

Diagnosis of tuberculous pleurisy with combination of adenosine deaminase and interferon- γ immunospot assay in a tuberculosis-endemic population

A prospective cohort study

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Abstract

The aim of this study was to identify the optimal cut-off value of T cell enzyme-linked immunospot assay for tuberculosis (T-SPOT.TB) and evaluate its diagnostic performance alone (in the peripheral blood) or in combination with the adenosine deaminase (ADA) activity test (in peripheral blood and the pleural fluid) in patients with tuberculous pleurisy.

Adult patients presenting with pleural effusion were included in this prospective cohort study. Tuberculous pleurisy was diagnosed by T-SPOT.TB in peripheral blood and a combination of T-SPOT.TB and ADA activity test in pleural fluid and peripheral blood. Receiver operating characteristic (ROC) curve in combination with multivariate logistic regression was used to evaluate the diagnostic performance of the assays.

Among a total of 189 patients with suspected tuberculous pleurisy who were prospectively enrolled in this study, 177 patients were validated for inclusion in the final analysis. ROC analysis revealed that the area under the ROC curve (AUC) for T-SPOT.TB in pleural fluid and peripheral blood was 0.918 and 0.881, respectively, and for the ADA activity test in pleural fluid was 0.944. In addition, 95.5 spot-forming cells (SFCs)/ 2.5×10^5 cells were determined as the optimal cut-off value for T-SPOT.TB in pleural fluid. Parallel combination of T-SPOT.TB and ADA activity test in pleural fluid showed increased sensitivity (96.9%) and specificity (87.5%), whereas serial combination showed increased specificity (97.5%). The combination of 3 assays had the highest sensitivity at 97.9%, with an AUC value of 0.964.

T-SPOT.TB in pleural fluid performed better than that in peripheral blood and the ADA activity test in pleural fluid for tuberculous pleurisy diagnosis. The optimal cut-off value of T-SPOT.TB in pleural fluid was 95.5 SFCs/ 2.5×10^5 cells. Combination of 3 assays might be a promising approach for tuberculous pleurisy diagnosis.

Abbreviations: ADA = adenosine deaminase, AFB = acid-fast bacterium, AUC = area under the receiver operating characteristic curve, CFP10 = culture filtrate protein 10, ELISPOT = enzyme-linked immunosorbent spot, ESAT-6 = early secretory antigenic target 6, IFN- γ = interferon-gamma, LR = likelihood ratio, LTBI = latent tuberculosis infection, NPV = negative predictive value, PBMCs = peripheral blood mononuclear cells, PFMCs = pleural fluid mononuclear cells, PPV = positive predictive value, QFT-GIT = QuantiFERON TB Gold In-Tube test, ROC = receiver operating characteristic curve, SFCs = spot-forming cells, TB = tuberculosis, T-SPOT.TB = T cell enzyme-linked immunospot assay for tuberculosis.

Keywords: adenosine deaminase activity, combination test, diagnostic performance, peripheral blood, pleural fluid, tuberculous pleurisy

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HYY and CYL should be considered as cofirst authors.

Authorship: Study conception and design: YPL, YPX, CSC; performance of biological experiments: PPK; data collection: SSS; statistical analysis: JRY, MY; preparation of the manuscript: HYY, CYL, LY. All authors read and approved the final manuscript.

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1. Introduction

Tuberculous pleurisy is a type of tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) infection. In endemic areas such as China, tuberculous pleurisy accounts for about 25% of all TB cases.^[1–3] Standard diagnosis of tuberculous pleurisy depends on positive MTB culture in the pleural fluid or pleural biopsy specimens.^[1,4] However, this approach has too many deficiencies that hinder its suitability for routine practice, such as long waiting time, trauma, and low sensitivity.^[5,6] Histological analysis based on closed pleural biopsy and medical thoracoscopy, which offers 80% to 100% positive diagnostic yield,^[7,8] is invasive and not feasible in all suspected tuberculous pleurisy cases.^[5]

