Tropical Biomedicine 37(2): 372–378 (2020)

Diagnostic values of different cytokines in identifying tuberculous pleural effusion

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Received 21 January 2020; received in revised form 27 March 2020; accepted 27 March 2020

Abstract. Interleukin (IL)-1 beta (IL-1β), IL-2, Interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) in the pleural fluid are valuable biomarkers in early diagnosis of Tuberculous Pleural Effusion (TPE). This study aimed to analyze the diagnostic values of some cytokines (IFN-γ, TNF-α, IL-1β, and IL-2) in pleural fluid for identifying TPE in Vietnam. We performed a cross-sectional study on tuberculosis (TB) patients with pleural effusion. Pleural IFN-γ, TNF-α, IL-1β, IL-2 were measured by ELISA® Kit (Abcam, USA) on Biotek system. Receiver operating characteristic curves (ROC), an area under the curve (AUC), sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and accuracy (ACC) of IFN-γ, TNF-α, IL-1β, IL-2 in identifying TBE were assessed. Among 386 patients, 234 (60,6%) had TPE, and 152 (39,4%) did not have TPE. The median of IL-2, TNF-α, and IFN-γ levels were significantly higher in TPE compared to the non-TPE group ($p<0.05$). AUC for IL-1β, IL-2, TNF-α, and IFN-γ were 0.54, 0.57, 0.62. 0.84 ($p<0.05$), respectively. The sensitivity of IL-1β, IL-2, TNF-α, and IFN-γ in the diagnosis of TPE were 82.1, 53.4, 77.8, and 80.3, while the specificity was 28,4, 69.7, 47.4, and 80.9, respectively. IFN-γ and TNF-α are potential biomarkers in diagnosing TPE.

INTRODUCTION

Tuberculosis (TB) has been well-recognized as a cause of great disease burden worldwide, especially in developing nations. The World Health Organization estimated that in 2017, 6.4 million new cases of TB were officially reported (World Health Organization, 2017). Vietnam ranked 15th out of 30 countries having the highest TB burden in the world, with 105,733 cases in 2017 (World Health Organization, 2017). Tuberculosis pleural effusion (TPE) is among the most pervasive cause of pleural effusion, accounting for 30% of total TB cases (Qiu *et al.*, 2006; Ni Zeng *et al.*, 2017).

Despite the availability of several microbiological approaches for diagnosis such as sputum microscopy, culture or cartridge-based nucleic acid amplification tests (CBNAAT), accurate detection of TPE is still a great challenge. The sensitivity of acid-fast bacilli (AFB) smear microscopy with Ziehl-Neelsen stain significantly varies from 20% to 70% in different studies (Qiu *et al.*, 2006; Trajman *et al.*, 2008; Light, 2010; McGrath & Anderson, 2010). Meanwhile, the sensitivity of mycobacterial culture was high but this method needs two to six weeks for analysis (Trajman *et al.*, 2008). Therefore, identifying screening and diagnosis methods to accurately detect TPE is particularly important for providing timely treatment and reducing the burden of TB in the community.

Mycobacterium tuberculosis (M. tuberculosis), which causes tuberculosis, has been known to interact with the host through alveolar macrophages and active inflammatory response, producing tumor necrosis factor-alpha (TNF-α), Interleukin (IL)-12,

IL-1, IL-6, and various cytokines. These molecules induce other cells monocytes, neutrophils, and lymphocytes to enter the lungs. CD4+ and CD8+ T cells secrete cytokines (including Interferon-gamma (IFN-γ) and TNF-α) to critically respond to *M. tuberculosis*. Recently, numerous studies suggested that cytokines are reliable pleural fluid biomarkers with high sensitivity and specificity for TPE diagnosis (Marie *et al.*, 2013). Therefore, this study aimed to analyze the diagnostic values of some cytokines (IFN-γ, TNF-α, IL-1β, and IL-2) in pleural fluid for identifying TPE in Vietnam.

MATERIALS AND METHODS

Study design and sampling method

This cross-sectional study was performed on 386 patients with pleural effusion at three hospitals including Hanoi Lung Hospital, Central Lung Hospital, and Thanh Nhan Hospital. Patients who 1) aged 15 years or above; 2) were confirmedly diagnosed with TPE according to the guideline of the Ministry of Health in Vietnam (No: 4263/2015/QD-BYT); and 3) agreed to become participants were conveniently recruited into the study. Exclusion criteria comprised 1) aged less than 15 years old; 2) being depleted, or pregnant women; and 3) refused to participate in the study or gave informed consent. The study protocol was approved by the Institutional Review Board of the Hanoi Department of Science and Technology (No: 4528/QD-UBND).

