Original Article

Diagnostic Utility of Interferon-Gamma Release Assay in Tuberculous Lymphadenitis

Xinchao Liu^{1†}, Susu Ye^{1†}, Wenze Wang², Yueqiu Zhang¹, Lifan Zhang^{1,3}, Xiaocheng Pan⁴, Ziyue Zhou⁴,
Miaoyan Zhang⁴, Jianghao Liu⁴, Zhiyong Liang², Xiaoqing Liu^{1,3*}

1 Department of Infectious Diseases, Peking Union Medical College Hospital

2 Department of pathology, Peking Union Medical College Hospital

3 Clinical Epidemiology Unit, International Epidemiology Network,

4. Department of Internal Medicine, Peking Union Medical College Hospital

Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China

Key words: Tuberculous lymphadenitis (TBL); T-SPOT.*TB*; diagnostic test; sensitivity; specificity

Objective The aim of this study was to evaluate the diagnostic performance of T-SPOT.*TB* for tuberculous lymphadenitis.

Methods Suspected tuberculous lymphadenitis patients between September 2010 and September 2018 who had both peripheral blood T-SPOT.*TB* test and lymph node biopsy were retrospective enrolled in this study. The cutoff value of T-SPOT.*TB* test for peripheral blood was set as 24 SFC/10⁶PBMC according to the testing kits. The gold standard for diagnosis of TBL is combination of tissue culture or acid-fast staining, or histological findings consistent with TBL and clinical or radiological response to anti-TB treatment. Diagnostic efficacy of T-SPOT.*TB* was evaluated, including sensitivity, specificity, accuracy, predictive values, and likelihood ratio.

Results Among 91 patients who met the inclusion criteria, we excluded 8 cases with incomplete clinical information and 6 cases who lost of follow-up. According to the gold standard, there were 37 cases of true TBL (9 confirmed TBL and 28 probable TBL), 30 cases of true non-TBL, and 10 patients of clinically indeterminate diagnosis which were excluded from the final analysis. T-SPOT.*TB* tests yielded 43 cases of positive response and 24 cases of negative response by. The sensitivity, specificity, accuracy, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR) and negative likelihood ratio (NLR) of peripheral blood T-SPOT.*TB* for diagnosing TBL were 89.2%, 66.7%, 79.1%, 76.7%, 83.3%, 2.676 and 0.162, respectively. The number of spot forming cells (SFCs) of T-SPOT.*TB* were [432(134-1264)/10⁶PBMCs] in TBL patients, higher than that in non-TBL patients [0 (0-30) /10⁶PBMCs] with significant statistical difference ($Z=-5.306$, $P<0.001$).

Conclusions T- SPOT.*TB* is a rapid and simple diagnostic test for TBL with a high sensitivity and negative predictive value.

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* Corresponding author phone 010-69155087; E-mail: liuxq@pumch.cn

† These authors contribute equally

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Tuberculosis (TB), an infectious disease and serious health hazard, caused 1.3 million deaths among HIV-negative people in 2017 according to the World Health Organization (WHO).¹ China bears a high burden of TB in the world. According to the estimation of WHO, in 2016 China had 895 000 new infections of TB, which ranked only after India and Indonesia. Tuberculous lymphadenitis (TBL) is the most common extra-pulmonary tuberculosis, which accounts for 4.0% to 5.1% of all tuberculosis and 14.5% to 50.0% of extra-pulmonary tuberculosis cases.²⁻⁵ The clinical manifestations of TBL can be atypical, and misdiagnosis is common. It usually requires tissue culture and pathological verification to make a definitive diagnosis. However, the cultivate yield from lymph node biopsy is usually extremely low, therefore it is paramount to develop a rapid, sensitive and less invasive diagnostic utility.

Interferon gamma release assay (IGRA), including QuantiFERON-*TB* Gold and T-SPOT.*TB*, is based on cellular immune response, and has been introduced into the diagnosis of tuberculosis in recent years. It was reported that IGRA used in the diagnosis of TBL had sensitivities of 78.8%-95.2% and specificities of 52%-95.5%.^{4,6-13} The great varieties of these results are likely due to the inability to acquire both IGRA and lymph node biopsy simultaneously, and the small sample size in each study. Some studies were designed for all entities of extra-pulmonary tuberculosis rather than TBL specifically, making them less specific for TBL evaluation.^{4,6,12,13} This study was designed to explore the diagnostic utility of T-SPOT.*TB* in TBL by using results from tissue culture, pathology and clinical follow-up.

