Evaluation of risk factors for false-negative results with an antigen-specific peripheral blood-based quantitative T cell assay (T-SPOT®.*TB*) in the diagnosis of active tuberculosis: A large-scale retrospective study in China

Chi Yang, Shaojun Zhang, Lan Yao and Lin Fan

Abstract

Objective: To investigate the diagnostic efficacy of an interferon-γ release assay, T-SPOT®.*TB*, for diagnosing active tuberculosis (TB) and to identify risk factors for false-negative results.
Methods: This retrospective study enrolled consecutive patients with active TB and with non-TB respiratory diseases to evaluate the risk factors for false-negative results when using the T-SPOT®.*TB* assay for the diagnosis of active TB. Patients with active TB were categorized as having confirmed pulmonary TB, clinically diagnosed pulmonary TB or extrapulmonary TB (EPTB).
Results: This study analysed 4964 consecutive patients; 2425 with active TB and 2539 with non-TB respiratory diseases. Multivariate logistic regression analyses identified the following five factors that were all associated with an increased false-negative rate with the T-SPOT®.*TB* assay: increased age (odds ratio [OR] 1.018; 95% confidence interval [CI] 1.013, 1.024); decreased CD8+ count (OR 0.307; 95% CI 0.117, 0.803); negative sputum acid-fast bacilli (AFB) smear staining (OR 1.821; 95% CI 1.338, 2.477); negative mycobacterial cultures (OR 1.379; 95% CI 1.043, 1.824); and absence of EPTB (OR 1.291; 95% CI 1.026, 1.623).
Conclusions: Increased age, decreased CD8+ count, negative sputum AFB smear results, negative sputum mycobacterial cultures and absence of EPTB might lead to an increased false-negative rate when using the T-SPOT®.*TB* assay.

Corresponding author:
Lin Fan, Clinic and Research Centre of Tuberculosis, Shanghai Key Laboratory of Tuberculosis, Shanghai Pulmonary Hospital, Tongji University School of Medicine, 507 Zhengmin Rd, Shanghai 200433, China.
Email: fanlinsj@163.com

Clinic and Research Centre of Tuberculosis, Shanghai Key Laboratory of Tuberculosis, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China

Introduction

Tuberculosis (TB) remains one of the most prevalent infectious diseases worldwide, with approximately 10.4 million new confirmed cases and 1.4 million deaths in the world in 2015.[1] Due to the infectiousness, prevalence and severity of TB, it is essential to make an early and accurate diagnosis to control its spread. However, as a consequence of the low rate of bacteriological confirmation, the diagnosis of active TB mostly relies on a combination of prior exposure to TB, clinical symptoms, radiological findings, together with a tuberculin skin test (TST), which might also be positive in patients who have previously received the Bacillus Calmette-Guérin vaccination.

Interferon (INF)-γ release assays, including T-SPOT[®].*TB*, QuantiFERON-TB Gold (QFT-G) and QuantiFERON-TB Gold In-Tube (QFT-GIT), were developed to overcome the limitations of the TST and were expected to be more effective tools for the diagnosis of TB infection.[2,3] The T-SPOT[®].*TB* assay uses a mixture of ESAT-6 and CFP-10 synthetic peptides as *Mycobacterium tuberculosis*-specific antigens, whereas QFT-GIT uses a mixture of synthetic ESAT-6, CFP-10, and TB7.7 peptides.[4]

The T-SPOT[®].*TB* assay has been widely applied throughout China in recent years as a diagnostic tool for TB infection. However, doubt and controversy exist over its accuracy and efficacy for the diagnosis of active TB in geographical areas with high TB burdens. In particular, the heterogeneity of diagnostic efficacy for active TB varies from 70–90%.[5–8]

As a consequence of using an immunodiagnostic method to identify TB infection, factors associated with the immune responsiveness, such as age and CD4+ T cell count,[9–25] might influence the performance of the T-SPOT[®].*TB* assay. Relatively few studies have examined the influence of these factors on the accuracy the T-SPOT[®].*TB* assay.[26]

This large-scale survey of the T-SPOT[®].*TB* assay aimed to investigate its diagnostic efficacy for active TB and to identify risk factors for false-negative results.

