Original Article

Risk factors for negative T-SPOT.TB assay results in patients with confirmed active tuberculosis: A retrospective study

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Abstract

Introduction: The interferon-γ release assays as potent adjunct tools for the quick detection of TB in high burden countries is feasible. In this retrospective study, we aimed to identify the risk factors for negative T-SPOT results in confirmed active tuberculosis.

Methodology: We consecutively enrolled 1,021 patients who were positive for acid-fast bacilli smear staining or culture-confirmed mycobacterial infection and simultaneously tested with the T-SPOT.TB assay. All of the included specimens were used to discriminate the Mycobacterium species using the biochip assay. We collected basic clinical characteristics and laboratory results for further analysis.

Results: Of the 1,021 patients enrolled in the study, 89 patients were identified as having nontuberculous mycobacteria (NTM). Ninety-nine patients were excluded from the analysis because of indeterminate T-SPOT.TB results, while the remaining 833 patients were identified as having Mycobacterium tuberculosis infection. In total, 159 patients had false-negative T-SPOT.TB results (19.1% of 833). The concordance rate between the T-SPOT.TB results and final diagnoses in females was always lower than that in males. Multivariate logistic regression analysis showed that female sex (OR 1.81; 95% CI 1.19, 2.7; p = 0.006), HIV coinfection (OR 6.83; 95% CI 2.73, 17.10; p = 0.003), acid-fast bacilli (AFB) smear-negative (OR 5.45; 95% CI 3.62, 8.19; p < 0.001), HIV coinfection (OR 6.83; 95% CI 2.73, 17.10; p < 0.001) were associated with negative T-SPOT.TB result.

Conclusions: Female is another independent risk factor of negative T-SPOT.TB results, besides to elder, HIV co-infection, acid-fast bacilli (AFB) smear-negative who are suspected of having active TB infection.

Key words: Active tuberculosis; IGRAs; T-SPOT.TB assay.

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Introduction

Tuberculosis (TB) is a severe global infectious disease that causes high mortality and morbidity, with 6.4 million newly reported cases and 1.3 million deaths around the world in 2017. China is the second highest TB burden country, accounting for 9% of 10.0 million people who developed TB disease across the globe. Approximately 1.7 billion people are estimated to have latent TB infection, and 5%-10% of these people will develop TB disease during their lifetime. Although the incidence of TB has been decreasing globally, it is still not fast enough to achieve the goals of the End TB Strategy [1]. Limited by available methods for detecting tuberculosis infection, gaps between the estimated number of new cases and the number actually reported cannot be overlooked. Therefore, rapid diagnostic methods and early effective treatment regimens are critical to narrow the gaps and thus to control further transmission among populations. Conventional culture-based methods are always the gold standard for identifying all TB cases, while limitations such as low-quality specimens and long delays in obtaining results do exist. The WHO recommends the advanced, accurate, molecular diagnostic method called the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) as an initial test for tuberculosis; this test has high specificity but often requires invasive procedures to obtain clinical specimens from disease sites [2]. Two commercially available blood diagnostic tests called interferon-γ release assays (IGRAs) include T-SPOT.TB (Oxford Immunotec, Abingdon, UK) and QuantiFERON-TB Gold (Cellestis, Carnegie, Australia). IGRAs detect interferon-gamma (IFN-γ) released by T-cells in the blood in response to Mycobacterium tuberculosis-specific antigens ESAT-6 and CFP-10. These specific antigens are not affected by exposure to Bacille Calmette–Guerin (BCG) vaccination or most nontuberculous mycobacteria [3]. However, IGRAs are
unable to differentiate latent TB infection (LTBI) from active TB disease and present inconsistent sensitivity and specificity in different populations with TB diseases [4,5]. Its unsatisfactory sensitivity may impact on interpreting IGRA results in highly suspicious TB patients. Previous studies have suggested that immunosuppression, underweight, older age, lymphocytopenia, HIV co-infection, and tuberculous meningitis were associated with false-negative T-SPOT.TB results in different clinical settings [6-9]. These inadequate studies had small sample sizes or were conducted in settings with varied burdens of disease. The investigation of the risk factors of the negative T-SPOT results in confirmed TB population would provide a more convincing interpretation of negative results. Further study is necessary due to the current studies lacking molecular diagnostic methods to differentiate NTM from M. tuberculosis, which impacts the T-SPOT results considerably. The main objective of our large-scale retrospective study was to identify the potential clinical characteristics associated with negative T-SPOT.TB assay results in patients with confirmed TB.

