primers rpoB rpoB-F (5'-TCGGCGAGCCCATACGTCG-3') and rpoB-R (5'-GCGTACACCGA-CAGCGAGCC-3') resulting PCR product 541 bp.

Sputum samples were first decontaminated using Petroff alkali obtained from WHO then pelleted by 1 mL. Of the pellet was taken 200 mL for cultures taken (Duplo) and incubated in a CO2 incubator for 2-8 weeks. After 8 weeks of assessed colony growth. From pellets than taken for culture, as much as 200 mL was also taken for PCR. DNA extraction used heating (boiling) method. Nucleic acid amplification performed a total of 35 cycles with stages as follows: pre-denaturation (98 °C, 5 min), denaturation (96 °C, 1 min), annealing (62 °C, 1 min), extension (72 °C, 1 min) and post extension (72 °C, 10 min). Amplification products electrophoresed and then read the results. Analysis of diagnostic test data shown in table form 2x2 then do the calculation values of sensitivity, specificity, positive predictive value, negative predictive value and accuracy of PCR for culture examination as a gold standard.

**RESULTS**

Total sample size was 11. Of 11 samples obtained sputum smear examination positive in 2 samples, positive growth on Lowenstein Jensen culture media as much as 1 sample, and PCR positive results with as many as 1 sample. The results of PCR and culture examination of sputum are shown in Table 1.

| Table 1. Tables PCR and culture on Lowenstein Jensen media. |
|------------------|------------------|
| Kultur M. tbc (+) | Kultur M. tbc (-) |
| PCR (+) | 1 | 0 |
| PCR (-) | 0 | 10 |

From the analysis obtained at 0% false positives and false negatives to 0%. From the above data obtained PCR targeting rpoB gene of *Mycobacterium tuberculosis* for diagnosis of pulmonary tuberculosis had a sensitivity of 100%, specificity 100%, positive predictive value 100%, negative predictive value 100% and 100% accuracy. Picture PCR results shown in Figure 1.
DISCUSSION

Gold standard for laboratory diagnosis of *Mycobacterium tuberculosis* is conventional culture method that has shortcomings, but requires a long scan times. Examination of the molecular methods such as PCR requires a faster time than culture, but both sensitivity and specificity still need to be validated.

In this research, PCR sensitivity was 100% and specificity of 100%. Negative predictive value was 100%, positive predictive value 100% and accuracy 100% (from 11 samples). In another study on the use of PCR methods for the diagnosis of pulmonary tuberculosis found varying sensitivity and specificity, the sensitivity of PCR was reported from 31.6% to 100% and specificity of PCR were reported by 81.8% to 100%. Although the results of this study showed good sensitivity and specificity, but are not eligible for the PCR and culture examination table on Lowenstein Jensen medium (Table 1) there is still a number of samples in column 0 2x2 table, so we need more samples in order to study more reliably. It is also still required internal and external validity to obtain a high degree of accuracy. Components of internal validity include BTA levels in the specimens (scanty, negative), PCR test with negative results on NTM (Non-tuberculous Mycobacteria) and the non Mycobacteria (Gram negative such as E. coli and others; Gram positive *S. aureus* and others). Components of external validity include large specimens (samples), the type specimen (a new pain, chronic, treated / untreated), generalization factor, and others.

In various studies with a variety of clinical specimens was also reported wide variations of sensitivity and specificity of PCR techniques for diagnosis. Sensitivity and specificity were reported from various studies difficult to compare, because the DNA extraction protocol (mycobacterium lyse method and DNA extraction procedures), the target nucleic acid sequence, the primary election, the amplification product detection system and the number and type of clinical samples used are different from each other (Boddinghaus et al, 1990; Thiery et al, 1990; Fries et al, 1991; Pierre et al, 1991 and Kolk et al, 1992). Sensitivity of PCR is highly dependent on the efficiency of DNA extraction procedures. Isolation of nucleic acids from mycobacteria is more difficult than other microorganisms because the thick peptidoglycan layer of the cell wall makes mycobacterium is resistant to a number of lysing buffer (Wards et al, 1995; Murray et al, 2002).

Because of his complex structure and impermeability of the cell wall, it is difficult to lyse mycobacteria cells. A common method used to isolate DNA produces low quantities (due to incomplete lysis of the cell wall) or low quality DNA from mycobacteria, therefore causing the sensitivity of the test is low.

Detection of DNA amplification method depends on the purity and quality of the template DNA. In this study, for extracting DNA from sputum samples used method of heating (boiling), where the heat is the only cause of the outbreak of the bacteria and release the DNA for PCR amplification process. In this research, the results of specific PCR revealed rpoB gene of *Mycobacterium tuberculosis*. One of the factors that determine the sensitivity of PCR is the amount of coffee contained the target sequence in the genome of a microorganism (Rish JA, et al, 1996; Thierry D. et al, 1990), the more the number of copies in the genome, the higher sensitivity of PCR (Kent L., 1995). RpoB gene contained only one copy in the genome of *M. tuberculosis* (Donnabella, 1994), in contrast to having 1-27 IS6110 copies in the genome of *M. tuberculosis* H37Rv (Rish JA, et al, 1996; Thierry D. et al, 1990). PCR sensitivity is also greatly influenced by the method of DNA extraction (Sjobring U, 1990). The existence of a number of contaminants in the process of DNA extraction, can decrease the efficiency of PCR (Ausubel FM et al, 1990).