Patients were diagnosed with TPE if 1) their pleural fluid, sputum or pleural biopsy had the *Mycobacterium tuberculosis* (MTB) via stain or culture; or 2) granulomatous inflammation presented on pleural biopsy. In the case that samples did not show any cause associated with pleural effusion, patients who had clinical and radiological evidence for TB, as well as a clinical improvement after anti-TB therapy were considered having TPE. Patients having malignant cells in pleural fluid or pleural biopsy were identified to have malignant effusion, while those having any exudative effusion associated with bacterial pneumonia, lung abscess or bronchiectasis were defined to have parapneumonic effusion.

Specimens and Measurement

We collected demographic and pleural fluid data (including protein, lactate dehydrogenase-LDH, cytology, aerobic and anaerobic culture), along with serum C-reactive protein (CRP), full blood count (FBC), and physicians' final diagnosis. Moreover, we performed microbiological (BACTEC MGIT 960 system, Becton Dickinson) or nucleic acid amplification tests – NAAT (transcription-reverse transcription concerted reaction - polymerase chain reaction) in pleural biopsy specimens/ pleural fluid/sputum, histopathological examinations in pleural biopsy specimens, and AFB staining in sputum.

We employed acid-fast bacilli (AFB) smear microscopy and culture (using the BACTEC MGIT system) to find *MTB*. Meanwhile, Adenosine Deaminase (ADA), CRP, LDH, and protein assay were performed on all the pleural effusion samples. A total of 15 milliliters (ml) of the pleural fluid sample were extracted and divided into two parts: 1) Seven milliliters were used for culture and CBNAAT; and 2) The remaining part was used to assess ADA, CRP, LDH, and protein assay. We evaluated storage conditions by analyzing ten pleural fluid samples within one hour after collection (at 22°C temperature), twenty-four hours after collection (at 4°C) or three months after collection (at -35 degrees C). IFN-γ, TNF-α, IL-1β, and IL-2 concentration were measured by ELISA® Kit (Abcam, USA) on the Biotek system according to the manufacturer's instructions.

Statistical analysis

Descriptive statistics were performed. Sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV) and accuracy (ACC) of IFN-γ, TNF-α, IL-1β, IL-2 in identifying TBE were assessed. The receiver operating characteristic curves (ROC) and area under the curve (AUC) were computed using the greatest sum of sensitivity and specificity. P-values ≤ 0.05 were recognized as statistical significance.

RESULTS

A total of 386 patients with pleural effusion were included in the study, of which 234 (60.6%) cases had TPE. There were 152 non-TPE (39.4%) cases, including 73 (48.0%) parapneumonic effusions, 49 (12.7%) malignant effusions, and 30 (7.8%) other diseases. As shown in Table 1, the non-TPE group had a higher mean age than that of the TPE group (63.7±16.6 vs. 47.4±20.3, $p<0.05$). The majority of patients were males and the sex difference between the two groups was not statistically significant ($p>0.05$).

Levels of LDH, protein, total cell count, and lymphocyte percentage in pleural fluid among TPE patients were higher than those of their non-TPE counterparts ($p<0.001$). Patients with TPE also had significantly higher median IFN-γ, TNF-α, IL-2 in pleural fluid (3.3 ng/L, 160,1 pg/mL, 47.0 ng/mL, respectively) compared to patients with non-TPE (0.5 ng/mL, 95.1 pg/mL, 42.9 ng/mL, respectively) ($p<0.05$). There was no significant difference in IL-1β between the two groups. Although the total number of white blood cells was significantly higher in the non-TPE group compared to the TPE group ($p<0.05$), the median of lymphocyte percentage did not differ between the groups. (Table 1).

As shown in Table 2. IFN-γ, TNF-α, IL-1β, IL-2 in pleural fluid were significantly higher in patients with TPE group than in patients with other diseases group (including cirrhosis, heart failure, kidney failure).

Four receiver operating characteristics (ROC) curves for IFN-γ, TNF-α, IL-1β, and IL-2 in the pleural fluid were illustrated in Figure 1. As shown in Table 3, areas under the ROC curve (AUCs) for IFN-γ, TNF-α, IL-1β, and IL-2 in the pleural fluid were 0.84, 0.62, 0.54, and 0.57, respectively. The optimal cut-off values were 1.2 ng/mL, and 78 pg/mL for IFN-γ, and TNF-α in pleural fluid, respectively (Table 3 and Figure 1).

