Use of Pleural Fluid Interferon-gamma Enzyme-linked Immunospot Assay in the Diagnosis of Pleural Tuberculosis

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ABSTRAK

Tujuan: *untuk melakukan penilaian terhadap uji diagnostik pemeriksaan interferon-gamma release assay (IGRA) metode enzyme-linked immunospot (ELISPOT), yaitu T-SPOT.TB, untuk deteksi TB pleura menggunakan spesimen sel mononuklear (MN) cairan pleura.* ***Metode:*** *sebanyak 48 pasien efusi pleura terduga TB dengan karakteristik cairan pleura eksudatif berdasarkan kriteria Light dan dominasi sel MN lebih dari 50% dilakukan pemeriksaan T-SPOT.TB, biakan TB media cair Mycobacterial Growth Indicator Tube (MGIT), dan aktivitas adenosine deaminase (ADA) cairan pleura. Penyebab lain efusi pleura seperti gagal jantung, gagal ginjal, sirosis hati, dan keganasan telah disingkirkan.* ***Hasil:*** *sebanyak 39 dari 48 subjek (81,25%) menderita TB pleura berdasarkan biakan MGIT positif dan/atau ADA lebih dari 40 U/L. Dari jumlah tersebut seluruhnya positif untuk T-SPOT.TB. Hasil uji diagnostik IGRA ELISPOT untuk diagnosis TB pleura adalah sensitivitas 100%, spesifisitas 88,89%, nilai prediksi positif 97,5%, dan nilai prediksi negatif 100%.* ***Kesimpulan:*** *IGRA ELISPOT menggunakan specimen cairan pleura merupakan metode diagnostik yang cepat dan reliabel sehingga bermanfaat untuk diagnosis TB pleura khususnya pada daerah endemis TB.*

Kata kunci: *interferon-γ release assay, ELISPOT, tuberkulosis pleura.*

ABSTRACT

Aim: *to evaluate the diagnostic value of an interferon-gamma release assay (IGRA) with enzyme-linked immunospot (ELISPOT) method, T-SPOT.TB, in the diagnosis of pleural TB using pleural fluid mononuclear cells (PFMC).* ***Methods:*** *forty-eight subjects, presumed to have pleural TB with exudative pleural effusion by Light's criteria, dominated by mononuclear cells, had their pleural fluid specimen tested with T-SPOT. TB, Mycobacterial Growth Indicator Tube (MGIT) culture, and adenosine deaminase (ADA) activity. Other causes of pleural effusion such as heart failure, renal failure, hepatic cirrhosis, and malignancy were excluded.* ***Results:*** *the sensitivity, specificity, positive predictive value, and negative predictive value of the IGRA ELISPOT assay using PFMC for the diagnosis of pleural TB were 100%, 88.89%, 97.5%, and 100%, respectively.* ***Conclusion:*** *IGRA with ELISPOT method performed on PFMC is useful for a rapid and reliable diagnosis of pleural TB in clinical practice, especially in area with high TB burden.*

Keywords: *interferon-γ release assay, ELISPOT, pleural tuberculosis.*

INTRODUCTION

Tuberculosis (TB) remains one of the leading causes of mortality in the world. Although TB is typically a disease of the lungs, which serves both as port of entry and also as the major site of disease manifestation, *Mycobacterium tuberculosis* has the ability to disseminate to various extrapulmonary sites. Tuberculous pleurisy, or pleural TB, is the second most common manifestation of extrapulmonary TB and a common cause of pleural effusion in endemic TB areas. To date, diagnosis of pleural TB relies on either insensitive (acid fast bacilli smears), unspecific (cell count, biochemical levels), or time consuming (culture) methods often leading to defer initiation of therapy. The paucity of bacilli in pleural fluid leads to low sensitivity of direct bacillary detection such as Ziehl-Neelsen staining, culture, and also PCR.[1-3] Therefore, a rapid, accurate diagnostic test is urgently needed for pleural tuberculosis.