Some other methods with promising potential are also used for tuberculous pleurisy diagnosis. Numerous studies suggested that adenosine deaminase (ADA) is one of the more reliable and cost-effective pleural fluid biomarkers of tuberculous pleurisy.^[1,9–12] However, ADA activity can be elevated in various conditions such as pulmonary empyema and rheumatoid arthritis.^[10] In addition, none of the available guidelines provide optimal cut-off values for ADA activity in pleural fluid, whereas 45 IU/L remains the commonly accepted cut-off for tuberculous pleurisy diagnosis.^[13,14] Furthermore, the T cell enzyme-linked immunospot test for TB (T-SPOT.TB) is a relatively new assay developed for tuberculous pleurisy diagnosis and is based on the detection of the interferon-gamma (IFN- γ) cytokine. IFN- γ is produced by effector T lymphocytes that are stimulated by culture filtrate protein 10 (CFP10) and early secretory antigenic target 6 (ESAT-6), which are secreted by MTB.^[15,16] Numerous studies investigated the diagnostic value of T-SPOT.TB in pleural fluid and peripheral blood.^[17–19] In peripheral blood, failure of T-SPOT.TB in differentiating active TB from latent TB infection (LTBI) was shown to be associated with low specificity in regions with high TB burden.^[20–22] Because antigen-specific T lymphocytes are recruited preferentially to pleural fluid, T-SPOT.TB in pleural fluid is superior to that in peripheral blood for tuberculous pleurisy diagnosis.^[5] Although the diagnostic values of single assays have been widely investigated, few studies focused on the diagnostic performance of different diagnostic tests in combination.

In this study, the diagnostic performance of a combination of T-SPOT.TB test in pleural fluid or peripheral blood and the ADA activity test in pleural fluid were evaluated in adult patients with tuberculous pleurisy at a single hospital in China. In addition, the optimal cut-off value for T-SPOT.TB in pleural fluid was determined. The efficacy of these assays was compared to establish a promising approach for tuberculous pleurisy diagnosis.

2. Materials and methods

2.1. Patients and study procedures

This was a prospective cohort study that was performed at the First Affiliated Hospital of Wenzhou Medical University, a tertiary teaching hospital in Zhejiang, China. All patients aged 16 years or older who presented with unilateral pleural effusion from September 2015 to June 2016 were enrolled in this study. Patients with compromised immune function and previous TB infection history were excluded from the study. In addition, patients who received anti-TB therapies were excluded. This protocol was approved by the institutional review board of the First Affiliated Hospital of Wenzhou Medical University (No: 228, 2017.1.5). All patients enrolled in this study provided written informed consent.

Routine clinical, biochemical, microbiological in pleural biopsy specimens or pleural fluid, histopathological examinations in pleural biopsy specimens, and acid-fast bacterium (AFB) staining in pleural biopsy specimens or sputum were performed. All clinical data were extracted from patient medical records by the investigators, who also tracked the treatment process and discharge diagnoses of all patients. For patients whose diagnosis was not established during hospitalization, a telephonic interview was conducted 6 months after discharge to evaluate treatment outcomes.

2.2. Mycobacterial culture

Specimens including solid pleural tissue and pleural fluid were transferred to BACTECMyco/F Lytic culture vials, and cultivation for MTB was performed by the automated BACTEC FX200 system (BD Biosciences, Sparks, MD), with a time lag of 20 to 40 days.

2.3. Clinical categories of tuberculous pleurisy

Patients were confirmed with tuberculous pleurisy diagnosis if the MTB culture using pleural fluid, pleural biopsy specimens or sputum, or AFB staining was positive, granulomatous inflammation was observed by histological evaluation of pleural biopsy specimens with positive AFB staining, or there was clinical improvement after anti-TB therapy in patients with negative AFB staining.^[23,24] Otherwise, patients were diagnosed with probable tuberculous pleurisy if the MTB culture using pleural fluid, pleural biopsy specimens, or sputum was negative, chronic inflammation was observed by histological evaluation of pleural biopsy with negative AFB staining, or clinical improvement and resolution of pleural fluid were achieved after anti-TB therapy without the use of corticosteroids (such as prednisone) and antimicrobials in the absence of evidence for other infectious pathogens for pleural disease.^[23,24]

2.4. T-SPOT.TB in pleural fluid and peripheral blood

T-SPOT.TB was conducted following the manufacturer's instructions (Oxford Immunotec Ltd, Oxford, UK). Peripheral venous blood (5 mL) and pleural fluid (40 mL) samples collected from patients were tested within 2 hours. For T-SPOT.TB in pleural fluid, samples were centrifuged and supernatants were decanted. The pellets were resuspended in 8 mL Roswell Park Memorial Institute 1640 medium (MD Pacific Biotechnology Ltd, Tianjin, China). Pleural fluid mononuclear cells (PFMCs) were obtained by separating the cell suspension using Ficoll-Hypaque density gradient. Subsequent steps were identical for T-SPOT.TB using pleural fluid and peripheral blood samples. In T-SPOT.TB, empty wells were utilized as negative controls, ESAT-6 and CFP10 as specific antigens, and the T lymphocyte mitogen phytohemagglutinin as positive control. Isolated peripheral blood mononuclear cells (PBMCs) and PFMCs were added to the wells of T-SPOT.TB plates (2.5×10^5 cells per well) that were pre-coated with a monoclonal antibody against IFN- γ and incubated at 37°C for 16 to 20 hours. An automated enzyme-linked immunosorbent spot (ELISPOT) reader was used to count spot-forming cells (SFCs). Criteria for a positive T-SPOT.TB in peripheral blood were the spots of any well of antigen (A or B) minus the negative control well ≥ 6 (when the negative control spots < 5); and any well of antigen (A or B) present 2 times more spots than negative control (when the negative control spots ≥ 5).