MATERIALS AND METHODS

Patients selection

This retrospective study was reviewed and approved by the Institutional Review Board of the hospital and patient information consent was waived. The study was designed to evaluate suspected TBL patients from Peking Union Medical College Hospital between September 2010 and September 2018. We retrospectively searched databases to retrieve all in- and outpatients with both results of lymph node biopsy pathology and peripheral blood T-SPOT.*TB*. Histological diagnosis was obtained for the lymphadenopathy from all cases either by thoracic/abdominal surgery or by superficial lymph node biopsy. Clinical information including demographic characteristics, symptoms, underlying conditions, sites of biopsy etc, was acquired through chart review. The inclusion criteria for patients are as follows: 1. Age greater than 18 years old; 2. at least one of the following manifestations: (1) symptoms of fever, weight loss, or night sweat; (2) peripheral lymph node swelling by physical examination; (3) lymph node enlargement confirmed by CT or MRI. Cases with insufficient chart information to make definitive diagnosis or lost follow up were excluded.

Two physicians specialized in infectious disease made categorization of patients' diagnosis independently based on patients' clinical manifestations, imaging findings, microbiology, pathology results, and therapeutic effect of anti-tuberculosis treatment in the follow up period without knowing T-SPOT.*TB* results. For cases whose definitive diagnosis could not be reached from the initial visit, we further reviewed medical records of the following three months, or we followed up by phone call to determine anti-TB treatment response of the patient. When the two physicians perceived different opinions, a third specialist would be consulted for the final diagnosis.

Diagnostic categorization for TBL

The gold standard for TBL was the combination of microbiology result, pathology result, and patients' response to anti-tuberculosis treatment. Patients were categorized into 4 groups according to protocols in the study of Liao CH *et al.*³ Confirmed TBL and probable TBL was considered as true TBL according to the gold standard.

1. Confirmed TBL: *Mycobacterium tuberculosis* (MTB) culturing of lymph node tissue is positive, or tissue Acid-fast staining is positive.
2. Probable TBL: histological findings are consistent with TBL (granulomatous inflammation and/or caseous necrosis and/or multinucleate giant cell), and the patient responds clinically and radiologically to anti-TB treatment.
3. Indeterminate TBL: do not meet the above diagnostic criteria or fail to reach a conclusive diagnosis during a 3-months follow-up.
4. Non-TB: alternative diagnosis is made, or there is clinical improvement without anti-TB therapy.

T-SPOT.TB test

T-SPOT.TB is generally ordered for patients who frequently present with fever, weight loss, night sweat, and elevated erythrocyte sedimentation rate (ESR), suspecting for TB infection. We collected 4 ml of heparin-anticoagulated venous blood from these patients, and blood samples were sent for T-SPOT.TB (Oxford Immunotec, Abingdon, UK) test within 6 hours by a laboratory personnel who was blinded to patients' clinical data. T-SPOT.TB utilized AIM-V (GIBCO™ AIM V Medium liquid, Invitrogen, US.) as negative control, phytohemagglutinin (PHA) as positive control, early secreted antigenic target 6-kDa protein (ESAT-6) and culture filtrate protein 10 (CFP-10) as specific antigens, respectively. Peripheral blood monocyte cells (PBMC) from each subject were separated and prepared at a concentration of $2.5 \times 10^6/\text{ml}$, and then plated (2.5×10^5 per well) on a plate precoated with antibody against interferon γ . Plates were incubated 16-18 h at 37°C in 5% carbon dioxide. After incubation, wells were developed with a conjugate against the antibody used and an enzyme substrate. Spot-forming cells (SFCs) were counted with an automated ELISPOT reader (AID-ispot, Strassberg, Germany), with each SFC representing an antigen-specific T cell secreting interferon γ . The response was considered positive if the antigen well contained 6 or more spots and twice the number of spots as seen in the negative control well based on the T-SPOT.TB kit (Oxford Immunotec, Abingdon, UK) operating instruction. The background spots number should be less than 10 of the negative control well for PBMC and greater than 20 of the positive control well for PBMC. The cutoff value of T-SPOT.TB test for peripheral blood was set as 24 SFC/ 10^6 PBMC according to the testing kits.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (SPSS Inc, Chicago, IL). Normal distribution was determined by Kolmogorov-Smirnov tests. Continuous data of normal distribution was expressed as mean $\pm$ standard deviation, while non-normal distributional data was expressed as medians and interquartile ranges. Classification data was expressed as percentage. T-test and Mann-Whitney U test were used to compare continuous variables of normal distribution and non-normal distribution, respectively. The 95% confidence interval (95% CI) was estimated from the binomial distribution. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the subjects