The diagnostic performance of some parameters in the diagnosis of TPE is shown in Table 4. On the basis of the cut-off value, the sensitivity, specificity, negative predictive values of ADA activity were 87.7%; 83.4%, 81.3%; IFN-γ were 80.3%, 80.9%, 72.7%; TNF-α were 77.8%, 47.4%, 58.1% respectively. Combined diagnostic parameters of ADA and

Table 1. Characteristics of patients

Characteristics	TPE group (n=234)	Non-TPE group (n=152)	*P*
Age, years (Mean ± SD)	47.4±20.3	63.7±16.6	0.004
Gender			
Male	148 (63.2%)	98 (64.5%)	0.50
Female	86 (36.8%)	54 (35.5%)	
Pleural fluid, median (IQR)			
LDH, U/L	430 (227)	157 (455)	0.001
Protein, g/L	53.6 (10.4)	23.5 (29.4)	<0.001
Total cell count, $\times 10^9$/L	3.03 (3.19)	0.62 (2.13)	<0.001
Lymphocyte, %	84 (20)	55 (45)	<0.001
ADA, U/L	59.9 (35.1)	25.1 (19.7)	<0.001
IFN-γ, ng/mL	3.3 (5.0)	0.5 (1.0)	<0.001
TNF-α, pg/mL	160.1 (218.2)	95.1 (187.2)	0.002
IL-1β, pg/mL	153.3 (256.5)	126.7 (247.2)	0.90
IL-2, pg/mL	47.0 (62.9)	42.9 (63.5)	0.03
Blood, median (IQR)			
Serum CRP, mg/L	44.6 (62.5)	17.8 (26.2)	<0.001
WBC, $\times 10^9$/L	7,5 (6,14-8,9)	9,4 (7,3-12,0)	<0.001
Lymphocyte, %	17.4 (9.2)	19.7 (8.8)	0.90

(TPE = tuberculous pleural effusion, SD = standard deviation, IQR = interquartile range, LDH = lactate dehydrogenase, ADA = adenosine deaminase, TNF = tumor necrosis factor, IFN = interferon, IL = interleukin, WBC = white blood cell, CRP = C-reactive protein).

Table 2. Compare activity, IFN-γ, TNF-α, IL-1β, IL-2 in pleural fluid in different diagnostic groups

Pleural fluid parameters	Tuberculous pleural effusion (n=234)	Parapneumonic effusions (n=73)	Malignant effusions (n=49)	Other diseases (n=30)	*P*
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	
IL-1β (pg/mL)	153.3 (261.5)	163.6 (337.3)	128.5 (246.1)	48.6 (134.4)	0.002
IL-2 (pg/mL)	47.0 (62.9)	44.2 (64.2)	38.0 (62.4)	32.2 (33.3)	0.02
TNF-α (pg/mL)	160.1 (218.2)	112.0 (211.2)	96.0 (266.4)	53.5 (98.4)	0.003
IFN-γ (pg/mL)	5.5 (7.7)	2.1 (6.1)	1.1 (1.6)	1.1 (1.5)	<0.001

(IQR = interquartile range, ADA= adenosine deaminase, TNF = tumor necrosis factor, IFN = interferon, IL = interleukin).

Table 3. Areas under the ROC curve for IFN-γ, TNF-α, IL-1β, IL-2 in pleural fluid

Pleural fluid parameters	AUC (95% CI)	*p*	Cut-off
IFN-γ	0.84 (0.78 – 0.88)	<0.001	1.2 ng/mL
TNF-α	0.62 (0.54 – 0.86)	0.002	78 pg/mL
IL-1β	0.54 (0.47 – 0.61)	0.200	–
IL-2	0.57 (0.51 – 0.64)	0.038	–

(ADA= adenosine deaminase, TNF = tumor necrosis factor, IFN = interferon, IL = interleukin, AUC = area under the curve, CI = confidence interval).