**Methodology**

**Study population**

This retrospective study was conducted at the First Affiliated Hospital of Zhejiang University between March 2012 and November 2017. We consecutively enrolled individuals with suspected tuberculosis as both outpatients and inpatients in any ward of the hospital.

**Study design and setting**

We included patients whose available specimens were identified either acid-fast bacilli (AFB) smear-positive or mycobacterium culture-positive. All of positive specimens underwent further biochip assay, which can identify 17 mycobacterial species within 6 hours. Active TB was defined as molecular diagnostic confirmation of infection with MTB by Biochip assay. Every patient went through T-SPOT.TB assay before anti-TB chemotherapy. We excluded TB patients with invalid blood samples, as well as those with NTM infection (Figure 1). We selected risk factors for negative T-SPOT.TB outcomes based on previous studies being accessible and reliable in clinical databases. Records were extracted from the electronic medical records database, including age, sex, range of TB disease, comorbidity, smoking and alcohol intake, underlying diseases and clinical laboratory findings. All patients were categorized into the following groups: Group one, active tuberculosis patients with positive T-SPOT.TB results; Group two, active tuberculosis patients with negative T-SPOT.TB results.

**Data sources and definitions**

Active TB was defined as follows: 1) Pulmonary TB: M. tuberculosis complex were identified by Biochip assays when sputum specimens with AFB smear-positive or culture-positive. 2) EPTB: patients with at least one specimen that was extracted from infected lesions other than the lung after biochip assays confirmed M. tuberculosis infection. Anemia was defined as a hemoglobin level lower than 120 g/L for males and 110 g/L for females. Patients with end-stage renal disease were defined as receiving renal replacement therapy. Patients with class C Child-Pugh scores were defined as having liver cirrhosis. Viral hepatitis including hepatitis A, B, and C virus infection.

**T-SPOT.TB assay**

The enzyme-lined immunosopt assay was based on the release of IFN-γ level in blood samples after stimulating by M. tuberculosis-specific antigens ESAT-6 and CFP-10 in vitro. Peripheral venous blood mononuclear cells separated immediately for T-SPOT.TB assay (Oxford Immunotec, Oxford, United Kingdom) which performed according to the manufacturer instructions. Then isolated peripheral blood mononuclear cells were seeded on precoated plates with anti-IFN-γ antibodies at 37°C in 5% carbon dioxide for 20 hours. After application of secondary antibody solution and substrate chromogen solution, the number of spot-forming cells in each well was

![Figure 1. The entry rules of study population.](image-url)
calculated by automated reader. The final reports were recorded under the same criteria recommended by the test kit manufacturer. One well-trained staff member performing the T-SPO T.TB assays was blinded to the results of other study tests using specimen codes. Data were input and captured through dedicated data systems.

**Biochip assay**

The commercial diagnostic kit includes a biochip, apparatus for sample preparation, chip hybridization, washing and data acquisition, and dedicated software for automated diagnosis (CapitalBio, Beijing, China). This method can rapidly distinguish 17 mycobacterial species (M. tuberculosis, M. intracellulare, M. avium, M. kansasii, M. chelonea/M. abscessus, M. gordonae, M. fortuitum, M. marinum/M. ulcerans, M. scrofulaceum, M. gilvum, M. terrae, M. nonchromogenicum, M. aurum, M. phlei, M. szulgai/M. malmoense, M. smegmatis and M. xenopi) directly in positive specimens in less than 6 hours. All samples were digested and purified with 2% acetyl-cysteine and sodium hydroxide (NALC-NaOH) for 15 minutes. At least 1.5 ml of the sample mixture was centrifuged for 5 minutes at 12000 rpm and the left sediment was diluted to 1 ml with a sterile saline solution and centrifuged for 5 minutes at 12,000 rpm one more time. Then added 100ul of the nucleic acid extract solution to the sediment, which was then transferred to a specific tube and continuously vortexed for 10 minutes. The suspension was placed in a dry bath at 95 °C for 8 minutes and then centrifuged at 12,000 rpm for 5 minutes. Amplified nucleic acid for asymmetric PCR before the hybridization. 9 ml of the hybridization buffer and 6 ml of the amplified product formed the mixture, extracted 13.5 ml of the mixture to the prepared slide. Chip hybridization was performed in a three-dimensional tilt stirrer BioMixer II hybridization oven and an automated SlideWasher. The microarrays on the slides were analyzed using a LuxScan 10K-B confocal laser scanner. The fluorescent intensities were measured though dedicated software named the mycobacteria identification array test system [10].