From patients who had both T-SPOT.*TB* and lymph node biopsy pathology reports available in our database, 91 patients with suspicious tuberculous lymphadenitis were included in this study. Among them, we excluded 6 cases who lost follow up and 8 cases with incomplete clinical information from analysis. The process of patient selection is illustrated in **Figure 1**. Of the remaining 77 patients, 28 were male and 49 were female; their age ranged from 18 to 75 years old, with mean of 47.9 ± 14.1 years old. The HIV serological antibody tests of all subjects were negative. There were 9 confirmed TBL cases, 28 probable TBL cases, 10 indeterminate TBL cases, and 30 non-TB cases. The clinical demographic characteristics of the enrolled patients are shown in **Table 1**.

Figure 1. Flowchart of enrolling patients in the study.

Table 1. Demographic and clinical characteristics of suspected TBL patients enrolled ($n=77$)

Characteristic	Confirmed TBL $n=9$	Probable TBL $n=28$	Indeterminate $n=10$	Non-TBL $n=30$
Age (years, mean $\pm$ SD)	43.1 $\pm$ 17.3	46.5 $\pm$ 16.0	49.9 $\pm$ 11.9	50.0 $\pm$ 12.0
Gender (n)				
male	5	7	4	12
female	4	21	6	18
TB history positive (n)	1	6	0	1
Co-Medication (n)				
Steroids	1	5	2	2

Immunosuppressive agent*	1	2	0	1
Underlying condition (n)	7(77.8%)	8(28.6%)	3(30%)	6(20%)
Cancer	1	1	1	2
Diabetes mellitus	2	2	1	2
Chronic liver diseases	2	1	1	0
Chronic kidney diseases	1	0	0	1
Autoimmune diseases	1	4	0	1
Blood tests				
Leukocyte				
($\times 10^9/L$, median IQR)	7.55 (6.04-8.41)	6.09 (4.84-7.67)	4.17 (3.98-6.23)	7.82 (4.64-11.78)
Lymphocyte	1.60	1.37	0.97	1.37
($\times 10^9/L$, median IQR)	(1.07-3.14)	(1.07-1.78)	(0.77-1.54)	(0.97-1.91)
Erythrocyte sedimentation rate	38 (13-57)	12 (4.51-19.75)	43 (23-69)	22 (14.25-39.25)
C-reactive protein (mg/dL, median IQR)	8.3 (4.1-49.0)	5.9 (1.7-18.8)	6.1 (2.8-10.3)	4.9 (2.1-32.4)
Site of lymph node biopsy* (n)	13	30	14	31
Postauricular	0	2	0	0
Submandibular	1	2	1	0
Cervical	2	7	3	8
Supraclavicular	3	5	1	4
Axillary	1	1	1	2
Mediastinal	4	8	4	13
Celiac	2	5	4	1
Inguinal	0	0	0	3
Histopathologic finding (n)				
Granuloma	9	28	10	26
Caseous necrosis	6	9	0	0
Multinucleate giant cell	4	16	4	6

*After administration of Thalidomide and Cyclosporin A. †Certain patient may have more than one lymph node biopsy.

TB, tuberculosis; TBL, tuberculous lymphadenitis; IQR, interquartile range.

The diagnostic performance of T-SPOT.TB for TBL

Of the enrolled 77 patients, according to the gold standard, 37 patients were true positive of TBL (9 confirmed TBL cases and 28 probable TBL cases), 30 cases were true negative of TBL. The indeterminate TBL cases (n=10) were excluded from the diagnostic test. Of 37 TBL cases, T-SPOT.TB was positive in 33 cases, with the sensitivity of 89.2% (95% CI, 73.6% - 96.5%). In 30 true non-tuberculosis patients, 20 cases were negative for T-SPOT.TB, with the specificity of 66.7% (95% CI, 47.1% - 82.1%) (**Table 2**). The accuracy of T-SPOT.TB for TBL was 79.1%. The positive predictive value, negative predictive value, positive likelihood ratio and negative likelihood ratio of T-SPOT.TB for TBL were 76.7% (95% CI, 61.0%-87.7%), 83.3% (95% CI, 61.8%-94.5%), 2.68 (95% CI, 1.59-4.49) and 0.16 (95% CI, 0.06-0.42), respectively.