Patients and methods

Patient population

This retrospective study analysed clinical data from all consecutive patients who were tested using the T-SPOT[®].*TB* assay and who were diagnosed as having active pulmonary TB or extrapulmonary tuberculosis (EPTB) at Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China between January 2013 and December 2014.

All active TB patients without HIV infection were recruited. The T-SPOT[®].*TB* assay results, acid-fast bacilli (AFB) staining, mycobacterial cultures and other relevant laboratory results and complications correlated with immune status, such as age, white blood cell (WBC) count, absolute

lymphocyte count, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), CD3+, CD4+, and CD8+ T cell counts, CD4+/CD8+ ratio, concomitant presence of diabetes mellitus, rheumatic disease or malignancy, were recorded for further analysis.

In addition, non-TB patients diagnosed with other respiratory diseases were enrolled as controls at the Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China between January 2013 and December 2014. The Institutional Review Board of Shanghai Pulmonary Hospital affiliated with Tongji University approved the study and waived the need for informed consent because no patients were at risk. Patient records were anonymized and de-identified prior to analysis.

Classification and diagnosis

Active TB was defined according to the World Health Organization guidelines as follows: (i) bacteriologically confirmed pulmonary TB (PTB): patients with *M. tuberculosis* complex isolated from clinical specimens by culture; (ii) clinically diagnosed pulmonary TB: patients with suspected PTB who were culture- and smear-negative, had pathological evidence of TB or had no pathological evidence of TB but improved following anti-TB treatment (any improvement in response to a course of broad-spectrum antibiotics) based on clinical evaluation and radiographic examinations for typical tuberculosis lesions; (iii) EPTB: patients with definite TB involving organs other than the lungs with at least one specimen with confirmed *M. tuberculosis* or histological or strong clinical evidence consistent with active EPTB, treated with a full course of anti-TB treatment at the clinician's discretion.[27] Non-TB patients diagnosed with other respiratory diseases were enrolled as controls.

Laboratory tests and examination

The T-SPOT®.*TB* assays were undertaken in accordance with the manufacturer's instructions (Oxford Immunotec, Abingdon, UK). All blood samples were collected from the median cubital vein in the morning, immediately prior to the tests to reduce potential interference. For those patients who had recently received a blood transfusion or those who had undergone positron emission tomography/computed tomography scans within 1 week of the test, a repeated test was recommended 2 weeks later. Peripheral blood mononuclear cells (PBMCs) were isolated from 2 ml heparinized venous blood using Ficoll-Hypaque gradient centrifugation (Sigma-Aldrich, St Louis, MO, USA) using an Eppendorf centrifuge (Eppendorf, Hamburg, Germany) at 400 g for 30 min at 20 °C. The PBMCs were seeded onto precoated IFN-γ EliSPOT plates (Oxford Immunotec) followed by incubation with a medium not containing an antigen (negative control), or a medium containing *M. tuberculosis*-specific peptide antigens from ESAT-6 (panel A) or *M. tuberculosis*-specific peptide antigens from CFP-10 (panel B), or a medium containing 5 μg/ml phytohaemagglutinin (positive control) (Sigma-Aldrich) in a 5% CO_2 atmosphere at 37 °C for 20 h in order to stimulate INF-γ secretion by the T cells.[28–30] The spot-forming cells (sfu number) were counted by an EliSPOT plate reader (AID GmbH, Strasberg, Germany). The results of the T-SPOT®.*TB* assay were considered positive if either panel A or panel B had ≥ 6 sfu number than the negative controls; and this number was at least two-times greater than the sfu number in the negative wells. All tests were performed before the patients received anti-TB medication. All sputum specimens were tested with AFB staining and all mycobacterial cultures were evaluated using the BD BACTEC™

