

Comparison of *Mycobacterium tuberculosis* Specific Antigen Stimulation Time for Performing Interferon Gamma mRNA Assay for Detecting Latent Tuberculosis Infection

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The tuberculin skin test (TST) and interferon gamma (IFN- γ) release assay (IGRA) have been widely used for diagnosis of latent tuberculosis infection (LTBI). In order to overcome limitations of current LTBI diagnostic methods, the development of a novel molecular assay which is able to measure the IFN- γ messenger RNA (mRNA) expression level after stimulation with *Mycobacterium tuberculosis* (MTB) specific antigen was recently developed. The ability of a molecular assay to detect MTB infection was similar to commercial IGRA however, the optimal incubation time for stimulating IFN- γ was not yet established. Therefore, in this study the direct comparisons of MTB Ag stimulation times (4 and 24 hrs) were performed for diagnosis of MTB infection. Data showed that the coincident rate between QFT-GIT IFN- γ ELISA and IFN- γ RT-PCR (4 hrs) was 88.35% and that of QFT-GIT and IFN- γ RT-PCR (24 hrs) was 70.85%. Based on a receiver operating characteristic (ROC) curve, the 4 hrs-MTB specific Ag stimulation time for IFN- γ RT-PCR had the significant *P* value, 95% CI value, and AUC (*P* < 0.0001, 95% CI=0.82 to 1.02, and AUC=0.9214) in comparison with 24 hrs-MTB specific Ag stimulation time (*P* = 0.009, 95% CI=0.06 to 0.94, and AUC=0.7711). These results show that 4-hr was the most optimal MTB Ag stimulation time for performing IFN- γ RT-PCR. Although semi-quantitative RT-PCR had a few analytical limitations, it might be useful as an alternative molecular diagnostic method for detecting MTB infection.

Key Words: *Mycobacterium tuberculosis* (MTB), Latent tuberculosis infection (LTBI), RT-PCR, MTB specific Ag stimulation time, IFN- γ mRNA

INTRODUCTION

Tuberculosis (TB) is mainly caused by *Mycobacterium*

tuberculosis (MTB) and remains a major infectious disease because it causes the highest mortality among the infectious diseases (Lier EA et al, 2007). Global TB control has been difficult because it is usually transmitted by airborne infection and recent globalization of the world population. According to the WHO report, therefore, one third of world population has infected with MTB (WHO, 2010). In the past, outbreaks of TB was mainly occurred in developing countries however, the TB occurrence in developed countries is increasing because of the increase of HIV co-infection, elderly population,

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and immunosuppressive therapy. Those factors elevate the possibility to develop the active TB disease.

Latent TB infection (LTBI) is a state in which MTB persists within its host without causing clinical symptom, and signs with acid fast bacilli (AFB) stain negative and chest X-ray normality (Mack U et al., 2009; Hanif E et al., 2012). Latently infected population has a potential risk of developing active disease according to the hosts' immune or nutritional condition in their lifetime, therefore, accurate diagnosis and therapy of LTBI might be the most important thing to control TB in developed countries (Comstock GW et al., 1974; Hong JY et al., 2012).

There are two available LTBI diagnostic methods; tuberculin skin test (TST) and interferon gamma release assay (IGRA), however, there is no gold standard diagnostic method for detecting LTBI. TST measures the hosts' delayed-type hypersensitivity after injection of MTB purified protein derivative (PPD). It has been widely used for over 200 years with inexpensive cost, however, it has shown false-positive results in NTM exposed and BCG vaccinated populations (Pouchot J et al., 1997). IGRA assesses the presence of MTB infection by detecting the *ex vivo* release of IFN- γ following stimulation with MTB-specific antigens (Ags), such as early secretory Ag target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) which are strong targets of T-cells in MTB infection, but also absence in all BCG strains and the major environmental NTM (Lalvani A., 2001).

