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Monitoring microbiological response to antituberculosis therapy by Real-Time PCR

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Abstract: Background: *M. tuberculosis* grows slowly and requires several weeks to detect in clinical specimens using standard culture techniques. Messenger RNA is more rapidly destroyed in cells than rRNA or genomic DNA, assays that target mycobacterial mRNA better reflect mycobacterial viability. Therefore we evaluated performance of mRNA for monitoring response to antituberculosis therapy using Real-Time PCR. Methods: Sputum specimens from 29 tuberculosis patients confirmed by positive culture were included in this study. Sputum specimens were collected before therapy, at 1st week, 4th week, 8th week and 16th week after initiating antituberculosis therapy to evaluate acid-fast bacilli (AFB), culture and mRNA level. Results: All 29 specimens were positive for culture and mRNA before initiating tuberculosis chemotherapy. Within 29 samples confirmed by positive culture, only 22 (75.9%) patients were positive by AFB. After 8 and 16 weeks of therapy, 27 (93.1%) and 28 (96.5%) were negative by culture, 26 (89.6%) and 28 (96.5%) were negative results for mRNA, and 15 (51.7%) and 29 (100%) were negative results for AFB. Conclusion: Rapidly decline of mRNA level correlated with rapid culture clearance after anti-tuberculosis therapy.

Keywords: *M. tuberculosis*, mRNA, Real-Time PCR

1. Introduction

Tuberculosis is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*) [1]. Tuberculosis continues to be a leading cause of mortality and morbidity worldwide [2; 3]. Despite continuous effort in the prevention, vaccination, monitoring and treatment of tuberculosis, the disease remains a major health problem in many countries particularly in developing countries including Indonesia [4; 5; 6; 7]. In 2012, an estimated 8.6 million people developed tuberculosis and 1.3 million died from the disease. Of the 8.6 million incident cases of tuberculosis estimated to have occurred; only 5.7 million were both detected and notified to national tuberculosisprogrammes or

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1. Introduction

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national surveillance systems. About 3 million people with tuberculosis were not diagnosed or because they were diagnosed but not reported [1; 8].

Mycobacterium tuberculosis grows slowly and requires several weeks to detect in clinical specimens using standard culture techniques. This may delay the microbiologic diagnosis of tuberculosis as compared to most other bacterial infections [9]. The conventional laboratory tests for the diagnosis of tuberculosis, which have been used for decades, are sputum smear microscopy and culture. Diagnosis based on culture is the reference standard but results take weeks to obtain [1; 10]. Detection of mycobacteria species by culture and microscopic methods remains difficult and time consuming therefore a fast and reliable diagnosis of tuberculosis would greatly improve the control of the disease [11]. Polymerase chain reaction (PCR) has proven to be a useful technique in the diagnosis of tuberculosis infection. Various researchers have recently described the rapid detection of *M. tuberculosis* by PCR, and many have reported a high degree of sensitivity in detecting *M. tuberculosis* in clinical samples by means of DNA amplifications [11; 12]. The problem with any PCR method is the risk of obtaining false-positive results due to contamination of clinical specimens with *M. tuberculosis* DNA product from laboratory [2; 13].

Since the discovery of messenger RNA (mRNA) as the intermediary in the transfer of genetic information from DNA to protein, it has been recognized that mRNAs tend to be highly unstable in the cell [14]. The relative stability of mRNAs contrasts with of other nucleic acid species in the cell, such as DNA, transfer RNA, and ribosomal RNA [15; 16]. Because the half-life of bacterial mRNA is extremely short compared to rRNA or genomic DNA, assays that target mycobacterial mRNA better reflect mycobacterial viability [17; 14]. The ability of mRNA-based assays to distinguish viable from nonviable organisms suggests that such assays should also be useful in monitoring the efficacy of anti tuberculosis therapy [9; 18; 19]. We have conducted a prospective study to detect *M.tuberculosis* mRNA from sputum specimens. We evaluated performance of mRNA for monitoring response to antituberculosis therapy using Real-Time PCR.