MGIT™ automated mycobacterial detection system (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Statistical analyses

Data obtained from medical records were analysed using MedCalc® software version 9.0.1.1 (MedCalc Software, Ostend, Belgium). Demographic and clinical characteristics were compared using χ^2-test. Age, WBC count, absolute lymphocyte count, CRP, ESR, CD3+ count, CD4+ count, CD8+ count, CD4+/CD8+ ratio, sputum AFB smear results, sputum mycobacterial cultures results, combined with malignancy, diabetes mellitus, rheumatic disease or EPTB were included in the univariate logistic regression analyses. Then, the variables with P-values < 0.2 in the univariate analyses were subjected to subsequent multiple logistic regression analyses to calculate their odd ratio (OR) and P-value. Factors with a P-value < 0.05 would be considered as risk factors. Furthermore, to avoid collinearity between the variables in multiple analyses, their variance inflation factors (VIF) were measured and factors with VIF > 5 would be regarded as having collinearity. Receiver operating characteristic (ROC) curve analysis was also conducted to determine the area under the curve (AUC), sensitivity and specificity for the T-SPOT®.TB assay for diagnosing active TB.

Results

This retrospective study enrolled 4964 patients; of whom, 2425 were diagnosed as having active TB. Of these, 640 had bacteriologically confirmed PTB, 1642 had clinically diagnosed PTB and 143 had EPTB. There were 2539 non-TB patients that were diagnosed with other respiratory diseases including the following three main respiratory diseases: 1327 (52%) were diagnosed with pneumonia, 373 (15%) with a pulmonary malignancy and 271 (11%) with bronchiectasis.

The demographic and clinical characteristics of the 2425 patients with active TB are shown in Table 1. Bacteriologically confirmed PTB patients had a significantly higher possibility of bronchus involvement than clinically diagnosed PTB patients ($P < 0.001$). However, clinically diagnosed PTB patients had a significantly higher possibility of involvement of the pleura, lymph nodes and central nervous system compared with the bacteriologically confirmed PTB patients ($P < 0.05$ for all comparisons). For other anatomical sites, there were no significant differences between these two groups. For EPTB, the three most frequent sites of involvement were the lymph nodes (53 of 143; 37%), bone and joint (19 of 143; 13%) and central nervous system (eight of 143; 6%).

With regard to the evaluation of the diagnostic accuracy of the T-SPOT®.TB assay for active TB, ROC curve analysis showed that the assay had a good diagnostic accuracy for active TB with an area under the ROC curve of 0.747 (95% confidence interval [CI] 0.734, 0.761; $P < 0.001$). The sensitivity and specificity at the recommended cut-off values (six spot-forming cells) were 75.3% (95% CI 73.6%, 77.0%) and 64.7% (95% CI 62.9%, 66.5%), respectively.

To investigate the risk factors for false-negative results with the T-SPOT®.TB assay, univariate logistic regression analyses were initially undertaken. Nine factors (age, CRP, ESR, CD3+ count, CD4+ count, CD8+ count, sputum AFB smear, sputum mycobacterial cultures and EPTB) were considered significant ($P < 0.2$) (Table 2). However, in the subsequent multivariate analyses, only five factors (age, CD8+ count, sputum AFB smear, sputum mycobacterial cultures and EPTB) that had P-values < 0.05 were identified as the risk factors for false-negative results with the

Table 1. Demographic and clinical characteristics of patients with active tuberculosis (TB) infection who were enrolled in this study to investigate the diagnostic efficacy of the T-SPOT®.*TB* assay for active TB and to identify risk factors for false-negative results.

