



The Adjusted Cut-Off Value of Interferon- Γ Release Assays (IGRA) to Diagnose LTBI in Indonesia

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Abstract

Latent Tuberculosis Infection (LTBI) is as a condition when Mycobacterium tuberculosis (MTB) persists in the body without causing symptoms while maintaining viability with the possibility to become active tuberculosis in the future. The detection of LTBI is an important strategy from WHO to end the chain of tuberculosis. LTBI can be diagnosed by using the IGRA test. IGRA uses specific antigens of MTB, early antigenic target-6 (ESAT-6), to induce interferon-gamma production by antigen-specific T cells. In the IGRA test, the cut-off value is explicitly needed to distinguish healthy people from LTBI patients. This study aimed to find IGRA's adjusted cut-off value in Indonesia. The subjects were 150 people, which were 50 healthy people, 50 subjects with active TB, and 50 with suspected LTBI. Isolated Lymphocytes derived from PBMC sand were cultured in a complete RPMI medium and induced by Esat-6 for six days. IFN-g levels were assayed from supernatants by the sandwich Elisa technique. Statistical analysis was used to determine cut-off values for IFN-g derived from healthy and diseased patients. The highest aptitude index (κ value) that could discriminate between healthy and LTBI groups was 0.925 at a concentration of 0.792 ng/ml of IFN-g, hence the cut-off value to diagnose LTBI was 0.792 ng/ml of IFN-g. Based on this value, out of 40 suspected cases of LTBI, up to 17 (42.5%) and 23 without LTBI (57.5%) were found. So, the conclusion was that the adjusted cut-off value for IFN-g to diagnose LTBI in Indonesia was 0.792 ng/ml.

Keywords: Cut off, Adjusted, IGRA, LTBI

1. INTRODUCTION

Tuberculosis (TB) is one of the world's dangerous communicable diseases. In 2020, an estimated 9.9 million people developed TB, and 1.3 million died from the disease (WHO, 2021). Indonesia is among the top 30 countries with the most TB cases, especially with complications or comorbidities. Following exposure to an infectious individual with pulmonary tuberculosis, up to 46% of close contacts become TST-positive (Reichler *et al.*, 2022). 5-10% of these individuals will develop the disease, the majority within the first five years. Most will show the 'latent phase' without clinical symptoms (WHO, 2021).

Latent tuberculosis infection (LTBI) conceptually denotes a state in which Mycobacterium tuberculosis persists within its host without causing symptoms or signs while maintaining viability with the possibility to replicate and cause active tuberculosis. Latent TB is defined by evidence of immunological sensitization by Mycobacterial proteins, and therefore LTBI diagnosis is conducted through evidence immune response has occurred (Esmail, E and Wilkinson, 2013; Fujikawa *et al.*, 2014). The standard test for diagnosis of LTBI is the TST. This involves the intradermal injection of purified protein derivative (PPD), which leads to a delayed-type hypersensitivity response causing a cutaneous induration at the injection site, which peaks at 48-72 h. PPD is a mixture of more than 200 antigens that are also shared by other mycobacteria. A positive TST

indicates previous exposure to *M. tuberculosis* or other non-tuberculous mycobacteria or prior BCG vaccination (Huang *et al.*, 2022; Pelzer *et al.*, 2022). Many studies have shown TST sensitivity is poor in the immunocompromised, and specificity is low that associated with cross-reactivity with non-tuberculous mycobacteria (NTM) and BCG) (Seyhan *et al.*, 2016).

Considering these limitations, many investigations screened LTBI using interferon-gamma release assay (IGRA) rather than the TST. IGRA uses specific antigens of *Mycobacterium tuberculosis*, such as early antigenic target-6 (ESAT-6), culture filtrate protein 10 (CFP-10), and TB 7.7, to stimulate whole blood to induce interferon-gamma production by antigen-specific T cells. Therefore, the IGRA results are not affected by the history of BCG vaccination (Lalvani and Pareek, 2013; Seyhan *et al.*, 2016)). There are two commercially available diagnostic tests incorporating specific antigens: the QuantiFERON-TB gold test (Cellestis Ltd., Australia) and the T-SPOT-TB assay (Oxford Immunotec, UK). Commercial diagnostic tests for latent TB have their own manufacturer's cut-off value, generalized for all nations in the world. Based on this research, the new adjusted cut-off value for IGRA test can be used more accurately because it was derived from Indonesian subject's blood samples.

In this research, we identify the new adjusted cut-off value for IGRA based on groups of patients with tuberculosis and healthy in Indonesia. This value can be used to identify latent TB from the group with suspected LTBI more accurately. The suspected LTBI were the subjects with history of TB's close contact without clinical symptoms and radiological findings.