2.5. Determination of adenosine deaminase activity in pleural fluid

ADA activity level in pleural fluid was detected using an ADA measurement kit (Kuake Biological Technology, Zhejiang, China) according to the manufacturer's instructions. The response was classified as positive if the ADA activity was ≥ 45 IU/L.

2.6. Statistical analysis

Data were analysed using SPSS Statistics version 23.0 (SPSS Inc., Chicago, IL). Differences between the tuberculous pleurisy and non-tuberculous pleurisy patients were analysed by Mann-Whitney *U* test or unpaired *t* test for continuous data and Pearson Chi-square for categorical data. $P < .05$ denoted statistical significance. Diagnostic performance was evaluated using positive likelihood ratio (+LR), negative likelihood ratio (-LR), sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). To assess the diagnostic performance of T-SPOT.TB in peripheral blood and pleural fluid and ADA activity in pleural fluid, receiver operating characteristic (ROC) curves were plotted. Concurrently, areas under the ROC curve (AUCs) were also calculated. In addition, optimal cut-off values were obtained by ROC analysis, and ROC analysis based on the multivariate logistic regression model was conducted to assess the diagnostic value of the combined assays. A serial positive test was defined as the positivity for T-SPOT.TB both in pleural fluid and peripheral blood in addition to a positive ADA activity in pleural fluid, whereas a parallel positive test was defined as positivity for either assay.

3. Results

3.1. Patient characteristics

As shown in the study flowchart (Fig. 1), among a total of 189 patients with suspected tuberculous pleurisy who were prospectively enrolled in this study, 12 patients were excluded; therefore, 177 patients were included in the final analysis.

Clinical characteristics of the 177 patients are presented in Table 1. According to the diagnostic criteria, 97 patients (55%) were classified as tuberculous pleurisy and 80 patients (45%) were classified as non-tuberculous pleurisy (lung cancer, $n=50$; pneumonia, $n=19$; pulmonary empyema, $n=4$; malignant mesothelioma, $n=3$; lymphoma, $n=3$; multiple myeloma, $n=1$). Overall, non-tuberculous pleurisy patients were significantly older than tuberculous pleurisy patients ($P < .001$). Significant differences were also found in the number of SFCs and ADA activity level ($P < .001$), whereas no significant difference between sexes was found ($P = .779$). Among the 97 patients in the tuberculous pleurisy group, 41 and 56 patients were further diagnosed as confirmed and probable tuberculous pleurisy, respectively. As summarized in Table 2, there were no significant differences in various characteristics between the confirmed and probable tuberculous pleurisy patient groups ($P > .05$).

3.2. Construction of the receiver operating characteristics curves

Three ROC curves for T-SPOT.TB in pleural fluid and peripheral blood and ADA activity in pleural fluid were plotted for the study population (Fig. 2A). The optimal cut-off values were determined as 95.5 SFCs/ 2.5×10^5 PFCs, 10.5 SFCs/ 2.5×10^5 PBMCs, and