IGRA uses IFN- γ monoclonal antibody and MTB-specific Ags. The cost of IGRA is expensive compared to TST, but the use of IGRA is recently increasing because the specificity and sensitivity were significantly higher in IGRA (Sester M et al., 2011; Diel R et al., 2010). However, it has shown several limitations. For instance, they are labor-intensive, sensitivity limitation, and IFN- γ ELISA (except IFN- γ ELISPOT) which does not consider the number of T cells contained in whole blood (Mandalakas AM et al., 2008). In order to overcome limitations of IGRA, a molecular diagnostic assay using IFN- γ mRNA real-time RT-PCR was developed (Kim et al., 2013). According to data from the

previous study, MTB specific Ag stimulation time could be shortened from 24 hr to 4 hr for performing real-time RT-PCR targeting IFN- γ mRNA. Additionally, the sensitivity and specificity of the molecular assay was similar to commercial IGRA (Kim et al., 2012). However, it has not shown a direct comparison of Ag stimulation time between 4 and 24 hr for IFN- γ molecular assay.

In this study, RT-PCR targeting IFN- γ mRNA was performed with whole blood samples from active TB patients and a non-TB group. For IFN- γ mRNA RT-PCR, whole blood was stimulated with MTB-specific Ags for 4 and 24 hr (two time point) and, the results of IFN- γ mRNA RT-PCR were compared with commercial IGRA (QFT-GIT) and clinical diagnosis.

MATERIALS AND METHODS

Study subjects

This study was carried out since May 2010 until Aug 2010 at Yonsei University Wonju Severance Christian Hospital with 39 study subjects (12 active TB and 27 non-TB) who have clinical symptoms of any infectious disease. Whole blood used in this study was collected from 39 study subjects. All subjects provided written informed consent, and the study was approved by the Institutional Ethics Committee of Yonsei University Wonju College of Medicine (approval no. 2010-21).

T-cell mitogen (PHA) and MTB specific Ag stimulation

Ag stimulation was performed using the commercial Ag tubes, QFT-GIT test (Cellestics, Vic, Australia), according to the manufacturer's instructions (Mori T et al., 2004; Harada N et al., 2008). Blood samples were collected into three QFT-GIT collection tubes (Nil, MTB specific Ags, and Mitogen). For IFN- γ mRNA RT-PCR, Ag stimulation was performed for both 4 hr and 24 hr at 37°C. For QFT-GIT IFN- γ ELISA, stimulation was performed for 16 to 24 hr at 37°C. Plasma was collected by centrifugation at 2,000 xg for 5 min and stored at -80°C until it was assayed QFT-Gold ELISA.

IFN- γ ELISA(QuantifERON-TB Gold In-tube test)

IFN- γ concentrations in plasma samples from each subject were determined using the QFT-GIT ELISA test according to the procedure enclosed in the product insert. The ELISA assay was carried out by trained staff at Yonsei University Wonju Severance Christian Hospital, Wonju, Republic of Korea. The test results were interpreted using QFT-GIT ELISA software (version no. 2.43; Cellestis Ltd., Vic, Australia) and the cut-offs for diagnosis in the manufacturer's instructions were used. Mitogen stimulation served as an intrinsic control for blood sample quality. An IFN- γ concentration of ≥ 0.35 IU/ml (after subtraction of nil control IFN- γ), following exposure to MTB Ags was considered positive for QFT-GIT; a concentration of < 0.35 IU/ml was considered negative. If the IFN- γ response to mitogen was < 0.5 IU/ml higher than that for the nil control, or > 8 IU/ml higher than that for the nil control, the result was deemed indeterminate (Mori T et al., 2004).

Total RNA isolation and cDNA Synthesis (reverse transcription)

After Ag stimulation, all RNA in the whole blood sample was isolated by the column and centrifugation. It needs red blood cells (RBCs) lysis prior to white blood cells (WBCs) lysis. Shortly, 3 volumes of RBC lysis buffer (Qiagen, Amsterdam, Netherland) were added to 1 volume of blood cell pellet and incubate on ice for 10 to 15 min. After incubation, samples centrifuged at 400 xg for 10 min at 4°C. After removal of supernatant, 2 volumes of RBC lysis buffer were added to remaining blood cell pellet and mixed very well. Samples centrifuged at 400 xg for 10 min at 4°C and removed the supernatant completely. After removal of RBC, the total RNA from WBCs was isolated with column-based commercial total RNA kits, QIAamp RNA Blood Mini kit (Qiagen, Amsterdam, Netherland) and easy-spin Total RNA Extraction kit (Intron Biotechnology, Seoul, Republic of Korea), were used according with manufacturer's recommendation.

