Folia Medica Indonesiana Vol. 47 No. 4 October - December 2011 : 224-229

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

ABSTRAK

Tuberkulosis (TB) merupakan salah satu masalah kesehatan masyarakat yang utama di seluruh dunia. Deteksi dari Mycobacterium tuberculosis patogen kompleks (MTBC) sedini mungkin memiliki dampak yang besar pada kontrol yang efektif dari penyebaran penyakit. Sulit untuk mendiagnosis infeksi Mycobacterium tuberculosis karena kurangnya rapid test, sensitif dan spesifik. Metode baru, yang mudah dan dapat diandalkan, diperlukan untuk mendiagnosis TB. Penelitian ini bertujuan untuk mengevaluasi akurasi polymerase chain reaction (PCR) teknik, menggunakan primer daerah gen rpoB dibandingkan dengan metode budaya di Lowenstein-Jensen media sebagai standar emas untuk mendeteksi Mycobacterium tuberculosis di sampel sputum. Dahak sampel dari pasien TB yang dicurigai diperiksa oleh budaya dan PCR, menggunakan gen target rpoB. Spesimen yang dicerna dan didekontaminasi dengan metode Petroff dimodifikasi (WHO). Sekitar dari 1,0 ml sedimen resuspended, masing-masing 100 l? Digunakan untuk menyuntik Lowenstein-Jensen miring di duplo dan 100 ul resuspended sedimen adalah proses untuk PCR. Mycobacterium tuberculosis diidentifikasi dengan menggunakan sepasang primer spesifik dirancang untuk memperkuat 541 bp urutan gen rpoB. Kesimpulan: PCR memiliki akurasi yang tinggi, sensitivitas 100% dan spesifisitas 100% untuk diagnosis TB paru. Kinerja alat tes Mycobacterium tuberculosis rpoB PCR memiliki nilai dalam diagnosis cepat tuberkulosis paru.

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.

Microscopic method is an efficient method in the national TB control program, but has a low sensitivity for early pulmonary TB, which can only be detected if the sputum smear at least 105/ml. Examination of culture is the gold standard method is more sensitive, can detect as many as 102 TB bacilli per ml and specific, but it takes a long time, from a few weeks to two months so needed a quick diagnosis so that effective case management. (Hofmann-Thiel, 2010).

The method of nucleic acid amplification by PCR has the potential to diagnose tuberculosis in a few hours with a level of sensitivity and specificity (58-100%). In various studies, it has been reported to yield false positive rate of 0.8% to 30%. *Mycobacterium tuberculosis* rpoB gene is a gene that encodes a subunit

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'-TCGGCGAGCCCATCACGTCG-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 º C, 5 min), denaturation (96 º C, 1 min), annealing (62 º C, 1 min), extension (72 º C, 1 min) and post extension (72 º 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.

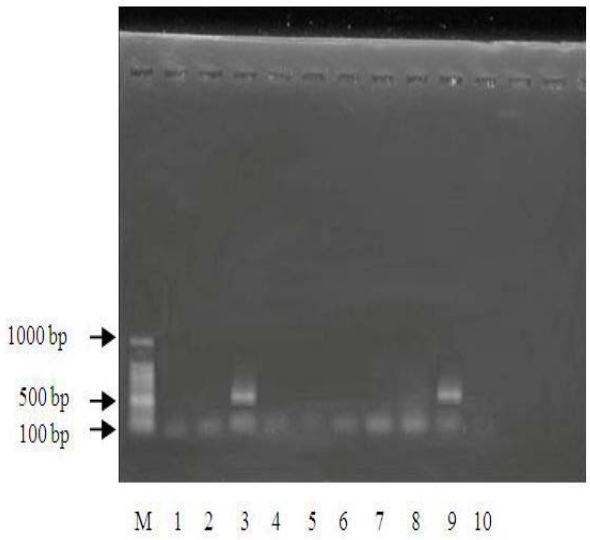

Figure 1. The results of clinical isolates DNA PCR

M 1 2 3 4 5 6 7 8 9 10
Lane M: 100 bp DNA ladder marker
Lanes 1-8: sputum specimens
Lane 3: positive (bands 541 bp)
Lane 9: DNA M. tuberculosis H37Rv (positive control)
Row 10: distilled water (negative control)