**Statistical analysis**

The data were entered into SPSS for Mac, version 25 (SPSS Inc, USA). Continuous data were described as medians and interquartile ranges (IQR). Continuous variables were analyzed with the Independent Samples t-test or Mann-Whitney U test. Categorical variables were compared with Pearson’s Chi-square test. The criterion for statistical significance was p < 0.05. We included variables with a p value of < 0.05 into multivariate logistic regression model with forward stepwise selection to identify independent risk factors significantly associated with the negative results of T-SPO T.TB assay.

**Ethical approval**

The ethics committee of the First Affiliated Hospital of Zhejiang University approved the study.

**Results**

A total of 1,021 specimens (sputum, n = 635; tracheal alveolar lavage fluid, n = 87; pleural fluid n = 30; peritoneal fluid, n = 4; pyogenic fluids, n = 13; urine, n = 42; cerebrospinal fluid, n = 22) were obtained from 1,021 patients. According to Biochip assay, 91 patients were confirmed nontuberculous mycobacteria (NTM) and 930 patients were confirmed tuberculosis, which included 97 patients with invalid T-SPO T.TB results. Thus, a total of 833 confirmed TB patients with Determinate T-SPO T.TB results and complete study information were included in the analysis. Overall, 674 (80.9%) had a positive T-SPO T.TB results, and 159 (19.1%) had a negative T-SPO T.TB results. Among the 833 patients, 96 patients had both positive microbiological outcomes and invasive procedures for histological confirmation.

The patients included in analysis had a median age of 53 years (IQR 37-66), and the patients with negative T-SPO T.TB results were older (median age 60 years [IQR 34.0-65.0]; p < 0.01) when compared with patients having positive results (median age 51.5 years [IQR 44.0-69.0]).

In total, 574 males (68.9%) and 259 females (31.1%) were enrolled, showing an over 2.2-fold male predominance. However, patients with negative T-SPO T.TB results were less likely to be male (p < 0.01) when compared with patients having positive T-SPO T.TB results. A total of 671 patients suffered from pulmonary TB (PTB), accounting for 82.1%, while 81 patients suffered from extrapulmonary TB (EPTB). Among the EPTB patients, the proportion of negative results (16.4%) compared with the proportion of positive results (8.2%) increased by a factor of 2 (p < 0.01). Other five factors including HIV co-infection (p < 0.01), any other autoimmune disease (p = 0.03), malignancy (p < 0.01), hypertension (p < 0.01) and anemia (p < 0.01) had statistically significant associations with negative T-SPO T.TB results.
Table 1. Univariate analysis of basic characteristics of confirmed active TB.