In the late 1990s, many studies about the *M. tuberculosis* genome have led to the identification of TB specific antigens. Among all antigens, early secretory antigenic target with 6 kDa molecular weight (ESAT-6) and culture filtrate protein with 10 kDa molecular weight (CFP10), which appear exclusively in *M. tuberculosis*, are the most immunodominant and also virulency determinant of mycobacteria. Other mycobacteria, such as *M. bovis* in Bacillus Calmette-Guerin vaccine and most environmental nontuberculous mycobacteria do not have these specific antigens therefore the use of ESAT-6 and CFP10 are beneficial for TB detection because it is more sensitive and specific.[4]

During active TB, antigen-specific T lymphocytes clonally proliferate and are recruited to the site of active infection. Those cells will release more interferon-gamma (IFN-γ) cytokine after rechallenge with TB specific antigens in vitro. In pleural TB, more CD4+ T lymphocyte subgroups are found in the pleural fluid than in peripheral blood, and also the level of IFN-γ secreted by T lymphocytes in pleural fluid are more than the level of IFN-γ in peripheral blood.[5]

Until now, the commercial platforms of IGRA are only validated for blood samples. Based on facts that more antigen-specific T lymphocytes are found in pleural fluid than those in peripheral blood, we were interested in evaluating the clinical utility of an *M. tuberculosis* antigen-specific IFN-γ using enzyme-linked immunospot (ELISPOT) method, performed on pleural fluid mononuclear cells (PFMC), for the diagnosis of pleural TB in a setting with high incidence of TB disease.

METHODS

A diagnostic study was performed in which 48 consecutive patients with presumed TB pleural effusion were enrolled at Cipto Mangunkusumo Hospital from May to September 2015. The study was approved by the Ethical Committee of Cipto Mangunkusumo Hospital/Faculty of Medicine Universitas Indonesia.

Subjects

Inclusion criteria were patients aged more than 18 years old with suspected pleural effusion based on clinical symptoms and ultrasound examination, with no history of antituberculosis medication in the last 6 months. Patients with heart failure, renal failure, hepatic cirrhosis, and malignancy were excluded. Written informed consents were obtained from all the subjects. Sample size was 48 subjects, determined using diagnostic test sampling formula.

Pleural fluid TB culture and ADA activity were used as the gold standard in this diagnostic study. Based on WHO guidelines of extrapulmonary TB management, microscopic examination is the gold standard for diagnostic. But it has limitations since the sensitivity is very low. So we combined with pleural fluid ADA activity to overcome this. In clinical practice in our centre, as Indonesia is a high TB burden country, when a clinically suspected pleural TB patients has elevated pleural fluid ADA activity and other causes of false elevation have been excluded, they will be treated with antituberculosis although TB culture is negative.

One hundred millilitres (75-125 mL) of pleural fluid was aspirated. First tube (25 mL) was for routine pleural fluid analysis such as white blood cell (WBC) count, mononuclear (MN) cell

count, protein and LDH levels). Determination of exudate was based on Light's criteria (fluid/serum protein ratio >0.5, fluid/serum LDH ratio >0.6, and LDH >2/3 upper limit of normal serum LDH).[6-7] If the results were not exudate, subjects would be excluded from the study. If the results were exudate, subsequent steps were ADA activity measurement, ELISPOT, and MGIT culture.

White blood cells count was performed using Sysmex XE-2100. MN cell count was performed using Wright stained cytocentrifuged slide which is presently considered as the "gold standard" for body fluid differential counting.The ELISPOT assay and MGIT culture was performed within 2 hours after the collection of pleural fluid.