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 reliable. 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; Piere 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

1. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhi K, 1990. Current protocols in moleculer biology. Vol. 2. New York: Greene Publishing Associates and Wiley-Interscience, John Wiley & Sons, 15.03-15.1.7.
2. Boddinghaus I, Rogall T, Flohr T, Blocker H, Bottger EC, 1990. Detection and identification of Mycobacteria by amplification of rRNA. J. Clin. Microbiol. 28: 1751-1759.
3. Damle AS, Kaundiya DV, 1986. Comparison of three methods of decontamination of sputum for mycobacterial culture. Indian Journal of Tuberculosis. July; 33(3): 124-8
4. Donnabella V, F. Martiniuk, D. Kinney, M. Bacerdo, S. Bonk, B. Hanna, W. N. Rom, 1994. Isolation of the gene for the beta subunit of RNA polymerase from rifampicin-resistant *Mycobacterium tuberculosis* and identification of new mutations. Am. J. Respir. Cell Mol. Biol. 11:639–643.
5. Filho LDA, Celemann WMR, Barreto CEN, Kritski AL, Fonseca Ls, 2002. Sensitivity of Amplicor MTB on direct detection of *Mycobacterium tuberculosis* in smear-negative specimens from outpatients in Rio de Janeiro. Brazilian J Microbiol; 33: 163-5
6. Fries JWU, Patel RJ, Piessehn WF, Wirth DE, 1991. Detection of untreated mycobacteria by using polymerase chain reaction and specific DNA probes. J. Clin. Microbiol. 29:1744-1747
7. Heep M., U. Rieger, D. Beck, N. Lehn, 2000. Mutations in the beginning of the rpoB gene can induce resistance to rifamycins in both Helicobacter pylori and *Mycobacterium tuberculosis*.Antimicrob. Agents Chemother. 44:1075–1077.
8. Hong SK, Kim BJ, Yun YJ, Lee KH, Kim EC, Park EM, Park YG, Bai GH, Kook YH, 2004. Identification of *Mycobacterium tuberculosis* by PCR-linked reverse hybridization using specific rpoB oligonucleotide probes. Journal of Microbiological Methods 59, 71-19.
9. Hofmann-Thiel S, Turaev L, Hoffmann H, 2010. Evaluation of the hyplex.sup.[R] .sup.TBC PCR test for detection of *Mycobacterium tuberculosis* complex in clinical samples.(Research article) (Report). BMC Microbiology, p.95
10. Jasaputra DK, Onggowidjaja P, Soeng S, 2005. Akurasi Deteksi *Mycobacterium tuberculosis* dengan Teknik PCR menggunakan "Primer X" dibandingkan dengan Pemeriksaan Mikroskopik (BTA) dan Kultur Sputum Penderita dengan Gejala Tuberkulosis Paru. JKM Vol.5, No 1, Juli, hal 7-13.
11. Kent L, McHugh TD, Billington O, Dale JW, Gillespie SH, 1995. Demonstration of homolog between IS6110 of *Mycobacterium tuberculosis* and DNAs of other Mycobacterium spp. J Clin Microbiol. 33: 2290-2293.
12. Kim BJ, Lee SH, Lyu MA, Kim SJ, Bai GH, Kim SJ, Chae GT, Kim EC, Cha CY, Kook YH, 1999. Identification of Mycobacterial species by Comparative Sequence Analysis of the RNA Polymerase Gene (rpoB). Journal of Clinical Microbiology, p.1714-1720
13. I Wayan Agus Putra, Eddy Surjanto, Suradi, Tjandra Yoga Aditama, 2008. Nilai Diagnostik Pemeriksaan Reaksi Rantai Polimerase Pada Tuberkulosis Paru Sputum Basil Tahan Asam Negatif. J Respir Indo Vol 28; no. 3
14. Kim BJ, Hong SK, Lee KH, Yun YJ, Kim EC, Park YG, Bai GH, Kook YH, 2004. Differential Identification of Mycobacterium Complex and Nontuberculous Mycobacteria by Duplex PCR assay Using the RNA Polymerase Gene (rpoB). Journal of Clinical Microbiology 42:1308-1312.
15. Lee H, Bang HE, Bai GH, Cho SN, 2003. Novel Polymorphic Region of the rpoB Gene Containing Mycobacterium Spesies-Specific Sequences and Its Use in Identification of Mycobacteria. Journal of Clinical Microbiology, p 2213-18.
16. Mertaniasih NM, Wiqoyah N, Kuntaman, Wahyunitisari MR, 2010. Metode Polymerase Chain Reaction (PCR) Regio Gen gyr B-*Mycobacterium tuberculosis* untuk Penegakkan Diagnosis Tuberkulosis secara Akurat dan Cepat.
17. Mokkadas E, Ahmad S, 2007. Development and Evaluation of a Multiplex PCR for Rapid Detection and Differentiation of *Mycobacterium tuberculosis* Complex Members from Non-Tuberculous Mycobacteria. Jpn.J.Infect.Dis., 60: 140-144.
18. Muhammad Asa ad Maidin, 2005. Harapan dan tantangan aplikasi reaksi rantai polymerase (PCR) multipleks dalam pemberantasan TB paru di Indonesia. Suplemen 26: 19-28
19. Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA, 2002. Medical Microbiology. 4th ed. Mosby Inc, St. Louise, Missouri, USA.366-368
20. Parekh KM, Inamdar V, Jog A, Kar A, 2006. A Comparative Study of The Diagnosis of Pulmonary Tuberculosis Using Conventional Tools and Polymerase Chain Reaction. Indian Journal of Tuberculosis; 53:69-76
21. Piere C, Lecossier D, Bonssongant Y, Boucart D, Joly V, Yeni P, Hence AJ, 1991. Use of reamplification protocol improve sensitivity of detection of *Mycobacterium tuberculosis* in clinical samples by amplification of DNA. J. Clin. Microbiol. 28: 712-717.
22. Rish JA, Eisenach KD, Cave MD, Reddy MV, Gangadharam PRJ, Bates JH, 1996. Polymerase chain reaction detection of *Mycobacterium*