| Characteristics                  | T-SPOT Positive (n = 674) | T-SPOT Negative (n = 159) | P Value* |
|----------------------------------|---------------------------|---------------------------|----------|
| **Gender (n,%)**                 |                           |                           |          |
| Male                             | 476 (82.9)                | 98 (17.9)                 | 0.03     |
| Female                           | 198(29.4)                 | 61(38.3)                  |          |
| **Age (median, IQR)**            | 51.5 (34.0-65.0)          | 60 (44.0-69.0)            | < 0.01   |
| **Smoking**                      |                           |                           |          |
| **Alcohol**                      |                           |                           |          |
| **AFB negative**                 |                           |                           | <0.01    |
| **Anatomical site of TB**        |                           |                           |          |
| PTB                              | 564 (83.7)                | 120 (75.5)                |          |
| EPTB                             | 55 (8.2)                  | 26 (16.4)                 |          |
| Both                             | 55 (8.2)                  | 13 (8.2)                  |          |
| Hypertension                     | 111 (16.5)                | 41 (25.8)                 |          |
| Diabetes mellitus                | 109 (16.2)                | 21 (13.2)                 |          |
| Anemia                           | 219 (32.5)                | 75 (47.2)                 | <0.01    |
| **Concomitant diseases**         |                           |                           |          |
| Viral hepatitis<sup>1</sup>      | 51 (7.6)                  | 15 (9.4)                  | 0.43     |
| End-stage renal disease<sup>2</sup> | 27 (4.0)              | 11 (6.9)                  | 0.11     |
| Liver cirrhosis                  | 20 (3.0)                  | 6 (3.8)                   | 0.6      |
| Transplantation<sup>3</sup>      | 21 (3.1)                  | 10 (6.3)                  | 0.06     |
| HIV co-infection                 | 14 (2.1)                  | 12 (7.5)                  | <0.01    |
| Autoimmune disease               | 36 (5.3)                  | 16 (10.1)                 | 0.03     |
| Malignancy                       | 65 (9.6)                  | 34 (21.4)                 | <0.01    |

AFB: acid-fast bacilli; PTB: pulmonary tuberculosis; EPTB: extrapulmonary tuberculosis; HIV: human immunodeficiency virus; IQR: interquartile range; <sup>1</sup>Viral hepatitis including hepatitis A, B, and C virus infection; <sup>2</sup>patients with end-stage renal disease receiving renal replacement therapy; <sup>3</sup>transplantation include: 1) renal translations 2) stem-cell transplantation 3) liver transplantation.

Table 2. Univariate analysis of laboratory findings of confirmed active TB.

| Laboratory findings (median, IQR) | T-SPOT Positive (n = 674) | T-SPOT Negative (n=159) | P Value* |
|-----------------------------------|---------------------------|--------------------------|----------|
| WBC count, *10<sup>9</sup>        | 6.7 (5.1-8.1)             | 6.5 (4.3-8.9)            | 0.73     |
| RBC count, *10<sup>9</sup>        | 4.29 (3.82-4.73)          | 3.97 (3.21-4.42)         | < 0.01   |
| Hemoglobin, g/L                   | 123 (109-137)             | 119 (93-131)             | < 0.01   |
| Lymphocytes, %                    | 19.05 (12.08-25.53)       | 18.0 (10.3-25.6)         | 0.68     |
| Total protein, g/L                | 66.95 (62.08-73.33)       | 64.9 (59.1-70.6)         | < 0.01   |
| Albumin, g/L                      | 37.34 (32.68-42.03)       | 36.40 (30.3-40.0)        | 0.05     |
| ESR, mm/h                         | 33.0 (13.0-63.0)          | 38.5 (15.0-69.0)         | 0.11     |

WBC: White blood cells; RBC: Red blood cells; ESR: Erythrocyte sedimentation rate; *p-values in Univariate analysis. continuous variables were calculated using the Mann-Whitney U test. p < 0.05 was the criterion for statistical significance.

Table 3. Multivariate logistic regression analysis of risk factor for false-negative T-SPOT.TB results confirmed active TB patients.

| Risk factors          | T-SPOT.TB negative results | OR | 95% CI | P value |
|-----------------------|----------------------------|----|--------|---------|
| Female                |                            | 1.81| (1.19, 2.75) | 0.006   |
| Age                   |                            | 1.02| (1.01, 1.03) | 0.003   |
| AFB negative          |                            | 5.45| (3.62, 8.19) | < 0.001 |
| HIV co-infection      |                            | 6.83| (2.73, 17.10) | < 0.001 |

AFB: acid-fast bacilli; HIV: human immunodeficiency virus.
Notably, transplantation showed a strong tendency towards statistical significance \((p = 0.06)\). Univariate analysis suggested that no significant differences were observed between the positive and negative groups in the distribution of smoking, alcohol, radiologic characteristics with effusion or nodules, and concomitant diseases such as end-stage renal disease, chronic liver diseases, cirrhosis, rheumatic disease, and diabetes mellitus. The details of the comparison of the clinical characteristics of patients with positive and negative results are summarized in Table 1.