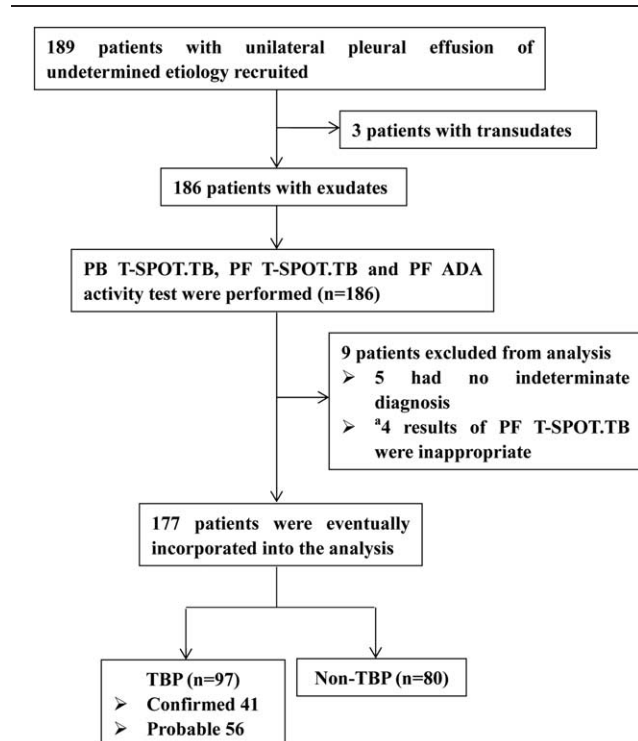


Figure 1. Flowchart of the study population. *As the number of spot-forming cells in positive-control wells was less than 20 for 3 samples, cell counts were not large enough to perform the test for 1 sample. ADA=adenosine deaminase; PB=peripheral blood; PF=pleural fluid; TBP=tuberculosis pleurisy; T-SPOT.TB=T cell enzyme-linked immunospot test.

29 IU/L for T-SPOT.TB in pleural fluid and peripheral blood and ADA activity in pleural fluid, respectively. Instead of the optimal cut-off values, 6 SFCs/ 2.5×10^5 PBMCs and 45 IU/L were selected as the cut-off criteria for T-SPOT.TB in peripheral blood and ADA activity level in pleural fluid, respectively. The AUCs for T-SPOT.TB in peripheral blood and pleural fluid and ADA activity level in pleural fluid were 0.881, 0.918, and 0.944, respectively.

3.3. Single performance of the T-SPOT.TB and the adenosine deaminase activity for tuberculous pleurisy diagnosis

As shown in Fig. 3, the mean number of SFCs was higher in the tuberculous pleurisy group than that in the non-tuberculous pleurisy group with the T-SPOT.TB in both pleural fluid and peripheral blood. In addition, the number of SFCs was significantly higher with the T-SPOT.TB in pleural fluid than that in peripheral blood within the tuberculous pleurisy group ($P < .05$), whereas no significant difference was observed in the non-tuberculous pleurisy group. As summarized in Table 3, the sensitivity and specificity of the T-SPOT.TB in pleural fluid were 88.7% [95% confidence interval (CI), 83.2–92.2] and 91.2% (95% CI, 84.7–95.6), respectively, whereas the sensitivity and specificity of the T-SPOT.TB in peripheral blood were 86.6% (95% CI, 80.2–91.6) and 72.5% (95% CI, 64.8–78.5), respectively. Finally, on the basis of a cut-off value of 45 IU/L, the sensitivity and specificity of the ADA activity in pleural fluid were 61.9% (95% CI, 56.3–64.4) and 95% (95% CI, 88.3–98.3), respectively.

Table 1
Clinical characteristics of 177 patients with suspected tuberculous pleurisy*

Characteristics	TBP group [†] (n = 97)	Non-TBP group (n = 80)	P
Age, y (mean ± SD)	42.0 ± 18.6	65.2 ± 14.1	<.001
Gender			
Male	65 (67)	52 (65)	.779
Female	32 (33)	28 (35)	.779
Underlying condition or illness			
Alcoholism	0 (0)	4 (5)	.040
Tobacco	19 (20)	24 (30)	.108
Liver cirrhosis	0 (0)	1 (1)	.452
Diabetes	6 (6)	9 (11)	.229
Hypertension	12 (12)	31 (39)	<.01
Congestive heart failure	0 (0)	0 (0)	1.000
Arrhythmia	1 (1)	1 (1)	1.000
COPD	0 (0)	3 (4)	.090
Chronic renal failure	2 (2)	1 (1)	1.000
Rheumatologic disease	2 (2)	0 (0)	.502
Hematologic disease	0 (0)	1 (1)	.452
Solid tumor	8 (8)	14 (18)	.063
Transplantation	0 (0)	0 (0)	1.000
HIV infection	0 (0)	0 (0)	1.000
No underlying disease	78 (80)	37 (46)	<.01
Prior TB treatment	0 (0)	0 (0)	1.000
Results of tests			
Albumin, median g/L (IQR)	34.3 (31.5–37.5)	34.4 (30.5–38.4)	.518
Sputum AFB	2/81 (2)	0/53 (0)	.518
Pleural tissue AFB	9/64 (14)	0/40 (0)	.012
PF TB culture	5/23 (22)	0/5 (0)	.550
PB T-SPOT.TB (IQR)	20 (13–20)	2 (0–6)	<.001
PF T-SPOT.TB (IQR)	453 (233–498)	2 (0–28)	<.001
PF ADA, median IU/L (IQR)	48 (38–60)	12 (9–18)	<.001
Histology of pleural (granuloma with necrosis)	‡39/43 (91)	§0/22 (0)	<.001

ADA=adenosine deaminase, IQR=interquartile range, PB=peripheral blood, PF=pleural fluid, TBP=tuberculosis pleurisy.