	Confirmed PTB $n = 640$	Clinically diagnosed PTB $n = 1642$	Statistical significance[a]	EPTB only $n = 143$	Total active TB $n = 2425$	Total non-TB $n = 2539$
Age, years	44.8 ± 18.9	43.2 ± 18.4	NS	44.1 ± 18.2	43.6 ± 18.5	53.7 ± 16.6
Sex, male	423 (66)	1037 (63)	NS	101 (71)	1561 (64)	1535 (60)
Anatomical site with TB involvement						
Bronchus	72 (11)	82 (5)	$P < 0.001$	0 (0)	154 (6)	0 (0)
Pleura	55 (9)	197 (12)	$P = 0.021$	0 (0)	252 (10)	0 (0)
Empyema	8 (1)	13 (1)	NS	0 (0)	21 (1)	0 (0)
Lymph nodes	13 (2)	101 (6)	$P < 0.001$	53 (37)	167 (7)	0 (0)
Bone and joint	3 (0)	23 (1)	NS	19 (13)	45 (2)	0 (0)
Central nervous system	3 (0)	34 (2)	$P = 0.005$	8 (6)	45 (2)	0 (0)
Larynx	0 (0)	1 (0)	NS	1 (1)	2 (0)	0 (0)
Chest wall	3 (0)	12 (1)	NS	3 (2)	18 (1)	0 (0)
Peritoneum	2 (0)	10 (1)	NS	3 (2)	15 (1)	0 (0)
Urinary tract	1 (0)	0 (0)	NS	5 (3)	6 (0)	0 (0)
Skin	0 (0)	0 (0)	NS	1 (1)	1 (0)	0 (0)
Infection sites >3	31 (5)	66 (4)	NS	50 (35)	147 (6)	0 (0)
With EPTB	91 (14)	351 (21)	$P < 0.001$	143 (100)	585 (24)	0 (0)
Concomitant diseases						
Diabetes mellitus	59 (9)	96 (6)	$P = 0.004$	8 (6)	163 (7)	246 (10)
Malignancy	19 (3)	43 (3)	NS	1 (1)	63 (3)	373 (15)
Rheumatic disease	2 (0)	20 (1)	$P = 0.047$	1 (1)	23 (1)	56 (2)
Coronary heart disease	5 (1)	28 (2)	NS	0 (0)	33 (1)	26 (1)
Hypertension	36 (6)	156 (10)	$P = 0.003$	10 (7)	202 (8)	319 (13)
Liver dysfunction	74 (12)	192 (12)	NS	20 (14)	286 (12)	155 (6)
Hypoxaemia	19 (3)	140 (9)	$P < 0.001$	3 (2)	162 (7)	268 (11)

Data presented as mean $\pm$ SD or *n* of patients (%).
[a]$P < 0.05$ between confirmed and clinically diagnosed PTB; χ^2-test.
PTB, pulmonary tuberculosis; EPTB, extrapulmonary tuberculosis; NS, no significant between-group difference ($P \geq 0.05$).

T-SPOT®.*TB* assay (Table 3). The remaining characteristics, such as CRP, ESR, CD3+ count, CD4+ count, CD4+/CD8+ ratio, WBC count, absolute lymphocyte count, the presence of diabetes mellitus, rheumatic disease or malignancy, were not significant predictive factors for false-negative T-SPOT®.*TB* assay results. There were no collinearities among the variables in the multiple logistic regression analyses (VIFs < 1.203).

Discussion

Previous studies suggested that age was a risk factor for false-negative results with the T-SPOT®.*TB* assay.[13,31] In the QFT-G and QFT-GIT tests, increased age was a risk factor for false-negative results in patients with active TB.[10,32,33] In line with these previous results,[10,13,31–33] the current univariate logistic regression analyses and the subsequent multiple analyses both indicated that age was a risk factor for false-negative results with an upward trend according to increasing age. To the best of our knowledge, the CD8+ count has not been previously identified as a risk factor for false-negative results with the T-SPOT®.*TB* assay. However, some relevant studies already reported that the CD8+ T cell response increased in subjects who had

Table 2. Univariate logistic regression analyses of risk factors for false-negative results with the T-SPOT®.TB assay in patients with active TB (n = 2425).