Complementary DNA (cDNA) was synthesized by

M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random hexamers (Invitrogen, Carlsbad, CA, USA) according with manufacturer's recommendations.

Reverse transcription PCR (RT-PCR)

For amplification of IFN- γ target gene and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) endogenous control gene, the amplification mixture contained primers (10 pmol/ μ l each), 1.5 units of *Taq* DNA polymerase (SolGent, Daejeon, Republic of Korea), 2 mM of MgCl₂, 250 μ M dNTP, and 3 μ l of cDNA in a final volume 20 μ l.

RT-PCR condition to amplify the IFN- γ gene consisted of an initial denaturation step of 5 min at 95°C ; 35 cycles comprising 30 sec at 95°C denaturation, 30 sec at 55°C primer annealing, and 30 sec at 72°C extension; and a final extension at 72°C for 7 min. RT-PCR condition to amplify the GAPDH gene consisted of an initial denaturation step of 5 min at 95°C; 23 cycles comprising 30 sec at 95°C denaturation, 30 sec at 55°C primer annealing, and 30 sec at 72°C extension; and a final extension at 72°C for 7 min.

Semi-quantitaion of PCR products

Following PCR amplification of IFN- γ and GAPDH gene, amplified PCR products were analyzed by 1.8% Tris-borate-ethylenediamine-tetraacetic acid disodium salt dehydrate (TBE) agarose gel electrophoresis and ethidium bromide (EtBr) staining. Stained gels were placed on the UV transilluminator (302 nm) and photographed with Molecular Image[®] Gel Doc[™] XR⁺ system (Bio-Rad, Hercules, CA, USA) then the results were analyzed by densitometer analyzing system, Quantity-one software (Bio-Rad, Hercules, CA, USA). The expression level of the IFN- γ gene was compensated with endogenous reference gene, GAPDH. The expression ratio (R) of target gene (IFN- γ) relative to a reference endogenous gene (GAPDH) in a MTB Ag control relative to nil control was calculated by following equation (Tetsuro H et al., 2000).

$$R = \text{density of target gene (MTB Ag - Nil)} / \text{density of endogenous control (MTB Ag - Nil)}$$

Statistical analysis

Data were analyzed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). Positivities and negativities for MTB infection, together with 95% confidence intervals (CIs), were calculated as proportions of positive and negative cases among active TB patients and non-TB (healthy) control subjects, respectively. Nonparametric statistics, Spearman's ranked correlation coefficient was employed. When testing for differences, a value of $P=0.05$ was used as the criterion for statistical significance.

RESULTS

Baseline characteristics of study subjects

A total of 39 study subjects were enrolled for the study. They were composed of 12 active TB patients and 27 non-TB subjects. The median age of active TB patients was 70 years (range 32-79) and that of non-TB subjects was 55 years (range 15-84). The male-to-female

Table 1. Baseline characteristics of study subjects

	Active TB	Non-TB
No. of subjects	12	27
Median age (Range)	70 (32~79)	55 (15~84)
M/F ratio	6 : 6	11 : 15

TB = tuberculosis; M = male; F = female.

rates were 6 : 6 and 11:15 for the active TB patients and non-TB subjects, respectively (Table 1).

Results of IFN- γ ELISA (QFT-GIT)

In order to measure the IFN- γ protein level after stimulation of MTB specific Ags, 3 ml of whole blood was collected from each study subject and, blood was treated with MTB specific Ags using commercial MTB specific Ag coated tubes, QFT-GIT test (Cellestics, Vic, Australia) for 16 to 24 hr. Ag treated blood was centrifuged (400 xg) and then plasma was used for performing ELISA to quantitate the IFN- γ protein level. The cut-off value (TB Ag control - nil control = 0.35 IU/ml) for this study was established by manufacturer's recommendation. Of 12 active TB patients, 11 (91.7%) were positives (0.35 to 25.41 IU/ml) and 1 (8.3%) was negative (-1.02 IU/ml) in QFT-GIT. Of 27 non-TB subjects group, 10 (37.0%) were positives (0.44 to 14.99 IU/ml), 4 (14.8%) were indeterminates (-15.00 to -0.06 IU/ml), and 13 (48.2%) were negatives (-2.04 to 0.07 IU/ml) in QFT-GIT (Table 2).