* Positive patients over total patients (%), unless otherwise indicated.

† Patients in TBP group include confirmed (n=41) and probable (n=56) patients.

‡ Thirty-nine cases showed granuloma with necrosis in pleural tissue.

§ No case showed granuloma with necrosis in pleural tissue.

3.4. Diagnostic performance of parallel and serial testing of pleural fluid and peripheral blood with T-SPOT.TB and pleural fluid for ADA activity

As summarized in Table 3, compared with single testing, parallel assessment of peripheral blood and pleural fluid with T-SPOT.TB

Table 2
Clinical characteristics of patients with confirmed and probable TBP.

Characteristics	Confirmed TBP group (n = 41)	Probable TBP group (n = 56)	P
Age, y (mean ± SD)	46.2 ± 19.4	39.8 ± 18.2	.176
PB T-SPOT.TB (IQR)	20 (12–35)	20 (13–20)	.860
PF T-SPOT.TB (IQR)	453 (248–500)	450 (170–495)	.388
PF ADA, median IU/L (IQR)	47 (39–58)	51 (38–63)	.585
PF LDH, median IU/L (IQR)	289 (218–494)	348 (256–603)	.175
Percentage of lymphocyte in PF	86 (75–94)	85 (69–95)	.590

ADA=adenosine deaminase, IQR=interquartile range, LDH=lactic dehydrogenase, PB=peripheral blood, PF=pleural fluid, TBP=tuberculous pleurisy.

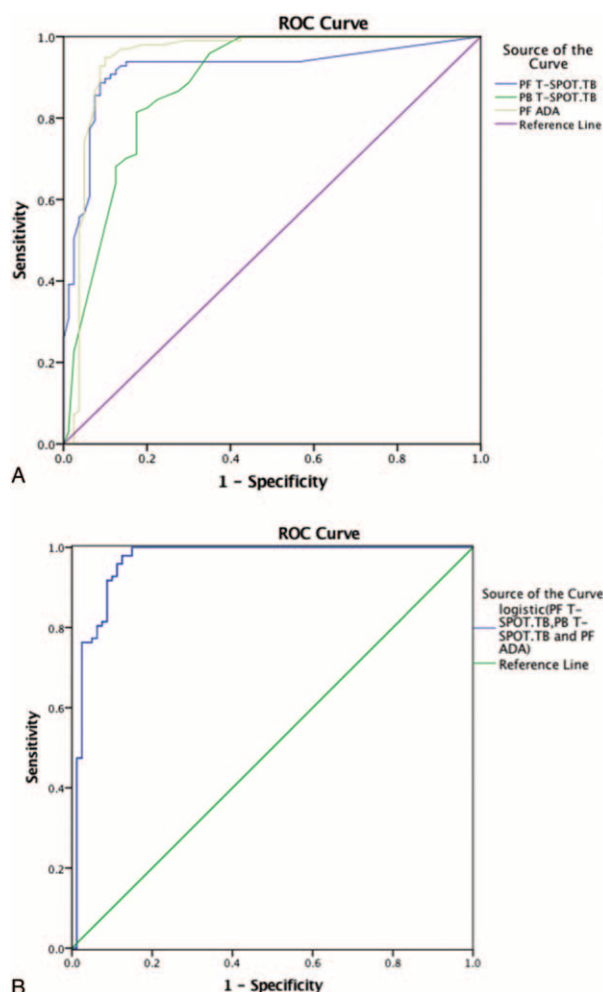


Figure 2. ROC curve for T-SPOT.TB in pleural fluid and peripheral blood in combination with the ADA activity test in peripheral blood. A, single test; B, combination. ADA=adenosine deaminase; PB=peripheral blood; PF=pleural fluid; ROC=receiver operating characteristic.

and pleural fluid for ADA activity was associated with increased sensitivity (93.8%/96.9%), decreased specificity (70%/87.5%), decreased PPV (79.1%/90.4%), increased NPV (90.3%/95.9%), decreased +LR (3.127/7.753), and decreased -LR (0.088/0.035), respectively. In contrast, serial assessment of peripheral blood and pleural fluid with T-SPOT.TB and pleural fluid for ADA activity showed decreased sensitivity (54.6%/54.6%), increased specificity (98.8%/97.5%), increased PPV (98.1%/96.4%), decreased NPV (64.2%/63.9%), increased +LR (43.711/21.856), and increased -LR (0.459/0.465), respectively. Overall, a slight increase in AUC was found for the combination testing with T-SPOT.TB and ADA activity in pleural fluid samples (0.959) compared with that for the T-SPOT.TB in peripheral blood in combination with the ADA activity in pleural fluid (0.950).