2. Methods

2.1. Sample Collection

Two hundred and fourty-three sputum samples from suspected tuberculosis patients were obtained from several primary health cares in Samarinda East Kalimantan from 2011 to 2013. Microscopy and culture assay were performed according to the standard diagnostic methods employed at the Department of Medical Microbiology and Molecular Biology Laboratory in MulawarmanUniversity, Samarinda, Indonesia. Sputum specimens were collected before therapy, at 1st week, 4th week, 8th week and 16th week after initiating anti tuberculosis therapy. Standard Ziehl-Neelsen techniques using carbolfushin stain were used to detect acid-fast bacilli (AFB) in sputum smears. For mycobacterial culture and Real-Time PCR for mRNA, sputum specimens were decontaminated with *N*-acetyl-L-cysteine (NALC). Treated specimens were incubated on Lowenstein-Jensen medium for 8 weeks at 37°C [9].

2.2. Specimen Processing

Sputum was stored at 4° C for no longger than 24 hour and was then homogenized by adding 1 ml of 2.5% NALC dissolve in phosphate buffer (67mM, pH 6.8) and vortex mixing with 4-mm glass beads. Specimens were divided into aliquots of 0.5ml of homogenate and added 100µl to lysis buffer L6 (50mM Tris-HCl, 5.25M GuSCN, 20mM EDTA, 0.1% Triton X-100) then frozen at -20°C until nucleic acid extraction described by Hatta et al. A separate aliquot of homogenized sputum was further processed with 2% NaOH-sodium citrate for microscopy and culture [20].

2.3. Nucleic Acid Isolation

Nucleic acid was extracted from homogenized sputum according to the diatom guanidiniumisothiocyanate (GuSCN) method described by Hatta et al [22; 23]. Homogenized sputum was mixed with 900µl of lysis buffer L6 (50mM Tris-HCl, 5.25M GuSCN, 20mM EDTA, 0.1% Triton X-100), vortexed vigorously, and centrifuged at 1,000 rpm for 5 min. To obtain the nucleic acid, samples were lysed by incubation for 15 minutes at 18°C and 20µl of diatom suspension was added. The diatom containing the bound nucleic acid was centrifuged at 12,000 x g for 15 seconds to obtain diatom pellet. The diatom pellet was then washed with washing buffer L2 (5.25M GuSCN in 0.1M Tris-HCl, pH 6.4), rinsed with 70% ethanol and acetone, and dried by incubation at 56°C for 10 minutes. The pellet was mixed with 60µl of 10 mMTris- HCl, pH 8.0, 1 mM EDTA buffer and the nucleic acid was eluted by incubation at 56°C for 10 minutes. After sedimentation of the diatom by centrifugation, the supernatant was collected and stored at -20°C until Real-Time PCR was performed [4].

2.4. Real-Time mRNA RT-PCR Assay

A Real-Time assay targeting the *M. tuberculosis*-specific antigen 85B gene was used. Inbrief, 25µl reaction mixtures containing 5µl extracted total nucleic acids, 0.5µM each primer was mixed with 25µl SYBR® Green for One-Step RT-PCR 2x Master Mix (Applied Biosystems). Reaction conditions were designed as follows: RT at 48°C for 30 min,initial denaturation at 95°C for 10 min, and 40 cycles of denaturation (95°C for 15s) and annealing/extension (60°C for 1 min). Primers (MTB-85B-693F: 5'-CGA CCC TACGCA GCA GAT C-3'; MTB-85B-758R: 5'-TTC CCG CAA TAA ACC CAT AGC-3') [7]. In this study, we were using an ABI PRISM 7300 real time PCR for mRNA quantification.

reported a high degree of sensitivity in detecting *M. tuberculosis* in clinical samples by means of DNA amplifications [11; 12]. The problem with any PCR method is the risk of obtaining false-positive results due to contamination of clinical specimens with *M. tuberculosis* DNA product from the PCR laboratory [2; 13]. The results of this study show that 85B mRNA rapidly decline in response to anti-tuberculosis chemotherapy (figure 1). The major drawback in PCR technique is that it detects the genome of both the live and dead *M. tuberculosis* from clinical specimen whereas the RT-PCR targeting the mRNA of *M. tuberculosis* detects only the live organism in the clinical specimen. RNA-based test is likely to reflect only nucleic acid from living organisms since the turnover rate of RNA, particularly mRNA [25]. The results of this study showed that Real-Time PCR technique using Primers (MTB-85B-693F: 5'-CGA CCC TAC GCA GCA GAT C-3'; MTB-85B-758R: 5'-TTC CCG CAA TAA ACC CAT AGC-3') was similar to the previous study, Jou et al reported that of mRNA sensitivity were 100% [26] while Negi et al. showed a sensitivity of 94.67% [27]. In a Real-Time reverse transcriptase PCR study by Mdivani et al showed a diagnostic sensitivity of 85.2% and specificity of 88.6% were reported. We conclude that mRNA RT-PCR assay may provide a rapid tool for monitoring anti-tuberculosis efficacy.