IFN- γ mRNA RT-PCR results between the blood samples from active TB patients and non-TB subjects group

In order to quantitate the IFN- γ mRNA expression levels after stimulation of MTB specific Ags, 3 ml of whole blood was collected from each study subject and blood was treated with MTB specific Ags using commercial MTB specific Ag coated tubes, QFT-GIT test for 4 hr. Ag treated blood was centrifuged (400 xg) and

Table 2. QFT-GIT results of study subjects

Study subjects (n)	Results of QFT-GIT (IGRA)		
	Positive, n (%)	Negative, n (%)	Indeterminate, n (%)
Active TB (12)	11 (91.7%)	1 (8.3%)	0 (0.0%)
Non-TB (27)	10 (37.0)	13 (48.2%)	4 (14.8%)

QFT-GIT = QuantiFERON-TB Gold In-tube test.

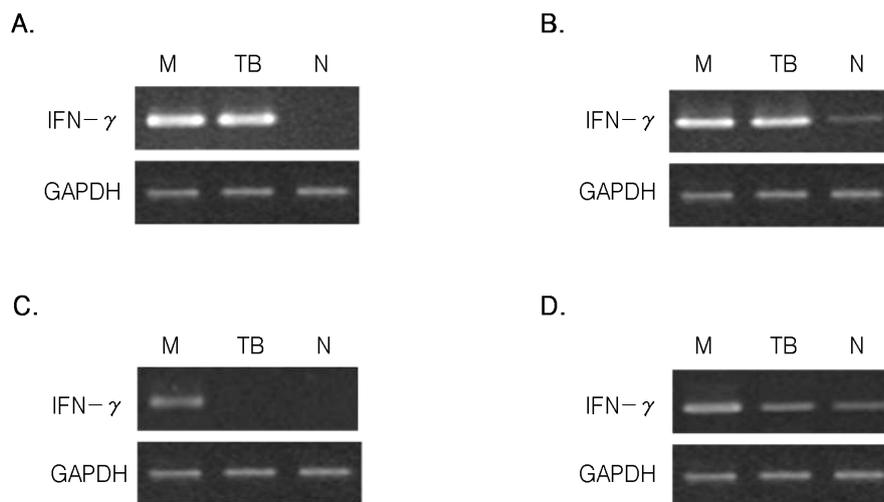


Fig. 1. The IFN- γ mRNA RT-PCR results in active TB patients blood samples (A, B) and non-TB subjects blood samples (C, D).

then blood cell pellets were used for performing IFN- γ mRNA RT-PCR. Separated blood cell pellets were lysed to extract total RNA using QIAamp RNA Blood Mini kit (Qiagen, Amsterdam, Netherland) and easy-spin Total RNA Extraction kit (Intron Biotechnology, Seoul, Republic of Korea), then extracted total RNA was used for reverse transcription using M-MLV RT and random hexamers (Invitrogen, Carlsbad, CA, USA). After performing the IFN- γ mRNA RT-PCR, the IFN- γ mRNA expression patterns between in active TB patients' and non TB (healthy) subjects' blood samples were compared (Fig. 1). The IFN- γ mRNA expression levels of MTB Ag control relative to nil control in active TB patients blood samples (Fig. 1-A, B) were significantly higher than that of non-TB subjects' blood samples (Fig. 1-C, D).