The laboratory findings showed that the number of RBCs \((4.29 \times 10^9/L \text{ vs } 3.97 \times 10^9/L)\), hemoglobin \((123 \text{ g/L vs } 119 \text{ g/L})\), total protein \((66.95 \text{ g/L vs } 64.9 \text{ g/L})\), and albumin \((37.34 \text{ g/L vs } 36.40 \text{ g/L})\) were significantly lower in the negative group than in the positive group. WBC count, lymphocyte ratio and ESR were not associated with negative T-SPOT.TB results (Table 2).

Multivariate logistic regression analysis revealed that female gender \((\text{OR}, 1.81; \text{95% CI, 1.19–2.75, } p = 0.006)\), age \((\text{OR}, 1.02; \text{95% CI, 1.01–1.03, } p = 0.003)\), AFB smear-negative result \((\text{OR 5.45; 95% CI, 3.62–8.19; } p < 0.001)\) and HIV co-infection \((\text{OR 6.83; 95% CI, 2.73–17.10; } p < 0.001)\) were the risk factors for negative T-SPOT.TB assay results. The remaining variables, such as culture negative, anatomical site of TB, hypertension, autoimmune disease and anemia were not associated with negative T-SPOT.TB results (Table 3).

**Discussion**

Our results indicated that the T-SPOT.TB assay produced negative results in 159 (19.1%) molecular method-confirmed tuberculosis patients. Older age, female, HIV co-infection and AFB smear-negative results were independent risk factors related to negative T-SPOT.TB results.

In line with the previous studies \([9,11]\), our study confirmed that older age significantly affects T-SPOT.TB results. Patients in the T-SPOT.TB false negative group were 10 years older than the positive group. Jeon et al. compared different age groups showing that the risk of false negative T-SPOT assay increased 10% \([12]\). A possible reason could be that the cellular immune response induced by M. tuberculosis specific antigens gradually weakens, leading to a downward trend in the IFN-\(\gamma\) concentration with increasing age \([6,13,14]\). In addition, elder patients with concomitant diseases and malnutrition status have been reported to lead false negative rate due to a greatly reduced immune response \([15]\).

The number of males in our study was greater than the number of females, showing a gender inequality in *M. tuberculosis* infection, which has been previously demonstrated in other studies. Specifically, men were more likely to contact and respond to *M. tuberculosis* infection \([16-18]\). Our data also confirmed that a negative T-SPOT.TB was less frequent among male than female. However, female has not been previously identified as a risk factor for false-negative results T-SPOT.TB assay. In our study, the age ranging from 45 to 60 years accounted for the highest at 31.1% in the female negative group, while men within the age range of 61-75 years accounted for 37.5% of the male negative group. The concordance rate of the T-SPOT.TB assay decreased with increasing age. Moreover, the concordance rate in females was always lower than that in males, and a dramatic difference was observed between the ages of 46 and 60 years with a high incidence of tuberculosis (Figure 2). It is worth
noting that a dramatic difference was observed between the ages of 46 to 60 years, which is the age group with the highest incidence of tuberculosis (Figure 3). EPTB is a factor that cannot be underestimated to explain this phenomenon [19]. In total, 14.3% of 259 females had EPTB compared with only 7.7% of 574 males, a nearly 2-fold higher rate. Patients with EPTB were much more likely to yield false negative T-SPOT.TB results, especially with TB meningitis [7-8,20]. Under different conditions, the immune response varies depending on the stimulation of the T cell population and the virulence of *M. tuberculosis* isolates. T-SPOT.TB assay relies on sensitized T-cells from a whole blood sample which produce the cytokine IFN-γ when incubated with TB antigens specific ESAT-6 and CFP-10. Gender differences in lymphocyte subsets have been detected in multiple ethnic groups. Males have relatively high frequency of CD8+ T cell counts [21], and greater CD8+ T cell response in tuberculosis exposure, which was associated with a reduced incidence of false negative T-SPOT.TB result [8,22,23]. Another possible explanation for the significantly higher negative rate in females could be that sex steroid in adult humans may affect the innate immune responses. Levels of estrogen fluctuate widely during perimenopause and are obviously lower after menopause. Estrogen receptors are expressed in diverse lymphoid tissue, and estradiol stimulates both cell-mediated and humoral immune responses [21]. The molecular mechanisms leading to sex differences in ER expression in particular immune cells are yet to be defined. The function of the immune system declines progressively with age, and the counterbalance between concentrations of sex steroid and immune cell activity was markedly more turbulent in females than males [24]. Although current study indicated that gender differences profoundly affect the accuracy of the immune-based T-SPOT.TB assay, apparently more significant with increasing age, the mechanisms remain unclear and need further studies [25].