References

[1] WHO: Global Tuberculosis Report 2013. WHO Press 2013: http://www.who.int/tb/publications/global_report/en/.

[2] Therese, L. K., Gayathri, R., Dhanurekha, L., Sridhar, R., Meenakshi, N., Madhavan, H. N., et al.: Detection of Mycobacterium tuberculosis directly from sputum specimens & phenotypic drug resistance pattern of M. tuberculosis isolates from suspected tuberculosis patients in Chennai. Indian J Med Res 2012, (135):778-782.

[3] WHO: Global tuberculosis control - surveillance, planning, financing. 2008.

[4] Hatta, M., Sultan, A. R., Dwiyanti, R., Sabir, M., Febrianty, A., Adhyka, A., et al: The Reproducibility of the multiplex RAPD-PCR assay in genotyping of Mycobacterium tuberculosis isolates from Sulawesi, Indonesia. American Journal of Clinical and Experimental Medicine, 2014, 2(2):14-21.

[5] Caminero JA: Multidrug-resistant tuberculosis: epidemiology, risk factors and case finding. Int J Tuberc Lung Dis 2010, 14(4):382-390.

[6] Golub JE, Mohan CI, Comstock GW, and Chaisson RE: Active case finding of tuberculosis: historical perspective and future prospects. Int J Tuberc Lung Dis 2005, 9(11):1183-1203.

[7] Barkan, D., Liu, Z., Sacchettini, J. C., & Glickman, M. S: Mycolic Acid Cyclopropanation is Essential for Viability, Drug Resistance, and Cell Wall Integrity of Mycobacterium tuberculosis. Chemistry & Biology 2009, 16:499–509.

[8] WHO: Countdown to 2015 Global Tuberculosis Report 2013 Supplement. Geneva: WHO Press 2013.

[9] Mdivani, N., Li, H., Akhalaia, M., Gegia, M., Goginashvili, L., Kernodle, D. S., et al.: Monitoring Therapeutic Efficacy by Real-Time Mycobacterium tuberculosis mRNA Detection in Sputum. ClinChem 2010, 55(9):1694–1700.

[10] Noodhoek GT, Kolk AHJ, Bjune D, and Catty JW: Sensitivity and specificity of PCR for detection of Mycobacterium tuberculosis: a blind comparison study among seven laboratories. Fine PEM 1994, 32:277-284.

[11] Hatta M, Sultan AR, Tandirogang N, Masjudi, Yadi: Detection and identification of mycobacteria in sputum from suspected tuberculosis patients. BMC Res Notes 2010, 3:72.

[12] Kox LFF, Leeuwen van J, Kuijper S, Jansen HM, Kolk AHJ: PCR assay bases on DNA coding for 16S rRNA for detection and identification of mycobacteria in clinical samples. J Clin. Microbiol 1995, 33:3225-3233.

[13] Desjardin, L. E., Perkins, M., Teixeira, L., Cave, M. D., & Eisenach, K. D: Alkaline Decontamination of Sputum Specimens Adversely Affects Stability of Mycobacterial mRNA. Journal of Clinical Microbiology 1996, 34(10):2435–2439.

[14] Rustad, Tige R., Minch, Kyle J., Brabant, William., Winkler, Jessica K., Reiss, David J., Baliga, Nitin S., Sherman, David R: Global analysis of mRNA stability in Mycobacterium tuberculosis. Nucleic Acids Research 2013, 41(1):509–517.