PFMC ELISPOT Assay

The ELISPOT kit (T-SPOT.TB; Oxford Immunotec Ltd, Oxford, UK) was performed according to the manufacturer's instruction with some modifications in order to have an adequate and good quality of pleural fluid mononuclear cells (PFMC). The sample preparation needs pleural fluid volumes of 50 mL to 100 mL. Volumes of 100 mL are more likely to provide sufficient cells although 50 mL are normally sufficient. Samples should be stored at room temperature or 2-8°C. They should not be frozen. Pleural fluid is transferred to a 50-mL Falcon tube and centrifuged at 465 g for 15 minutes at room temperature. After centrifugation, check if there is a good cell pellet at the bottom of the tube. Pipette the supernatant carefully to ensure that there is no loss of cells. If the pellet is small, centrifugation can be repeated. If the pellet is contaminated with red blood cells, then carry out a "hypertonic shock" as follows. Resuspend the pellet in 2 mL sterile or distilled water and incubate for 50-60 seconds. To restore the normal osmolarity, add 2x PBS to the suspension. After that, dilute the suspension with 1x PBS or RPMI medium to 20-30 mL and centrifuge the suspension again at 465 g for 15 minutes. If the pleural fluid is contaminated with many red blood cells, a density gradient centrifugation similar to the density gradient centrifugation used in the standard T-SPOT.TB test can be performed, such as the Ficoll procedure and Leucosep method. If the contamination of the pellet with red blood cells is low, resuspend the pellet in one mL of RPMI using a pipette and make up to 20-30 m, then centrifuge again at 465 g for 15 minutes, remove supernatant carefully. After that, resuspend the pellet in 0,7 mL AIM-V medium and process the T-SPOT.TB test according to the manufacturer's instruction for peripheral blood mononuclear cells (PBMC).T-SPOT.TB uses two TB specific antigens: ESAT-6 and CFP10. Panel A contains ESAT-6 and Panel B contains CFP10. The response of stimulated cultures was considered positive when the test well (Panel A or Panel B) contained at least six more spots and had twice the number of spots shown in the control well. The background number of spots in negative control wells was below 10 spots per well in all patients.[8]

TB Culture Using Mycobacterial Growth Indicator Tube

Twenty five millilitres of pleural fluid was centrifuged at 3500 g for 15 minutes. The pellet was treated with an equal volume of NaOH 4% for 15 minutes at room temperature and neutralized with sterile phosphate buffer. The suspension was inoculated for isolation of acid fast bacilli by culture in MGIT (BD Bactec MGIT 960 system).

Determination of ADA Activity in Pleural Fluid

Adenosine deaminase (ADA) activity levels were detected using an ADA measurement kit with enzymatic colorimetric method (Mindray Bio-Medical Electronics, Shenzhen, China) by following the manufacturer's instructions.

Statistical Analysis

Data results were analyzed by using MS Excel for Mac 2011 and IBM SPSS Statistics ver. 23.0. Normality of data was analyzed using Kolmogorov-Smirnov and Shapiro-Wilk analysis. The difference between two groups was analyzed by using unpaired t test or Mann-Whitney test (age, cell count, biochemistry levels, number of spots, ADA) and chi square (gender). Statistical significance defined as p value <0.05. Diagnostic values such as sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were counted from 2x2 table. Positive MGIT culture and/or ADA activity $\geq$40 U/L were used as the gold standard of pleural TB.

Table 1. Clinical and laboratory characteristics of the patients

Characteristics	Pleural TB (n=39)	Non pleural TB (n=9)
Age (years), mean (SD)	44.9 (16.5)	51.1 (18.4)
Gender (Male/Female)	18/21	7/2
Unilateral effusion on ultrasound	37	4
WBC count (/µL)	1530 (80 – 64.680)**	672.2 (388.3)*
MN count (/µL)	1203 (64 – 58.212)**	546 (307.8)*
Fluid protein (g/dL), mean (SD)	4.7 (0.95)	4.4 (0.71)
Protein ratio, mean (SD)	0.67 (0.11)	0.64 (0.07)
Fluid LDH (U/L)	613 (212 – 51,413)**	647.8 (395.25)*
LDH ratio, median (range)	1.24 (0.59 – 28.28)	0.79 (0.6 – 3.58)
T-SPOT.TB		
- Panel A – negative control (spot)	38 (4 – 314)**	-2 (1 – 12)**
- Panel B – negative control (spot)	27 (0 – 328)**	1.8 (4.7)*
ADA (U/L)	87.6 (48.9 – 461.5)**	17.95 (10.17)*

* mean (SD), ** median (range)

RESULTS

Of the 48 patients with presumed pleural TB, with mean age of 49 years (range 19–80 years), 39 patients were confirmed as pleural TB based on positive MGIT culture and/or ADA activity ≥40 U/L as the gold standard. Characteristics of both groups can be seen in **Table 1**. Based on the gold standard, patients can be divided into two groups: pleural TB (39 patients) and non pleural TB (9 patients).

The median proportion of cell count in pleural effusion fluid was 1530 cells/µL (range 80–64 680 cells/µL) in the group of patients with confirmed pleural TB and 672.2 (SD 388.3) cells/µL in the group of patients with no pleural TB. The median proportion of MN cell count was 1203 cells/µL (range 64–58 212 cells/µL) in the pleural TB group and 546 (SD 307.8) cells in no pleural TB group. Both of cell count and MN cell count were statistically different between two groups.