2. METHODS

Participants

The protocol for this research was approved by the Ethics Committee, Faculty of Medicine, Andalas University. Written consent was obtained from all subjects. Contact investigation of 150 subjects comprising 50 subjects with active TB, suspected latent TB, and Health subjects. Subjects were chosen by inclusion and exclusion criteria. The active TB subjects were patients with Tuberculosis proven by laboratory and radiological findings (beside the history and physical examination). The suspected LTBI were the subjects with history of TB's close contact (at least 3 months) without clinical symptoms and radiological findings. The healthy ones were control group who have no clinical symptoms, laboratory or radiological findings. Blood samples were sent to Biomedical Laboratory in the Faculty of Medicine, Andalas University, for evaluation.

PBMC isolation and ESAT-6 stimulation

Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation of venous blood samples using the ficoll method. They were resuspended at 2×10^6 cells/mL in RPMI media supplemented with 10% FBS and 2% Penicillin - Streptomycin. Per previous standardization, PBMC was stimulated with five ug/ml Esat-6.

The cells were incubated for six days at 37°C under 5% CO₂. The culture supernatants were then collected, and IFN-γ was measured.

Elisa for IFN-γ

IFN-γ was detected in cellular supernatants using standards and ELISA reagents obtained from e-Bioscience (San Diego, USA). IFN-γ level was measured using a sandwich ELISA

technique according to the manufacturer’s instructions. The ELISA technique used recombinant human cytokine to get a dose-response curve with a detection range from 4-500 pg/ml. All experimental samples were tested in duplicates.

Statistical analysis

Data was represented by box blots illustrating the 25th and 75th quartiles, with the median as a horizontal bar. Statistical analysis was performed using One-way ANOVA as required. P values ≤ 0.05 were considered to be statistically significant. Sensitivity, specificity, and Kappa values were also performed, all using the Statistical Package for Social Sciences software (SPSS).

3. RESULT

IFN-γ concentration

In this research, the concentration of IFN-γ derived from patients with active TB was higher than that found in the other groups, namely 1,044 ± 0,23 ng/ml, whereas in the group suspected of LTBI was 0,745 ± 0,28 ng/ml and the healthy group was 0,455 ± 0,20 ng/ml. Statistical analysis showed significant difference levels of IFN-γ between the three groups (p = 0.000). Post hoc analysis with LSD showed differences found between the groups of patients with suspected latent (p = 0.000), patients with healthy (p = 0.000), and healthy with suspected latent (p = 0.000).

Cut off value

Determining the cut-off levels of IFN-γ by lymphocyte cell culture is done by identifying the levels of IFN-γ, which gives the highest kappa score. In this research, the highest kappa score that can distinguish between healthy and diseased groups was 0.925 for an IFN-γ concentration of 0.792 ng/ml, so the level of IFN-γ 0.792 ng/ml was determined as the new adjusted cut-off value.

Table 1. IFN-γ level from PBMCs culture that stimulated with esat-6 in three groups

No	Diagnose	IFN-γ level (ng/ml)	p
1.	Active TB	1,044 ± 0,23	0.000
2.	Healthy subjects	0,745 ± 0,28	
3.	Suspected LTBI	0,455 ± 0,20	

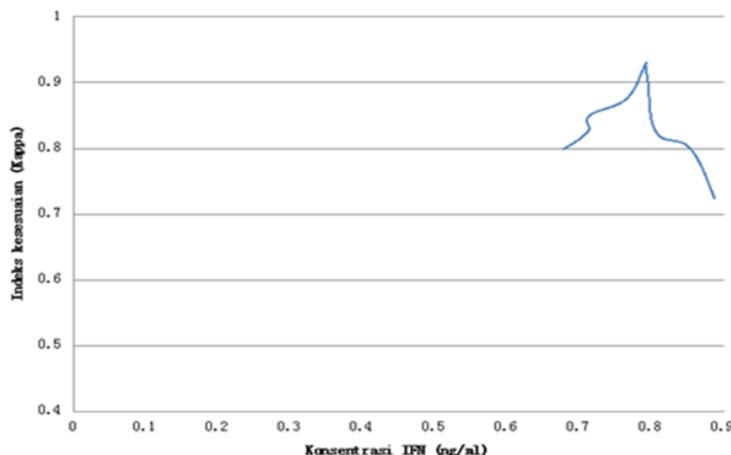


Fig 1. Determination cut-off point based on kappa score

Identification of LTBI

Based on the cut-off value by IFN- γ concentration, we identified 22 (44%) subjects as LTBI and 28 (56%) subjects as not LTBI.

4. DISCUSSION

According to WHO, latent tuberculosis infection (LTBI) is a sustained immune response stimulated by *Mycobacterium tuberculosis* antigens without evidence of clinically active tuberculosis (WHO, 2021). The detection and treatment of LTBI are now essential components of the World Health Organization's Strategy to End TB, especially in developing countries like Indonesia (Kiazyk and Ball, 2017). Latent tuberculosis infection can progress to active tuberculosis or reactivate it. The risk is significantly increased in immunocompromised people, such as HIV patients or other immunodeficiency, children, and people with a history of close contact with someone who has bacteriologically confirmed active TB. The explanation of underlying reasons for the reactivation of LTBI is incomplete, but the theory is based on bacterial, host, and environmental factors (Narasimhan *et al.*, 2013). However, the lifetime risk of reactivation in otherwise healthy individuals with LTBI has been reported to be approximately 5% to 15% (WHO, 2021).