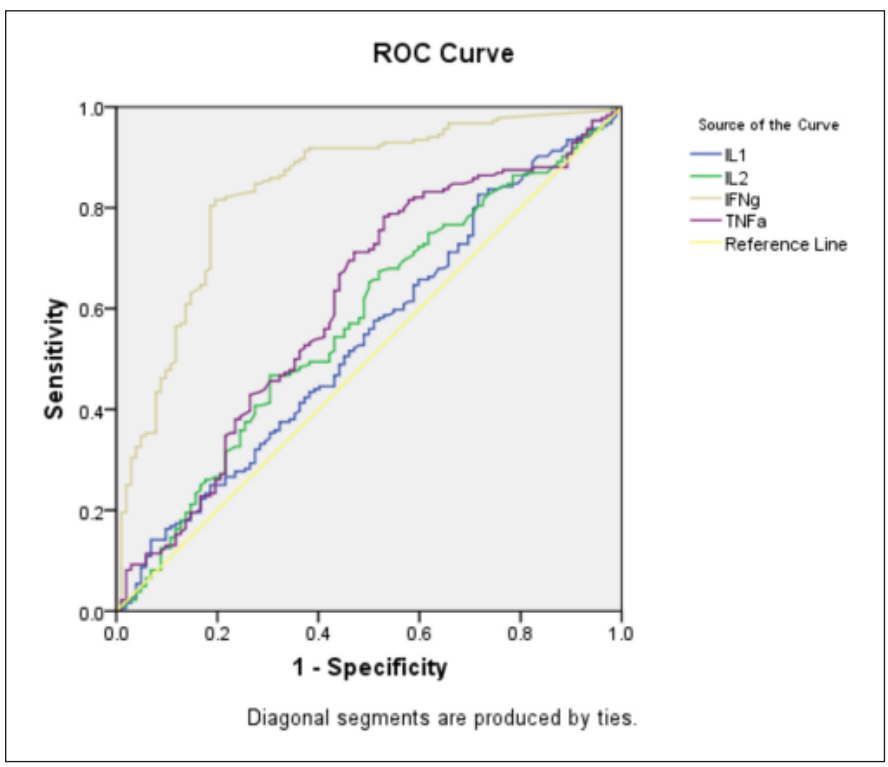

Figure 1. ROC curve for, IFN-γ, TNF-α, IL-1β, IL-2 in pleural fluid.

Table 4. Diagnostic parameters of tuberculous pleural effusion

Parameters (cut-off values)	n	Se (%)	Sp (%)	PPV (%)	NPV (%)	ACC (%)
IFN-γ (≥ 1.2ng/mL)	386	80.3	80.9	86.6	72.8	80.6
TNF-α (≥ 78pg/mL)	386	77.8	47.4	69.5	58.1	65.8
ADA (> 37.5U/L)	386	87.7	83.4	89.2	81.3	86.0
ADA and IFN-γ[a]	386	93.5	61.8	81.5	84.0	82.2
TNF-α and IFN-γ[b]	386	92.4	44.1	74.9	76.2	75.1
Sputum AFB smear	257	7.5	100	100	40	41.5
Culture						
Pleural fluid	386	17.6	100	100	43.5	45.0
All specimens	386	31	100	100	47.8	52.3
TRC Rapid MTB						
Pleural fluid	63	2.8	100	100	43.5	33.9
All specimens	72	13.6	100	100	42.4	42.5
PCR						
Pleural fluid	85	36.5	81.8	75	48.1	54.3
All specimens	102	43.4	79.4	76.7	47.4	51.2
Histopathological	138	63.3	100	100	56.7	72.3

Se = sensitivity, Sp = specificity, PPV = positive predictive values, NPV = negative predictive values, ACC = accuracy, AFB = acid-fast bacilli, TRC = transcription-reverse transcription concerted reaction, MTB = Mycobacterium tuberculosis, PCR = polymerase chain reaction.

[a] Combined diagnostic parameters of ADA and IFN-γ, positive when either of these parameters are greater than or equal to the cut-off value, negative when both of them parameters are lower than to the cut-off value.

[b] Combined diagnostic parameters of TNF-α and IFN-γ, positive when either of these parameters are greater than or equal to the cut-off value, negative when both of them parameters are lower than to the cut-off value.

IFN-γ, TNF-α and IFN-γ in the pleural fluid were associated with increased sensitivity and NPV (93.5% and 84%, 92.4% and 76.2% respectively) .

As summarized in Table 4, compared with other parameters (AFB smear, culture, nucleic acid amplification testing for MTB, and histopathologic examination), ADA activity and IFN-γ in pleural fluid yielded higher sensitivity and NPV.

DISCUSSION

Given that a number of TPE cases have not been diagnosed in many low and middle-income countries due to insufficient resources, identifying potential biomarkers to detect TPE is critically important. This study contributes to the current knowledge about the diagnostic values of IFN-γ, TNF-α, IL-1β, and IL-2 in diagnosing TPE among Vietnamese TB patients.