Pleural fluid protein, pleural fluid LDH, fluid/serum protein ratio, and fluid/serum LDH ratio between two groups were not statistically different.

Of the 39 patients in pleural TB group, all of them (100%) were positive for T-SPOT.TB (**Table 1**). The median spot of (Panel A – negative control) was 38 (4–314) spots and (Panel B – negative control) was 27 (0–328) spots. These results were statistically different with the results in non pleural TB group with p-value <0.001.

The median levels of ADA were significantly higher in pleural TB group than in non pleural TB group as shown in **Table 1**.

From **Table 2**, sensitivity, specificity, PPV, NPV, LR+, and LR- of T-SPOT.TB using PFMC for the diagnosis of pleural TB are 100% (95% CI 90.97-100%), 88.89% (95% CI 51.75-99.72%), 97.5% (86.84-99.94%), 100% (63.06-100%), 9 (1.42 – 57.12), and 0, respectively.

Tabel 2. 2x2 table of diagnostic test

		Gold standard		Total
		Positive	Negative	
T-SPOT.TB	Positive	39	1	40
	Negative	0	8	8
	Total	39	9	48

DISCUSSION

Because TB disease can be difficult to diagnose, IGRA such as T-SPOT.TB has recently become popular as supportive diagnostic method for TB. However, IGRA cannot distinguish between active and latent TB infection (LTBI) or healed TB if performed on peripheral blood samples. In patients with pulmonary affection, bronchoalveolar cells but not PBMCs showed reactivity towards mycobacterial antigens. Thus,

it is suggestive that whilst only a small number of antigen-specific T cells are found in peripheral circulation, highly activated, antigen-specific effector T cells accumulate at disease site and rapidly produce Th-1-type cytokines.[5]

However, recently it was demonstrated that enumeration of MTB-specific mononuclear cells from the site of the infection by ELISPOT can distinguish between active TB, LTBI, or other disease with a high diagnostic sensitivity and specificity. In smear-negative pulmonary TB, the mean numbers of ESAT-6 and CFP10 spots in lung mononuclear cells were 9.6- and 7.9-fold higher than in PBMCs. In a smaller study on pleural TB where only ESAT-6 antigen was used, the mean number of ESAT-6 spots in PFMC was 15-fold higher than in PBMCs.[8] In the present study, the possibility of a reliable and rapid diagnosis of pleural TB using a commercially available MTB-specific ELISPOT in a routine clinical practice was further evaluated.

Average age of subjects in this study was 49 years (range 19 – 80 years). It is similar with an epidemiological analysis from the United States with the mean age of 7549 patients was 49 years. Epidemiologically, pleural TB predominates in men with an overall male-to-female ratio of 2:1.[9] In this study, the number of male subjects was slightly more than the number of female subjects.

Of 48 patients, 39 patients (81.25%) were diagnosed as having pleural TB, based on positive MGIT culture and/or ADA activity more than 40 U/L. The median/mean of pleural fluid WBC count was 1530 cells/μL in pleural TB group and 672 cells/μL in non pleural TB group (**Table 1**). Both of them are statistically different. In TB infection, cells, especially antigen-specific T cells, are recruited and clonally expand at the site of infection.[5] This causes an increase in WBC count. The median/mean of MN cells count was 1203 cells/μL in pleural TB group and 546 cells/μL in non pleural TB group, and both groups are statistically significant. Although neutrophils may be the predominant cells in the pleural cavity in the initial stage, T lymphocytes predominate thereafter. The compartmentalized inflammatory process increases the permeability of pleural capillaries. Along with impaired lymphatic clearance due to parietal pleural involvement, this leads to pleural fluid formation and accumulation.[9] Protein as inflammatory products is accumulated in pleural fluid. A pleural fluid protein concentration greater than 5 g/dL is found in 70% patients.[9] The enzyme lactat dehydrogenase (LDH) is found in the cells of many body tissues, especiallly heart, liver, lungs, brain, and skeletal muscle. When disease or injury affects the cells containing LDH, the cells lyse and LDH is spilled, causes an increase in LDH activity in pleural TB effusion.[10] In this research, pleural fluid protein, fluid/serum protein ratio, pleural fluid LDH, and fluid/serum LDH ratio were not statistically different between two groups. This is particularly because in this research we only include patient with exudative pleural fluid based on Light's criteria.