Comparison of the correlation coefficient analysis between IFN- γ mRNA expression and protein level (4 hr vs. 24 hr)

To investigate the correlation between IFN- γ mRNA expression (4 and 24 hr) and protein levels (24 hr) after stimulation with MTB specific Ags (ESAT-6, CFP-10 and TB7.7), the IFN- γ mRNA expression levels were compared to IFN- γ protein levels in each sample. The IFN- γ mRNA expression levels were measured by RT-PCR after MTB specific Ag stimulation for both 4 and 24 hr and, IFN- γ protein levels were measured by IFN- γ ELISA after MTB specific Ag stimulation for 24

hr. The correlation coefficient between IFN- γ mRNA expression and IFN- γ protein level was analyzed by Spearman correlation coefficient test. The Spearman r and p value between IFN- γ mRNA expression (4 hr) and protein levels (24 hr) was 0.7 and <0.0001 respectively (Fig. 2-A). The Spearman r and P value between IFN- γ mRNA expression (24 hr) and protein levels (24 hr) was 0.4 and 0.0097 (Fig. 2-B). These results show that the correlation between IFN- γ mRNA expression (4 and 24 hr) and protein levels (24 hr) after MTB specific Ag stimulation was statistically significant. However, in order to perform the IFN- γ mRNA RT-PCR, 4 hr was more optimal Ag stimulation time.

Comparison of IFN- γ mRNA RT-PCR (4 and 24 hr) results with IFN- γ ELISA (QFT-IT) results

To compare the IFN- γ mRNA RT-PCR results in different MTB specific Ag stimulation time (4 and 24 hr), IFN- γ mRNA RT-PCR was performed after MTB specific Ag stimulation in two time points (4 and 24 hr) and, the results were compared with commercial IFN- γ assay (QFT-GIT). Among 20 QFT-GIT positive samples, 18 (90.0%) were positive and 2 (10.0%) were negative when performed RT-PCR after Ag stimulation for 4 hr, and 11 (55.0%) were positive and 9 (45.0%) were negative when stimulating with MTB specific Ag for 24 hr. Among 4 QFT-GIT indeterminate samples, 2 (50.0%) were positive and 2 (50.0%) were negative when