Table2. Diagnostic yields of T-SPOT.TB for the suspected tuberculous lymphadenitis

T-SPOT.TB	Diagnosed by gold standard		Total
	TBL	Non-TBL	
Positive	33	10	43
Negative	4	20	24
Total	37	30	67

The number of IFN - γ - secreting T cells(SFCs, Spot Forming Cells) in peripheral blood

The number of **ESAT-6** antigens in true TBL group and true non-TBL group were 236 (48-508) SFCs / 10^6 PBMCs and 0(0-0) SFCs / 10^6 PBMCs, respectively ($Z=-5.274$, $P < 0.001$) (**Figure 2**). The number of **CFP-10** antigens in the TBL group and non-TBL group were 148 (36-690) SFCs / 10^6 PBMCs and 0 (0-28) SFCs / 10^6 PBMCs, respectively ($Z=-4.630$, $P < 0.001$). The *tuberculosis mycobacteria* specific antigen T-SPOT.TB SFCs, which was the sum of ESAT-6 and CFP-10 SFCs, of the TBL group and non-TBL group were 432(134-1264) SFCs / 10^6 PBMCs and 0 (0-30) SFCs / 10^6 PBMCs, respectively ($Z=-5.306$, $P < 0.001$).

Figure2. The number of mycobacterium tuberculosis specific IFN- γ secreting T cells in peripheral blood of TBL patients and Non-TBL patients.

Significant difference in the number of ESAT-6, CFP-10 and total T-SPOT.TB spots between TBL group and the non-TB group (all $P < 0.001$). The segmentation line indicates the threshold value of T-SPOT.TB (24 SFCs/ 10^6 PBMC). Results below the threshold were all present as 0 in the final report. ESAT-6, early secreted antigenic target 6-kDa protein; CFP-10, culture filtrate protein 10.

DISCUSSION

Tuberculosis is an important threatening for public health. In 2017 it caused up to 1.3 million and 3.0 million deaths in HIV negative and positive patients respectively.¹ Tuberculous lymphadenitis is one of the most common extra pulmonary tuberculosis. The clinical manifestations of tuberculous lymphadenitis are mostly non-specific, which makes the definitive diagnosis quite challenging. At present, the most commonly used test for *Mycobacterium tuberculosis*

infection is still the tuberculin skin test. But its low sensitivity and low specificity,^{14,15} potential influenced by bacille Calmette-Guerin (BCG) vaccination and environmental mycobacterial infection, not being applicable in immunosuppressed patients, and severe tuberculosis patients, have limited its universal clinical application.

Ever since Koch discovered *Mycobacterium tuberculosis* in 1882, acid-fast staining has been the cornerstone of tuberculosis diagnosis.¹⁶ The number of *Mycobacterium tuberculosis* in tissue samples needs to be greater than 10⁴/ml to yield a positive acid-fast staining result. But tuberculous lymphadenitis usually contains very few *Mycobacterium tuberculosis*, which makes the sensitivity of acid-fast staining in diagnosing TBL extremely low (0-40%).^{17,18} Also, non-tuberculous mycobacterium can be acid-fast positive. We found only 9 out of 37 TBL patients (24.3%) were positive for acid-fast staining in this study. *Mycobacterium tuberculosis* culture and identification is still diagnostic gold standard, which usually takes 4-6 weeks to isolate, and the positive rate of culturing can be as low as 30% - 67.2%.¹⁹ Polymerase chain reaction (PCR) can be a useful method in diagnosing *Mycobacterium tuberculosis* infection, with sensitivities and specificities of 88%-90.3% and 82%-100%, respectively,²⁰⁻²² and the result can be obtained in a few hours. However, PCR in diagnosing TBL requires invasive methods for sampling, professional staff and advanced laboratory facilities. Furthermore, it cannot determine whether the *Mycobacterium tuberculosis* in tissue is alive or not.