tuberculosis in formalin-fixed tissue. Am J Respir Crit Care Med.153: 1419-1423.
23. Sjobring U, Mecklenburg M, Andersen AB, Miorner H, 1990. Polymerase chain reaction for detection of *Mycobacterium tuberculosis*. J Clin Microbiol. 28: 2200-2204.
24. Sulistyaningsih E, 2007. Polymerase Chain Reaction (PCR): Era Baru Diagnosis dan Manajemen Penyakit Infeksi. Biomedis, Juni Vol. 1 No. 1
25. Thabrani Z, Aditama TY, Dawud Y, Jusuf A, Prasetyo S, Liuswa, 2001. Pemeriksaan Reaksi Rantai Polimerase dan hubungannya dengan mikroskopis BTA dan biakan konvensional pada penderita tuberculosis paru di RSUP Persahabatan. J Respir Indon; 21: 56-65
26. Thiery D, Brisson-Noel A, Vincent-Levy-Frebault V, Nguyen S, Guesdon J, Gicquei B, 1990. Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS6110, and its application in diagnosis. J. Clin. Microbiol. 28:2668-2673
27. Wards BJ, Collins DM, De Lisle GW, 1995. Detection of Mycobacterium bovis in tissues by polymerase chain reaction. Veterinary Microbiology, 43, 227-240