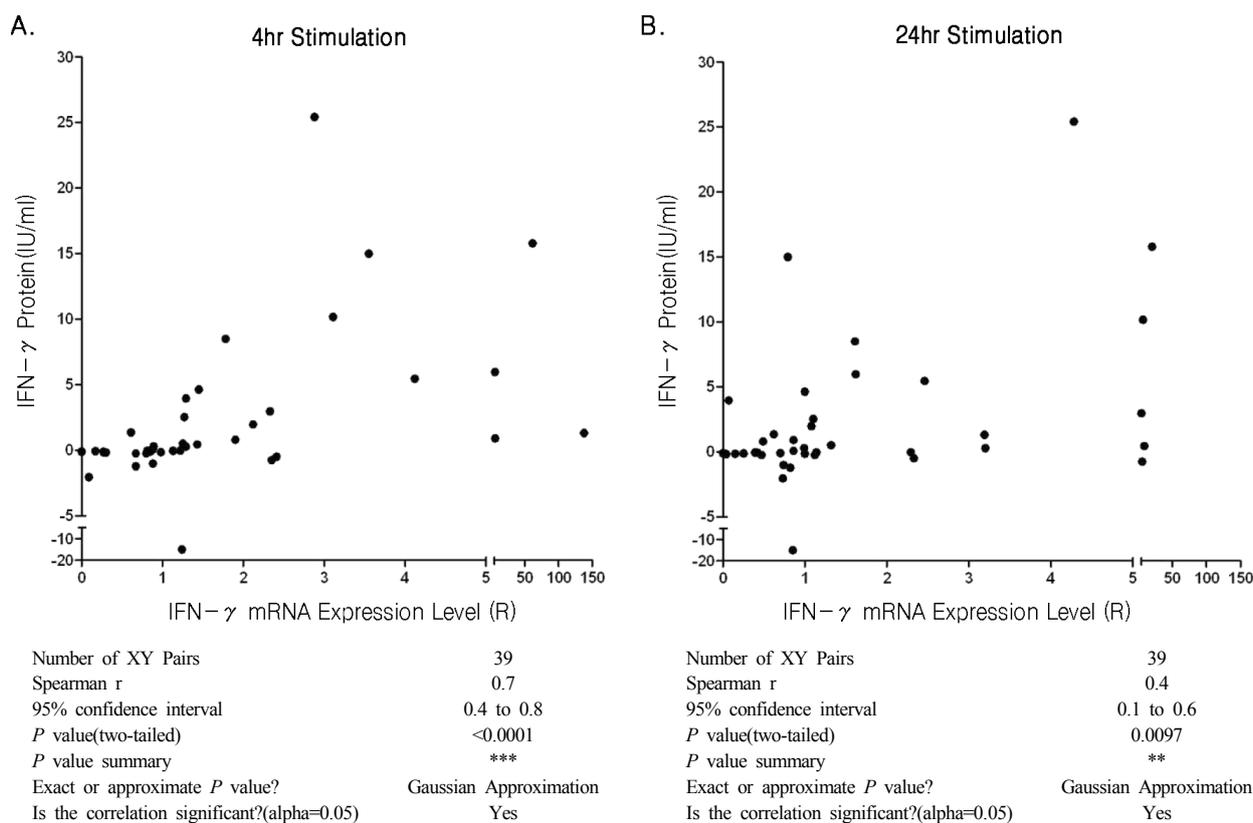


Fig. 2. The correlation coefficient analysis between IFN- γ mRNA expression (4 hr) and protein level (A), and IFN- γ mRNA expression (24 hr) and protein level (B) after stimulation of MTB specific antigens.

Table 2. Comparison of IFN- γ mRNA RT-PCR results with QFT-GIT results (QFT-GIT positive, QFT-GIT indeterminate, QFT-GIT negative) according to the MTB specific antigen stimulation time (4 and 24 hr).

IFN- γ ELISA (QFT-GIT)	No. of cases	IFN- γ RT-PCR (4 hr)		IFN- γ RT-PCR (24 hr)	
		Results	No. of detection	Results	No. of detection
Positive	20	Positive	18	Positive	11
		Negative	2	Negative	9
Indeterminate	4	Positive	2	Positive	1
		Negative	2	Negative	3
Negative	13	Positive	2	Positive	2
		Negative	12	Negative	13
Total	39		39		39

performing RT-PCR after Ag stimulation for 4, and 1 (25.0%) was positive and 3 (75.0%) were negative when performing RT-PCR after Ag stimulation for 24 hr. Among 15 QFT-GIT negative samples, 2 (13.3%) were

positive and 13 (86.7%) were negative when performing RT-PCR after Ag stimulation for both 4 and 24 hr (Table 3). The coincident rate between QFT-GIT and IFN- γ RT-PCR (4 hr) was 88.4%, and The coincident rate