Patient characteristic	T-SPOT®.TB positive n = 1820	T-SPOT®.TB negative n = 605	Univariate analysis Odds ratio (95% confidence interval)	Statistical significance
Sex, female	652/1820	212/605	0.966 (0.797, 1.172)	NS
Age, years	40 (26–57) (n = 1820)	48 (33–62) (n = 605)	1.017 (1.012, 1.022)	P < 0.001
CRP, mg/l	8.7 (2.7–37.7) (n = 1650)	6.0 (2.0–31.5) (n = 497)	0.997 (0.995, 1.000)	P = 0.032
ESR, mm/h	30 (12–62) (n = 1713)	27 (10–58) (n = 532)	0.997 (0.994, 1.000)	P = 0.025
CD3+ count	0.658 (0.584–0.734) (n = 867)	0.643 (0.557–0.726) (n = 328)	1.383 (1.051, 1.820)	P = 0.021
CD4+ count	0.362 (0.296–0.430) (n = 867)	0.359 (0.286–0.424) (n = 328)	1.789 (1.121, 2.856)	P = 0.015
CD8+ count	0.221 (0.165–0.288) (n = 1642)	0.217 (0.157–0.283) (n = 521)	0.356 (0.159, 0.797)	P = 0.012
CD4 +/CD8+ ratio	1.62 (1.13–2.31) (n = 1642)	1.67 (1.13–2.33) (n = 521)	0.990 (0.927, 1.058)	NS
WBC, 10^{12}/l	5.99 (4.92–7.39) (n = 1712)	6.02 (4.90–7.89) (n = 533)	0.983 (0.952, 1.015)	NS
Absolute lymphocyte count, 10^{12}/l	1.42 (1.07–1.82) (n = 1712)	1.48 (1.09–1.89) (n = 533)	0.923 (0.806, 1.056)	NS
Diabetes mellitus	123/1820	40/605	0.977 (0.675, 1.413)	NS
Malignancy	48/1820	15/605	0.939 (0.522, 1.688)	NS
Smear negative	1469/1820	538/605	1.919 (1.451, 2.537)	P < 0.001
Cultures negative	1437/1820	520/605	1.631 (1.263, 2.105)	P < 0.001
Rheumatic disease	15/1820	8/605	1.613 (0.680, 3.822)	NS
Without EPTB	1365/1820	475/605	1.218 (0.976, 1.519)	P = 0.081

Data presented as n of patients (%) or median (interquartile range).
CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; WBC, white blood cell; EPTB, extrapulmonary tuberculosis; NS, not significant ($P \geq 0.2$).

Table 3. Multivariate logistic regression analyses of risk factors for false-negative results with the T-SPOT®. *TB* assay in patients with active TB ($n = 2425$).

| Patient characteristic | False-negative results with the T-SPOT®.*TB* assay | | |
	Odds ratio	95% confidence interval	Statistical significance
Age, years	1.018	1.013, 1.024	$P < 0.001$
CRP, mg/L	1.000	0.997, 1.003	NS
ESR, mm/h	0.998	0.994, 1.002	NS
CD3+ count	2.651	0.940, 7.477	NS
CD4+ count	0.423	0.077, 2.326	NS
CD8+ count	0.307	0.117, 0.803	$P = 0.016$
Smear negative	1.821	1.338, 2.477	$P < 0.001$
Culture negative	1.379	1.043, 1.824	$P = 0.024$
Without EPTB	1.291	1.026, 1.623	$P = 0.029$

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; EPTB, extrapulmonary tuberculosis; NS, not significant ($P \geq 0.05$).

recent exposure to TB patients,[34] and this stronger CD8+ T cell response was also observed in a bovine model of *M. tuberculosis* infection.[35] Compatible with these previous findings,[34,35] this current study demonstrated that a higher CD8+ count correlated with a decreased false-negative rate, suggesting a stronger CD8+ T cell response in patients with active TB. As for the CD3+ count, CD4+ count and CD4+/CD8+ ratio, no such correlation was established, which suggests that CD8+ T cells may play a more significant role than CD3+ and CD4+ cells in *M. tuberculosis* infection.