3.5. Diagnostic performance with the combination of pleural fluid T-SPOT.TB, pleural fluid ADA activity, and peripheral blood T-SPOT.TB

Figure 2B shows the ROC curve for the 3 assays in combination. The probability of tuberculous pleurisy was expressed using the

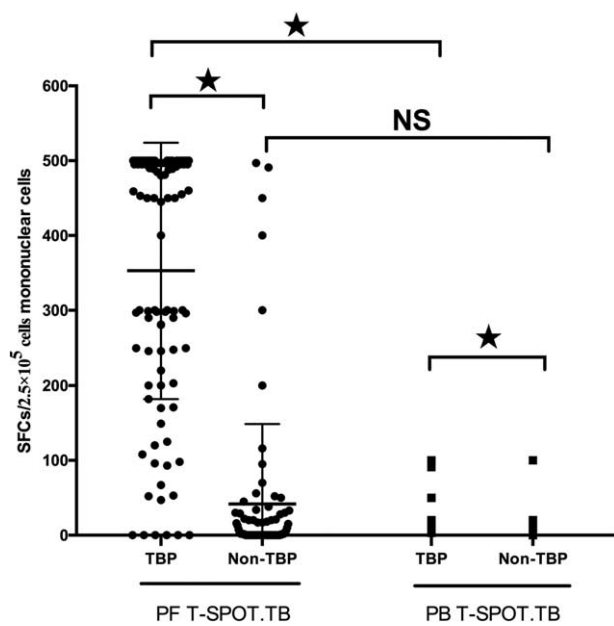


Figure 3. Scatter plots of SFCs using T-SPOT.TB and T-SPOT.TB in pleural fluid and peripheral blood between the tuberculous pleurisy and non-tuberculous pleurisy groups. Group comparison was performed by the Mann-Whitney *U* test. NS=not significant; PB=peripheral blood; PF=pleural fluid; SFCs=spot-forming cells; TBP=tuberculous pleurisy. **P* < .001.

following logistic equation: $Y = -3.725 + 0.051 \times ADA + 0.009 \times \text{pleural fluid T-SPOT.TB} + 0.042 \times \text{peripheral blood T-SPOT.TB}$. The cut-off value of *Y* for tuberculous pleurisy diagnosis was 0.368. As summarized in Table 3, the sensitivity, specificity, PPV, NPV, and AUC of the 3 assays in combination were 97.9% (95% CI, 93.4–99.6), 87.5% (95% CI, 82.0–89.6), 90.5% (95% CI, 86.3–92.0), 97.2% (95% CI, 91.1–99.5), and 0.964.

4. Discussion

The diagnosis of tuberculous pleurisy in clinical settings remains a significant challenge.^[25] The diagnostic performance of single assays such as the measurement of ADA activity or T-SPOT.TB often shows low sensitivity. However, the diagnostic performance of T-SPOT.TB in pleural fluid and peripheral blood in combination with the assessment of ADA activity in pleural fluid has not been well studied. In this study, the optimal cut-off value of T-SPOT.TB in pleural fluid was 95.5 SFCs/2.5 × 10⁵ cells. Compared with the single assays, both the 2-component and 3-component tests showed improved diagnostic performance for tuberculous pleurisy.

Our data showed that higher values for all diagnostic parameters (i.e., +/-LR, PPV, NPV, AUC) were obtained using T-SPOT.TB in pleural fluid compared with those using T-SPOT.TB in peripheral blood; this was in line with the findings by Zhang et al^[19] and Losi et al.^[24] The higher value of the T-SPOT.TB in pleural fluid samples might benefit from a larger number of antigen-specific T lymphocytes and higher IFN-γ levels produced by these cells^[26,27].

The optimal cut-off value of T-SPOT.TB in pleural fluid was calculated as 95.5 SFCs/2.5 × 10⁵ cells, corresponding to 382 SFCs/10⁶ cells. A study conducted in Peking Union Medical College Hospital found a far lower optimal cut-off value (56