23,24

In recent years, IFN- γ release assays (IGRA) have been developed for screening tuberculosis infections, including whole blood IFN- γ enzyme-linked immunosorbent assay (QuantiFERON-TB Gold) and enzyme-linked immunospot assay (T-SPOT.TB). T-SPOT.TB is a new immunological diagnostic tool for the detection of *Mycobacterium tuberculosis* infection. It is not influenced by BCG vaccination and most infections of non-tuberculous mycobacteria. The sensitivity of T-SPOT.TB is also high in that one specific T lymphocytes can be detected in every 1 million peripheral blood mononuclear cells and the entire detection process only takes 2 days.²⁵ According to the peripheral blood T-SPOT.TB result in current study, the number of spot forming cells was significantly higher in TBL patients than in the non-TBL patients. Peripheral blood T-SPOT.TB showed a good sensitivity (89.2%) but a relative low specificity (66.7%) for diagnosis of TBL. These results were comparable with that in previous reports where T-SPOT.TB in diagnosing TBL had sensitivities of 81% - 95%, specificities of 52% - 80%.¹⁰⁻¹³ It was reported that false negative T-SPOT.TB results might be associated with elderly age, serious comorbidities such as liver cirrhosis, and other underlying diseases.^{26,27} Thus, T-SPOT.TB should be interpreted discreetly in these population. In the current study, 4 cases of 37 TBL patients were T-SPOT.TB negative.

We found that peripheral blood T-SPOT.TB had a good negative predictive value (83.3%) for TBL, which was high, and this result was consistent with the study of Jia *et al*¹⁰ who reported a negative predictive value as high as 95.9%. Therefore, T-SPOT.TB may have a great value in excluding the diagnosis of TBL. Of the non-TBL group, 10 patients had positive T-SPOT.TB results (FPR 33.3%), but only one of them had a history of tuberculosis. We speculate that these cases may have latent tuberculosis infections. Gao and colleagues reported a high prevalence of latent tuberculosis in China,²⁸ with the QuantiFERON-TB positive rate being 13% -20%, which may explain the relatively low specificity of T-SPOT.TB in diagnosing TBL.

In this study the number of T cells that responded to ESAT-6 in the TBL group appeared to be higher than the number of T cells that responded to CFP-10, but the difference was not statistically significant. Similar findings were reported in female genital tuberculosis patients ²⁹ and in active TB infection populations. ³⁰ However, as these studies are not specifically targeting patients of TBL, more TBL specific researches are needed.

TBL is generally considered to be prevalent mainly in children. However, to our clinical experience in recent year, it is commonly seen in people of 20-40 years old, especially in women. The gender preference was documented in this study as well. The reason women are more susceptible to TBL is still unclear. Tumor necrosis factor and interleukin-10 may play a role ³¹. CD4⁺ lymphocyte counts, endocrinal factors, socioeconomic factors, and cultural factors might also be involved in the gender difference.³² The most commonly involved lymph node of TBL in our study was mediastinal lymph nodes (32.4%), followed by cervical lymph nodes (24.3%), supraclavicular lymph nodes (21.6%). Jia *et al.* found that the most commonly involved lymph node was cervical lymph nodes 86.7%, followed by axillary lymph nodes 6.0% and inguinal lymph nodes 3.6%. ¹⁰ The inconsistencies in this aspect may be largely associated with differences in patients characteristics of the healthcare facilities, where the hospital in our study receives more complicated and severe cases with swollen deep lymph nodes.

Our study has several limitations. This is a retrospective study and the sample size is relatively small, which may introduce bias that leads to either over or under-estimation of the final outcome. In addition, as the healthcare institution where study was conducted is the National Center for Difficult and Complicated Cases, patients in this study are generally severe or complicated cases, the results should be applied with caution when T-SPOT.*TB* being applied to hospitals with different patient characteristics. It is necessary to perform multi-centric large sample-sized studies to further evaluate the diagnostic value of T-SPOT.*TB*.

In summary, the study suggests that T-SPOT.*TB* may be considered as a simple, rapid, highly sensitive tool for TBL diagnosis with good negative predictive value.

Conflict of interest statement

All authors declared no conflict of interests.

Article information

Partial results of this study were presented by poster at IDWeek conference 2017, San Diego.

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