In the current study, the sputum AFB smears and mycobacterial cultures were both identified as risk factors for false-negative results with the T-SPOT®.*TB* assay, which suggested heavier burdens of *M. tuberculosis* might be associated with a greater immune response. Considering the difficulty of the diagnosis of smear-negative TB and the reduced capability of the T-SPOT®.*TB* assay in these patients, the development of new tools should be undertaken with a matter of urgency in order to improve the diagnosis of smear-negative TB.

The sensitivity of the QFT-GIT assay may be lower when levels of inflammatory markers increase, and CRP is usually regarded as a predictive factor of negative QFT-GIT results by univariate analyses.[11] A previous study also stated that the probability of indeterminate and negative QFT-GIT results increased with increasing CRP level as determined by multivariate logistic regression analyses.[10] The current multivariate logistic regression analyses failed to identify CRP level and another inflammatory marker (i.e. ESR) as predictors of false-negative results with the T-SPOT®.*TB* assay.

Although there was already a report suggesting an association between diabetes mellitus and lower levels of *M. tuberculosis*-specific IFN-γ response in both TB and non-TB patients,[21] many studies have failed to establish a significant association between diabetes and the accuracy of the T-SPOT®.*TB* assay results in TB patients.[13,36,37] A previous study found no significant differences in the qualitative or quantitative T cell IFN-γ response to *M. tuberculosis*-specific antigens as measured using the T-SPOT®.*TB* assay between diabetic and non-diabetic patients with

culture-confirmed TB.[21] Similarly, the sensitivity of the T-SPOT®.TB assay did not decrease in TB patients with diabetes mellitus.[36] Furthermore, another study failed to detect a significant association between diabetes mellitus and false-negative T-SPOT®.TB assay results in both PTB and EPTB.[13] This current study failed to find a correlation between false-negative T-SPOT®.TB assay results and diabetes mellitus; however, it identified EPTB as a risk factor of false-negative results, which needs to be evaluated in further studies.

As lymphocytopenia can decrease the production of IFN-γ, it was previously identified as an independent risk factor that can cause false-negative QFT-GIT results.[11,38–40] However, the current data suggested that lymphocytopenia did not influence the false-negative rate with the T-SPOT®.TB assay. Similarly, malignancy was also previously reported to be a risk factor for decreased QFT-GIT sensitivity,[11,18,40,41] however, the current study failed to confirm this effect on the false-negative rate of the T-SPOT®.TB assay.

According to the current results, the T-SPOT®.TB assay had a good accuracy for the diagnosis of active TB with a high AUC of 0.747 (sensitivity 75.3%; specificity 64.7%). In some studies, the accuracy of the T-SPOT®.TB assay for active TB has been shown to be variable, with a sensitivity of approximately 70–90% and a specificity of approximately 85–100%.[4,8,9,36] As for China, the sensitivity was approximately 86% and the specificity was approximately 66% while distinguishing active TB from non-tuberculosis pulmonary diseases.[22] The heterogeneity was partly due to limited case numbers and different characteristics of the included populations. The large consecutive data set ($n = 4964$) included in this current study should provide a more accurate evaluation of the diagnostic performance of the T-SPOT®.TB assay. The results showed that the sensitivity was consistent with recent findings, while the specificity was lower than those reported in some published studies.[4,7,42–47]

In conclusion, this current study demonstrated that the T-SPOT®.TB assay was a valuable method for identifying patients with active TB. However, the results of this assay should be carefully interpreted in older patients, in those with lower CD8+ counts, in patients without EPTB, and in patients with negative sputum AFB smears and negative sputum mycobacterial cultures.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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