SFCs/10⁶ serous effusion mononuclear cells) for the diagnosis of tuberculous serositis^[5]; the inclusion of tuberculous peritonitis and tuberculous pericarditis patients in addition to tuberculous pleurisy patients might be a contributing factor for this difference. Kang et al^[18] and Liu et al^[28] reported comparable cut-off values of 75 SFCs/2.5 × 10⁵ cells and 216 SFCs/10⁶ cells, respectively. However, both studies demonstrated higher sensitivity and specificity (90.5% and 93.3% by Kang et al,^[18] 96.3% and 94.5% by Liu et al,^[28] respectively) with T-SPOT.TB. These observed diagnostic differences might be due to the exclusion of probable tuberculous pleurisy patients, limited number of patients, or different prevalence rates. On the basis of the cut-off value of 95.5 SFCs/2.5 × 10⁵ cells, the specificity of T-SPOT.TB in pleural fluid was determined to be 91.2%, indicating a false-positive probability of 8.8%. Seven patients (2 with parapneumonic effusions and 5 with lung cancer) exhibited a false-positive result. Mazurek et al^[29] reported that certain bacteria, such as *Mycobacterium kansasii*, *Mycobacterium szulgai*, and *Mycobacterium marinum*, also express MTB, CFP-10, and ESAT-6 proteins. Therefore, they eventually can cause false positivity in T-SPOT.TB in pleural fluid via the stimulation of IFN-γ secretion. Hematologic malignancies and empyema might also lead to false-positive results via the induction of IFN-γ levels in pleural fluid.^[30] Future studies are necessary to identify factors associated with false positivity with T-SPOT.TB.

Although a larger AUC and a higher specificity were observed with the ADA activity in pleural fluid than in those in T-SPOT.TB in pleural fluid, the sensitivity (61.9%) was extremely unsatisfactory. Liao et al^[17] inferred that parapneumonic effusion was the most likely reason for the low sensitivity (55.5%) of ADA activity in pleural fluid. They reported that the ADA activity levels in patients with parapneumonic effusion were higher than those in patients with tuberculous pleurisy and that a large portion of the patients with pneumonia were positive for ADA activity in pleural fluid.^[17] Among other reasons underlying the limited utility of ADA activity in pleural fluid for TBD diagnosis are the nonspecific inflammation and the immune response in TB,^[31] higher false negativity rates in individuals with human immunodeficiency virus infection and liver cirrhosis,^[6] and occasional false positivity in patients with malignancy-related fluid or bacterial pneumonia.^[6]

Compared with single assays, 2 assays in combination were shown to improve diagnostic power for tuberculous pleurisy in a number of studies: QuantiFERON TB Gold In-Tube test in combination with nested polymerase chain reaction in regions with high TB prevalence,^[32] ADA activity level in combination with lymphocyte percentage in three different prevalence scenarios,^[25] and ADA in combination with IFN-γ activity in Taiwan.^[33] In addition, serial and parallel combination of T-SPOT.TB in PBMCs and serous effusion mononuclear cells were performed to diagnose TB serositis, with evident improvement in the specificity and +LR using the serial combination test.^[19] The current study is unique, as we tested serial and parallel combinations of T-SPOT.TB in pleural fluid and peripheral blood with the ADA activity test in pleural fluid. Our findings also revealed that serial combination of tests had increased specificity, PPV and +LR. Furthermore, a combination of T-SPOT.TB in pleural fluid with the ADA activity measurement in pleural fluid exhibited greater diagnostic performance, as determined by all parameters, compared with the combination of T-SPOT.TB in peripheral blood with the ADA activity in pleural fluid, which has been rarely evaluated.

Table 3
Single and combined diagnostic parameters of PF T-SPOT.TB, PB T-SPOT.TB, and PF ADA activity test.

Parameters	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	+LR (95% CI)	-LR (95% CI)	AUC (95% CI)
PF ADA activity test	61.9 (56.3–64.4)	95.0 (88.3–98.3)	93.8 (85.4–97.9)	67.3 (62.5–69.6)	12.371 (4.817–39.001)	0.402 (0.360–0.494)	0.944 (0.901–0.987)
PB T-SPOT.TB	86.6 (80.2–91.6)	72.5 (64.8–78.5)	79.2 (73.4–83.8)	81.7 (73.0–88.5)	3.149 (2.280–4.259)	0.185 (0.108–0.305)	0.881 (0.828–0.934)
PF T-SPOT.TB	88.7 (83.2–92.2)	91.2 (84.7–95.6)	92.5 (86.8–96.2)	86.9 (80.7–91.0)	10.133 (5.437–20.828)	0.124 (0.081–0.198)	0.918 (0.872–0.963)
PB T-SPOT.TB and PF ADA activity test (parallel test)	93.8 (88.1–97.3)	70.0 (63.1–74.2)	79.1 (74.3–82.1)	90.3 (81.4–95.8)	3.127 (2.388–3.778)	0.088 (0.036–0.188)	0.950 (0.909–0.991)
PB T-SPOT.TB and PF ADA activity test (serial test)	54.6 (50.0–55.6)	98.8 (93.1–99.9)	98.1 (89.7–99.9)	64.2 (60.5–65.0)	43.711 (7.211–850.408)	0.459 (0.444–0.538)	0.964 (0.926–0.992)
PF T-SPOT.TB and PF ADA activity test (parallel test)	96.9 (92.1–99.2)	87.5 (81.7–90.2)	90.4 (85.9–92.5)	95.9 (89.6–98.9)	7.753 (5.041–10.139)	0.035 (0.009–0.096)	0.959 (0.926–0.992)
PF T-SPOT.TB and PF ADA activity test (serial test)	54.6 (49.6–56.3)	97.5 (91.4–99.6)	96.4 (87.5–99.4)	63.9 (59.9–65.3)	21.856 (5.769–128.584)	0.465 (0.439–0.551)	0.959 (0.926–0.992)
Logistic (PF ADA activity test, PF T-SPOT.TB, PB T-SPOT.TB)	97.9 (93.4–99.6)	87.5 (82.0–89.6)	90.5 (86.3–92.0)	97.2 (91.1–99.5)	7.835 (5.187–9.537)	0.024 (0.004–0.081)	0.964 (0.934–0.995)