In this study, our findings suggested that IFN-γ in the TPE group was much higher than the non-TPE group, which was in line with previous work (Chen *et al.*, 2016). In fact, IFN-γ is a critical cytokine to both innate and adaptive immunity, and serves as a primary activator of macrophages, in addition to stimulates natural killer cells and neutrophils. Furthermore, in our study, with 1.2 ng/mL of pleural fluid as the cut-off point, IFN-γ yielded 80.3% sensitivity, 80.9% specificity and 0.84 AUC, indicating a "good" predictive factor for TPE diagnosis. Prior meta-analysis indicated that IFN-γ in pleural fluid had 89% sensitivity, 97% specificity and 0.99 AUC for the diagnosis of TB (Porcel, 2016). The sensitivity and specificity in this study were not as high as in other studies which might be because the non-TPE group was mostly parapneumonic effusion, resulting in the increase of IFN-γ in the body's immune system. This result was similar to Li M. et al.'s findings with the cut-off value of 103.65 ng/L, 80.9% sensitivity, 81.4% specificity, and 81.1% accuracy (Li *et al.*, 2014).

The involvement of TNF-α in protection against *M. tuberculosis* was confirmed in previous studies (Ehlers, 2003; Lin *et al.*, 2007; Mootoo *et al.*, 2009). Mingying Li used 30.3 ng/L as a cut-off value of TNF-α, which had 83% sensitivity, 97% specificity and

90% accuracy in differentiating TPE from malignant pleural effusions (Li *et al.*, 2014). In our study, TNF-α concentration in the pleural fluid of the TPE group was significantly higher than that of the non-TPE group. Moreover, we found that the AUC for TNF-α in diagnosing TPE was 0.62 (p=0.01). However, this diagnostic value is quietly "poor" compared to prior research. Nonetheless, the combination of multimarker in our study improved the diagnostic value for TPE significantly. In particular, when combination ADA with IFN-γ, TNF-α with IFN-γ, sensitivity and NPV were higher than each test alone.

Notably, we found that IL-1β was not able to diagnose TPE (AUC = 0.54; p = 0.2, and AUC = 0.57; p = 0.038, respectively). Our study showed that there was no difference in IL-1β concentration between TPE and non-TPE groups, which was in line with the previous studies (Orphanidou *et al.*, 1996). Although no difference in IL-1β was found between the TPE and non-TPE groups, the IL-1β concentration in other diseases group was significantly lower than the TPE, parapneumonic effusions, and malignant effusions groups. One of the possible reasons for the change of proinflammatory cytokine levels was the use of antibiotics. The use of antibiotics such as cefuroxime and gentamicin increase the production levels of IL-1β and IL-6, whereas the elimination of bacteria with ciprofloxacin- and imipenem-cilastatin reduces cytokine production (Frieling *et al.*, 1997). Antibiotic use is probably a confounding factor when many patients have received different antibiotics before admission, which was difficult to clarify when antibiotic use in Vietnam has been a universal problem. In addition, the use of some anti-inflammatory drugs has been shown to reduce pro-inflammatory cytokines (IL-6, MIP-1α and IL-1β) through inhibition of NF-B (Annamanedi *et al.*, 2014).

IL-2 is also known as a T-cell growth factor, which is required for T-cell proliferation and other activities to regulate the immune response. However, several studies showed that IL-2 had low sensitivity and specificity in diagnosing TPE (Akarsu *et al.*, 2005; N. Zeng *et al.*, 2017). Another study in China found a difference in the IL-2 concentration between TPE and other groups, but the sensitivity and specificity of IL-2 were not high (67% and 76%, respectively) (Lin *et al.*, 2007). In our study, there was a small difference in IL-2 concentration between the TPE group and the other three groups of causes (AUC = 0.57; p = 0.038). This was similar to the study in China which found that the diagnostic values of IL-2 in endemic regions were not high but it was substantially better in places having a low prevalence of tuberculosis (Tural Onur *et al.*, 2015).

CONCLUSIONS

Our study shows that IFN-γ and TNF-α are potential biomarkers in diagnosing TPE. Combining test results, clinical symptoms, imaging diagnosis and pleural fluid characteristics, a preliminary diagnosis of whether this patient is likely to have tuberculosis or not could be provided, contributing to early TPE detection.

Funding:
This research received no funding.

Conflicts of Interest:
The authors declare no conflict of interest.

Author contributions:
All authors participated in study design, data collection and analysis, writing and editing manuscript.

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