[15] Jain, C: Review Article: Degradation of mRNA in Escherichia coli. IUBMB Life 2002, 54:315–321.

[16] Moore, D. F., Curry, J. I., Knott, C. A., & Jonas, V: Amplification of rRNA for Assessment of Treatment response of Pulmonary Tuberculosis Patients during Antimicrobial Therapy. Journal of Clinical Microbiology 1996, 34(7):1745–1749.

[17] Fenhalls G, Stevens L, Moses L, Bezuidenhout J, Betts JC, HeldenPv P: In situ detection of Mycobacterium tuberculosis transcripts in human lung granulomas reveals differential gene expression in necrotic lesions. Infect Immun. 2002, 70(11):6330–8.

[18] Aellen S, Que YA, Guignard B, Haenni M, Moreillon P: Detection of live and antibiotic-killed bacteria by quantitative Real-Time PCR of specific fragments of rRNA. Antimicrob Agent Chemother 2006, 50(6):1913–20.

[19] L. Li, C. S. Mahan, M. Palaci, L. Horter, L. Loeffelholz, J. L Johnson, R. Dietze, S. M. Debanne, M. L. Joloba, A. Okwera, W. H. Boom, K. D. Eisenach: Sputum Mycobacterium tuberculosis mRNA as a Marker of Bacteriologic Clearance in response to antituberculosis Therapy. J. Clin. Microbiol 2010, 48(1):46–51.

[20] Desjardin, L. E., Perkins, M. D., Wolski, K., Haun, S., Teixeira, L., Chen, Y: Measurement of Sputum Mycobacterium tuberculosis Messenger RNA as a surrogate for response to chemotherapy. AM J Respir. Crit. Care Med 1999, 160:203-210.

[21] Garg, Sanjay K, Tiwari, R. P, Tiwari, Dileep, Singh, Rupinder, Malhotra, Dolly, Ramnani, V. K, Prasad, G.B.K.S, Chandra, Ramesh, Fraziano, M, Colizzi, V, Bisen, Prakash S: Diagnosis of tuberculosis: Available technologies, limitations, and possibilities.Journal of Clinical Laboratory Analysis 2003,17(5): 155–163.

[22] Jaiswal, Suresh, Prakash, Jay and Sharma, Bhoopendra: Standard Diagnostic Procedure forTuberculosis: A Review. Research and Reviews: A Journal of Life Sciences2013, 1-10.

[23] Kivihya-Ndugga Lydia, van Cleeff Maarten, Juma Ernest, Kimwomi Joseph, Githui Willie, Oskam Linda, SchuitemaAnja, van Soolingen Dick, Nganga Lucy, Kibuga Daniel, Odhiambo Joseph, Klatser Paul: Comparison of PCR with the Routine Procedure for Diagnosis of Tuberculosis in a Population with High Prevalenceof Tuberculosis and Human Immunodeficiency Virus. Journal of Clinical Microbiology 2004, 42(3): 1012–1015

[24] GholoobiAida, Masoudi-KazemabadAli, MeshkatMojtaba, MeshkatZahra: Comparison of Culture and PCR Methods for Diagnosis of Mycobacteriumtuberculosis in Different Clinical Specimens. Jundishapur J Microbiol2014, 7(2): e8939

[25] Von Gabain, A., J. G. Belasco, J. L. Schottel, A. C. Y. Chang, and S. N.Cohen: Decay of mRNA in Escherichia coli: investigation of the fate of specific segments of transcripts. Proc. Natl. Acad. Sci. USA 1983, 80:653-657.

[26] Jou, N.-T., Yoshimori, R. B., Mason, G. R., Louie, J. S., &Liebling, M. R. Single-Tube, Nested, Reverse Transcriptase PCR for Detection of Viable Mycobacterium tuberculosis. Journal of Clinical Microbiology 1997,35(5):1161–1165.

[27] Negi, SS. , Anand, R, Pasha, ST, Gupta, S, Basir, SF, Khare, S, Lal, S. Diagnostic Potential of IS6110, 38KDA, 65KDA, 85B Sequence-Based Polymerase Chain Reaction in the Diagnosis of Mycobacterium tuberculosis in Clinical Samples. Indian Journal of Medical Microbiology 2007, 25(1):43-9