Compatible with previous findings [14,26], our study indicated that HIV co-infection was one of independent risk factors. In current study, the sensitivity of T-SPOT.TB assay in HIV co-infected TB patients was 33.3%. Patients with HIV co-infected had a more than 6-fold increased risk of false-negative T-SPOT results compared to HIV non-infected patients. HIV infection causes secondary destruction of TB-associated immune response, which varies to immunodeficiency and HIV viral loads [27,28]. Cai et al. found that the number of spot-forming cells (SFCs) reactive to ESAT-6 and CFP-10 in the T-SPOT.TB assay were related to the degree of immunodeficiency [29]. Edward et al. found that low CD4+ count in combination with high/normal CD8+ count was associated with false-negative results [30].

The negative AFB smear results was an independent risk factor. The patients with false negative T-SPOT.TB results had significantly higher AFB negative rates (44.0%) than the AFB positive group (13.6%). Negative AFB smear indicates a relatively low *M. tuberculosis* burden, which might trigger a weaker immune response against the TB infection. Ewer et al. reported that the T-SPOT.TB assay correlated closely with *M. tuberculosis* exposure degree than TST [31]. Considering the lack of TB culture results from all patients, the current study cannot prove that negative TB culture is a risk factor for false negative T-SPOT.TB result, which has been mentioned previously [8]. Additionally, our studies have found anemia and a low concentration of total protein were the factors associated with negative T-SPOT.TB results. Anemia may occur in various diseases, such as inflammatory bowel disease, end-stage renal disease, cirrhosis, malignant tumor and malnourished state, all of which represent functional immunosuppressed conditions [32].

Our study had several limitations. First, as an independent comprehensive hospital, most patients in complex conditions had received primary health care at the hospital location and might have been sicker and misdiagnosed before attending our hospital. The spectrum of disease in our hospital is different from TB-specific hospitals and local primary medical institutions. We could not avoid selection bias because we used a single center sample. The retrospective design studied only bacteriologically or histologically proven TB patients. Our conclusions may not be generalizable to individuals with a lower mycobacterial load. Second, additional large-scale research on transplant recipients would help to identify associations among the T-SPOT.TB assay results. Third, nearly half of HIV co-infected TB patients showed negative results, but the number of recruited patients was limited. Further study should be dynamically focused on HIV co-infection patients’ T-SPOT.TB results to determine the real diagnostic value among the specific population. Finally, we explored only partial clinical information, and other experimental errors might lead to result differences that were not included in our study.

**Conclusions**

Currently widely used T-SPOT.TB assay is a valued adjunct immunodiagnostic method for quickly
identifying patients with TB infection. Our study confirmed that older age, negative AFB smear results, HIV co-infection that mentioned in previous studies are associated with negative T-SPOT results. Furthermore, our findings also identified gender difference may exert impact on T-SPOT.TB assay, and female is an additional independent risk factor results in negative T-SPOT.TB results among suspected TB patients. Clinical physicians cannot hesitate to conduct further tests for highly suspicious TB patients with these risk factors, in case of delayed treatment initiation leads to poor outcomes [11].

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