–LR = negative likelihood ratio, +LR = positive likelihood ratio, ADA = adenosine deaminase, AUC = area under the ROC curve, CI = confidence interval, NPV = negative predictive value, PB = peripheral blood, PF = pleural fluid, PPV = positive predictive value.

Research on the efficacy of these 3 tuberculous pleurisy diagnostic assays in combination has been limited to date. Combination of 3 biomarkers (interleukin 27, ADA, and IFN- γ) was able to yield high sensitivity and specificity.^[34] He et al^[35] evaluated the diagnostic value of a flow chart applying T-SPOT.TB, ADA activity, and medical thoracoscopy, which also had a high diagnostic power. One meta-analysis also showed that the cut-off value of ascitic ADA is 39IU/L, with high sensitivity (93%–100%) and high specificity (92%–100%).^[36] Gheorghita et al^[37] reported that the sensitivity and specificity of ascitic ADA could achieve 100% and 97%, and 98.4% and 95.9% for serum CA-125, but have not used them in combination. To the best of our knowledge, this was the first investigation that adopted logistic regression analysis to assess the performance of the combination of T-SPOT.TB in pleural fluid and peripheral blood with ADA activity in pleural fluid. Of note, compared with the single and 2-component combination tests, the highest sensitivity (97.9%) and AUC value (0.964) were found in this 3-assay combination, indicating its high diagnostic performance for tuberculous pleurisy. Florea et al^[38] have evaluated 1 PCR/ESI-MS method for the detection of *M. tuberculosis* drug resistance. PCR/ESI-MS also has a high sensitivity and specificity, but the high costs would limit its use.

A few of limitations should be considered in the present study. First, medical thoracoscopy and pleural biopsy were not routinely performed due to various reasons, including old age, refusal of consent from selected patients, and complications. A total of 56 tuberculous pleurisy patients did not have the results of histopathological evaluation, and MTB could not be cultured from pleural biopsy samples. However, all 56 patients were successfully treated with regular anti-TB regimens. Therefore, these patients were included in the tuberculous pleurisy group, which might have a slight effect on the results. Second, only 4 patients with empyema and high ADA activity levels were eventually included in the analyses, which might impact the diagnostic performance of the ADA activity test in pleural fluid samples. Third, the pleural effusion TB culture method has too many deficiencies to be suitable for clinical practice, such as long time waiting and poor sensitivity. So, a total of 28 cases including 23 TB pleurisy and 5 non-TB pleurisy perform pleural fluid TB culture according to patients' condition. The premise of empirical anti-TB treatment is ADA levels that are higher than 45IU/L, so some patients with tumors (confirmed) and TB pleurisy (not confirmed) may not be diagnosed, due to not performing the TB culture and not starting empirical anti-TB treatment. The design of our study may overestimate the sensitivity and the specificity of ADA. Fourth, the diagnostic prediction model we adopted was not validated, and prospective studies are warranted to validate this prediction model. Patients with pleural effusion of unknown aetiology with microbiological and histopathological results should also be included to determine whether patients with tuberculous pleurisy are suitable for this model.

In conclusion, T-SPOT.TB in pleural fluid performed better than the ADA activity test in pleural fluid and T-SPOT.TB in peripheral blood for tuberculous pleurisy diagnosis. The optimal cut-off value for T-SPOT.TB in pleural fluid was 95.5SFCs/ 2.5×10^5 cells. In addition, a combination of T-SPOT.TB and the ADA activity test in pleural fluid and peripheral blood and the ADA activity test in pleural fluid demonstrated the best diagnostic performance for tuberculous pleurisy. Future prospective studies are necessary to improve the diagnostic utility of these methods.

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