The LTBI screening methods widely used worldwide are The TST (Tuberculin skin test) and the IFN- γ release assay (IGRA). However, based on the latest studies, screening or identification for a person with LTBI is not recommended using Mantoux or tuberculin skin test (TST) because it has many limitations. The most significant limitations are that there may be false-positive results due to exposure to *Mycobacterium* other than tuberculosis (TB), such as *Mycobacterium Bovis*. TST also can come out positive because of BCG vaccination (Pai *et al.*, 2014; He *et al.*, 2022).

In contrast to the TST, the IGRA test is a more specific laboratory in vitro test using Early Secretory Antigenic Target-6 (ESAT-6) and optimized by MTB-specific antigens. ESAT-6 is a specific antigen of the immunodominant *Mycobacterium tuberculosis* (M.tb). Notably, IGRA shows no cross-reactivity with *Bacillus Calmette-Guérin* (BCG) vaccine and non-tuberculous mycobacterial (NTM) infection. IGRA can detect the presence of tuberculosis by measuring interferon- γ (IFN- γ) secretion from lymphocytes in response to ESAT-6 (Burhan *et al.*, 2019; Lalvani and Whitworth, 2019).

The cut-off value of interferon-gamma release assays has been the subject of debate and research worldwide, especially in countries with high TB case rates. A single cut-off value given in the manufacturer's instructions is consistently applied to diagnosing latent tuberculosis infections. However, as IGRA measures antigen-stimulated IFN- γ release in whole blood, the results of these assays are inherently continuous variables. Non-specific variability and reproducibility of continuous data may require adjustment of test results using other cut-off values. Several other studies have also adjusted the T-SPOT or QFT cut-off values to improve the diagnostic accuracy of these two assays. Also, in countries and regions with different rates of tuberculosis infection, the optimal cut-off value may differ from the manufacturer's recommended value. It is necessary to find the optimal cut-off value suitable for such situations (Perneger *et al.*, 2007; Shen *et al.*, 2022).

In this study, we found the cut-off value of IFN- γ to differentiate LTBI patients from healthy people was 0.792 ng/ml. The cut-off value was measured from the concentration of IFN- γ in healthy people, people with LTBI, and patients with active TB after being induced with ESAT-6 as the specific antigen of *Mycobacterium Tuberculosis*. This cut-

off value is the standard point for the response of IFN- γ that can be used to diagnose LTBI in healthy people with increased risks (Zellweger *et al.*, 2020).

A study by Shen *et al.*, (2022) was also about finding adjusted cut off value of IGRA test to increase the sensitivity and specificity of commercial IGRA tests that are already globally widespread, T-SPOT and QFT. Shen *et al.* used receiver operating characteristic (ROC) analysis to evaluate the diagnostic potential of the T-SPOT and QFT assays in diagnosing LTBI and Active TB patient. The adjusted cut off value was needed in order to reduce the false positive rate of IGRA test.

The specificity of IGRA test increased from 90.3% and 94.1%, respectively, and the sensitivity increased 43.1% to 41.6% with the adjusted cut off values, which were 2.5×10^5 cells for T-SPOT and 4.0 IU/ml for QFT. The cut off values in Shen *et al.* study were obtained after carrying out the IGRA test on certain samples with similar characteristics.

The cut off value also needs to be adjusted in pediatric patients who are suspected of having LTBI due to differences in the physical condition and metabolism of children. Research about cut-off point for IGRA test in children with suspected LTBI was also conducted by Anindya (2019), and the study obtained a cut-off of 0.115 for sensitivity of 62.5% and specificity of 75%.

Although there are currently commercial IGRA tests such as T-SPOT and QFT which have their manufacturer's cut-off values, both of the tests had relatively low specificity and sensitivity for detection of LTBI. This is due to the high number of false positive results in these commercial test results in high LTBI endemic countries such as China and Indonesia (Metcalf *et al.*, 2011). Therefore, it is necessary to determine the cut-off value of IGRA for each country with its characteristics so that IGRA examination results become more accurate (Nemes *et al.*, 2019). However, the limitation of this study was that the tests we carried out to measure IGRA's cut off value still used tools and materials that were quite expensive with a long time test duration, so the test still needed to be more effective than commercial tests such as T-SPOT and QFT with their own cut off values given by their manufacturers. The T-SPOT and QFT use practical assay KIT the assay kit (Oxford Immunote Ltd., Oxford, UK), and the results do not take long to come out, making it more efficient to use in screening or diagnosing LTBI (Borge *et al.*, 2019).

5. CONCLUSION

The cut-off value found in this study is 0.792 ng/ml. The cut-off value was obtained after calculating the concentration of IFN- γ from healthy people, LTBI patients, and patients with active TB after being induced with ESAT-6. This cut-off value can be used to screen patients with LTBI. It is expected to be more accurate than commercial tests because it has been adjusted with the characteristics of samples from Indonesian people

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