In this study digestion and decontamination methods used were Petroff method using 4% NaOH. Although this method is more toxic to mycobacteria than NALC-NaOH method, but if the procedure is done properly and carefully then obtained a good rate of contamination (2-5%). Moreover, this method is relatively simple and easy to obtain the reagent. Even at a U.S. research Damle (1986) obtained NALC-NaOH method has a number of contamination greater than Petroff method. When the digestion and decontamination procedures mycobacteria in specimens killed all the cultures became negative and thus false negative occurs.

Specificity of PCR results showed a variation between laboratories, it is caused by differences in laboratory work procedures, differences in the level of cross-contamination and the primary election (Parekh et al., 2006). Various studies used different gene targets (KM Parekh et al, 2006; Khorshidi A. et al, 2009; Mertaniasih NM. Et al, 2010; Jasaputra et al, 2005), and so far there has been no analysis of the accuracy of the PCR method with the target gene rpoB *Mycobacterium tuberculosis* for diagnosis of pulmonary tuberculosis. So far the research on the detection and identification of *Mycobacterium tuberculosis* by rpoB gene targets using PCR-Restriction Fragment Length Polymorphism (Lee H, 2000), Comparative Sequence Analysis rpoB gene (BJ Kim, 1999), PCR-linked reverse hybridization with oligonucleotide probes specific rpoB (Hong SK, 2004),
Duplex PCR (Kim BJ, 2004) and Multiplex PCR (Mokkadas E, 2007).

Selection of the primary target genes "conserved" and also influence the accuracy of specific PCR. RpoB gene encodes a subunit β RNA polymerase, which produces RNA molecules in the cell. So, is one of the genes rpoB 'guard cells' (housekeeping gene), which is very important, which is closely related to the vitality of the cells and therefore the target of the drug rifampin is bactericidal for M. primary tuberculosis and M. leprae. Therefore, it is reasonable to assume that the genetic structure of the rpoB gene is highly conserved in the same species. However, unlike the 16S rRNA or other rRNA primary structure is functionally important, rpoB gene appears to be more tolerant to changes in the DNA sequence without causing a change in protein function. In particular, the region of DNA that is not associated with the active protein appears to be more polymorphic and did not cause major functional defect, so that there are regions of DNA are highly conserved and variable regions of DNA relative to the rpoB gene (Lee H, 2003).

Resistance to rifampin occurs due to mutations in the RNA polymerase subunit β encoded by the gene rpoB. Almost all of the mutations in the rpoB gene occur in a small area of less than 100 bp (hot spot area), with possible mutations outside the area by 5% (Heep M, 2000). Although the target for PCR amplification area contains frequent mutation, when the primary sticking point sequence mutations then a germ-free can still be identified as M. tuberculosis. Intra-species variation rpoB sequences M. tuberculosis reported less than 1%, and mutations in addition to the hot spot region (81 bp) where most of the mutations occur, it is extremely rare (Kim et al, 1999).

Differences laboratory work procedures greatly affect the specificity of PCR. These components are below play a role in the process of PCR: DNA Print / target DNA, oligonucleotide primers, Deoxynucleoside Triphosphate (dNTP), the enzyme DNA polymerase, buffer solution, the number of PCR cycles, and the PCR step. Specificity of the results is affected by the possibility of specimen contamination during processing in the laboratory, if the primers used were not specific or if conditions are not optimal PCR amplification allowing non-specific products. Contamination of DNA fragments even in very small amounts can cause the error to have received unwanted amplification products or even specific. The most frequent sources of contaminants is another specimen or from a previous amplification procedure. For this reason, the laboratory should have a separate room for the various stages of PCR procedures and quality control measures must follow very strict. False-negative results may also occur if there is material in the specimen that inhibits nucleic acid extraction or amplification (Sulistyaningsih E, 2007).

In this research, a sample of sputum smear negative culture positive, this can happen due to the microscopic examination of smear cannot identify the species. Because niacin test sample was culture negative on the growth of colonies of the sample is expressed as Mott. In this study, no positive PCR results obtained with smear negative. In this research, I Wayan Agus Son et al (2008) stated that of the 74 patients with smear-negative pulmonary TB found 61 patients with positive PCR results. Thabrani in research Friendship Hospital get the 36% of patients with negative sputum smears obtained microscopically positive PCR.

Filho 1996 in Rio de Janeiro in the study of microscopic smear negative pulmonary TB PCR positive gain of 44.9%. PCR positive with a negative smear results possible because PCR can detect 1-10 bacteria while the smear can detect if there is a minimum of 105 bacteria per ml sputum smear result, when PCR contamination or germs dormant / dead (Muhammad Asaad Maidin, 2005). RpoB gene encodes a subunit β RNA polymerase, which produces RNA molecules in the cell (transcription). When bacteria expressing the gene rpoB it means the bacteria are doing replication and active metabolic process occurs, which indicates the germs alive and breeding. Thus, if the active bacteria in the disease process the levels of rpoB gene region will increase thereby increasing the detection of *Mycobacterium tuberculosis*.

With sensitivity and specificity values were high in this study, it is expected examination PCR nucleic acid amplification method with *Mycobacterium tuberculosis* rpoB gene targets can be useful for diagnosing pulmonary tuberculosis quickly and accurately, with research continuing to conduct internal and external validity for the accuracy of the method high.

**CONCLUSION**

Due to the sensitivity and specificity values were high in this study, the examination of nucleic acid amplification by PCR method targeted rpoB gene of *Mycobacterium tuberculosis* is expected to be useful for diagnosis of pulmonary tuberculosis is fast and accurate, but the research is expected to continue with internal and external validity to achieve goals accuracy of this PCR method in the diagnosis of pulmonary tuberculosis.
REFERENCES