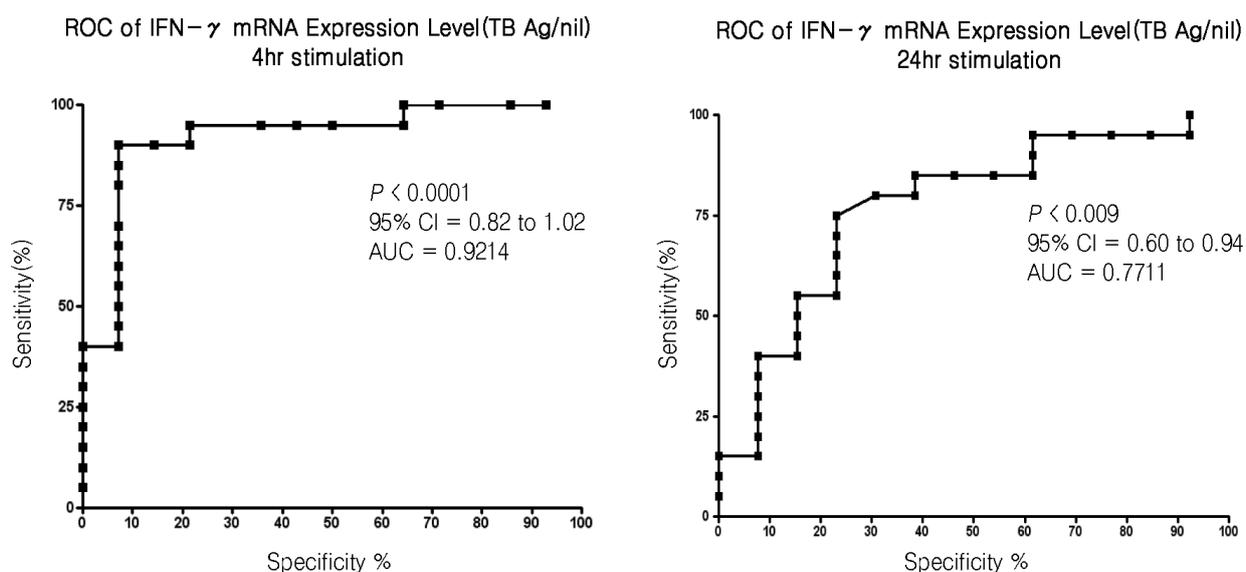


Fig. 3. Comparison of receiver operating characteristic (ROC) curve for IFN- γ mRNA expression levels both at 4 and 24 hr after MTB specific Ag (ESAT-6, CFP-10, TB7.7) stimulation.

between QFT-GIT and IFN- γ RT-PCR (24 hr) was 70.9%. This shows that IFN- γ RT-PCR (4 hr) has the higher coincident rate (17.5%).

To investigate the further statistical significance, receiver operating characteristic (ROC) curve was carried out. The 4 hr MTB specific Ag stimulation time for IFN- γ RT-PCR had the significant P , 95% CI, and AUC value ($P < 0.0001$, 95% CI=0.82 to 1.02, and AUC = 0.9214) than 24 hr MTB specific Ag stimulation time ($P=0.009$, 95% CI=0.60 to 0.94, and AUC = 0.7711) (Fig. 3). These results show that 4 hr stimulation time was the most optimal time for IFN- γ RT-PCR compared with the results with IFN- γ ELISA (QFT-GIT).

DISCUSSION

This study reports that quantitation of IFN- γ mRNA expression level using RT-PCR after MTB specific Ag stimulation at early time point was useful for detecting MTB infection. Although the usefulness of measuring IFN- γ mRNA expression for detecting LTBI was demonstrated from the previous study (Bibova et al., 2012; Kim et al., 2013), there was not a direct comparison between Ag stimulation times for performing

RT-PCR. Therefore, the results of IFN- γ mRNA RT-PCR in different Ag stimulation time points (4 and 24 hr) were compared. Additionally, semi-quantitative RT-PCR was used for measuring IFN- γ mRNA expression level.

Results from this study have shown that when comparing the Ag stimulation times between 4 and 24 hr, the data showed that 4 hr provides more reliable information of MTB infection than 24 hr. The coincident rate with IFN- γ protein ELISA (QFT-GIT) and the statistical values from ROC curve and Spearman correlation test were high in RT-PCR after 4 hr stimulation with MTB specific Ag. These results were identical with the data from the previous study (Kim et al., 2013) which reported that the IFN- γ mRNA expression was measurable at early time point (4 hr). Furthermore, quantitation of IFN- γ mRNA expression with semi-quantitative RT-PCR was available too alike quantitative real-time PCR TaqMan assay. Therefore, it might be useful at a laboratory which does not have real-time PCR instrument. However, it has several limitations for accurate and rapid quantitaion of gene expression. As semi-quantitative RT-PCR is the end-point analysis, gene expression level was measured at after target gene amplification. Furthermore, it might be

more labor intensive than real-time RT-PCR because quantitation of gene expression by semi-quantitative RT-PCR has to be followed agarose gel electrophoresis, gel staining, and densitometer analysis.

Overall, we conclude that optimal MTB Ag stimulation time for IFN- γ mRNA RT-PCR was 4 hr. Although semi-quantitative RT-PCR has several limitations than quantitative real-time PCR, quantitation of IFN- γ mRNA expression by semi-quantitative RT-PCR could be useful for the diagnosis of MTB infection.

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