The ELISPOT assay was positive in all 39 patients (100%) with pleural TB. In pleural TB patients, the number of immunospots in the pleural fluid ELISPOT assay was much higher than the number of immunospots in non pleural TB patients. The median value in pleural TB group was 38 for the ESAT-6 antigen (range 4–314) and 27 for the CFP10 antigen (range 0–328) (**Table 1**). The median value of immunospots in non pleural TB group was -2 for ESAT-6 antigen (range 1–12) and 1.8 (SD 4.7) spots for CFP10 antigen. Both groups showed significantly difference with p-value of <0.001. This is because the number of antigen-specific T cells in pleural TB is higher and these cells secrete more IFN-γ when stimulated.

A false-positive result of pleural fluid ELISPOT was obtained for one patient, probably due to the non-specific IFN-γ present in the effusion before *M. tuberculosis* antigen stimulation which trapped at the bottom of the well immediately after the fluid was added.[11] Furthermore, ESAT-6 and CFP10 are absent from all BCG strains and from non-tuberculous mycobacteria with the exception of *M. kansasii*, *M. szulgai*, and *M. marinum*.[12]

In the diagnosis of pleural TB, ADA activity is highly sensitive, simple, speed, and relatively cheap. They cause the widespread implementation and routine utilization of this assay. In this research, ADA activity was higher in pleural TB group than in non pleural TB group

with p-value of <0.001 (**Table 1**). Increased ADA activity in pleural TB is due largely to increased activity of the ADA isoenzyme ADA-2, together with the fact that only cells in which ADA-2 has been found are monocyte and macrophage. Stimulation of those cells by live phagocytosed microorganisms will cause cells to release ADA.[4,8]

In this research, the sensitivity, specificity, positive predictive value, and negative predictive value of the IGRA ELISPOT assay using PFMC for the diagnosis of pleural TB were 100%, 88.89%, 97.5%, and 100%, respectively.A meta-analysis of 19 studies showed that pooled sensitivity and pooled specificity for the pleural fluid IGRA assay were 72% (95% CI, 55% - 84%) and 78% (95% CI, 65% - 87%), respectively.[13]

As sensitivity of T-SPOT.TB was 100%, theoretically this test is appropriate for screening purpose. As specificity of T-SPOT.TB was only 88.89%, positive test results should always be furtherly evaluated with caution because there is a chance of 11.11% of false positive. As PPV of T-SPOT.TB was 97.5%, if a patient with presumptive pleural TB gets positive result, the chance of this patient to be really sick is 97.5%. As NPV of T-SPOT.TB was 100%, negative result of patients with presumptive pleural TB always ruled out a diagnosis of active pleural TB.

There are some limitations involved in the use of IGRA in the diagnosis of pleural TB using pleural fluid specimen. First, the T-SPOT.TB assay is validated for PBMC sample therefore the cut-off level for PBMC is well defined while the cut-off level for PFMC sample has not yet been defined. Several previous reports have used various cut-off levels derived in heterogeneous settings, this must be a problem in routine clinical practice. A large, comparative study is required to determine the appropriate cut-off levels for positivity, and this may be different in areas with a low or high incidence of TB. Second, pleural TB could only be confirmed by TB culture in less than half of cases (data not shown). However, we have implemented a strict exclusion criteria. In our clinical practice, considering that our country has a high TB-burden setting, presumptive pleural TB patients with exudative pleural fluid, high ADA activity, with other common causes of pleural effusion (liver failure, kidney failure, malignancy, congestive heart failure) are already excluded, will be given TB therapy although they have negative TB culture.

CONCLUSION

The IGRA ELISPOT assay, T-SPOT.TB,using pleural fluid samples showed a high diagnostic accuracy for diagnosing pleural TB in a high burden setting of TB infection. It suggests that T-SPOT.TB assay may be an adjunctive but useful method in the diagnosis of pleural TB.

ACKNOWLEDGMENTS

The research was financially supported by CiptoMangunkusumo Hospital Research Grant 2015.

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