Journal of Clinical Tuberculosis and Other Mycobacterial Diseases 26 (2022) 100290

Contents lists available at ScienceDirect

Journal of Clinical Tuberculosis and Other Mycobacterial Diseases

journal homepage: www.elsevier.com/locate/jctube

Effect of adjusted cut-offs of interferon-γ release assays on diagnosis of tuberculosis in patients with fever of unknown origin

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ARTICLE INFO

Keywords:
Interferon-γ release assay
Tuberculosis
T-SPOT.TB
QuantiFERON-TB Gold

ABSTRACT

Background: Tuberculosis (TB) is a leading cause of fever of unknown origin (FUO). In recent years, interferon-γ release assays (IGRAs) have been widely utilized and the cut-off values given by the manufacturers are set in countries where rates of TB are not as high.

Methods: A prospective cohort study was conducted in a Chinese general hospital to evaluate the diagnostic performance of T-SPOT.TB (T-SPOT) and QuantiFERON-TB Gold (QFT) in detecting active TB (ATB) in a high TB endemic area. Test results were compared with the culture and clinically confirmed diagnosis. Further, we explored an alternative method of interpreting IGRAs by increasing the cut-off values.

Results: The sensitivity and specificity of T-SPOT in detecting ATB were 85.3% (95% CI 81.6–94.0%) and 71.8% (95% CI 67.3–76.0%), respectively. The sensitivity and specificity of QFT were 72.3% (95% CI 62.8–80.1%) and 77.0% (95% CI 72.7–80.8%), respectively. Receiver operating characteristic analysis was used for evaluation of different cut-off values. When the cut-off values were adjusted as 125 spot-forming cells (SFCs)/ $2.5*10^5$ cells for T-SPOT and 4.0 IU/ml for QFT, the specificity could be improved to > 90.0% (90.3% and 94.1%, respectively), and the sensitivity were 43.1% and 41.6%, respectively. The new adjusted cut-off values were validated in another independent validation cohort.

Conclusion: The adjusted cut-off values of the two assays considerably improved the diagnostic value when applied to FUO patients in clinical settings.

1. Introduction

Fever of unknown origin (FUO) is a challenging problem in clinical practice. It was first defined in 1961 as fever higher than 38.3 °C lasting higher than 3 weeks, with uncertain diagnosis after 7 days of medical observation [1]. FUO has many possible causes, which have now been classified as infectious diseases, non-infectious inflammatory diseases, malignancies, other conditions, and unknown etiologies. Infectious diseases remain the most common cause of FUO in recent years [2,3]. Tuberculosis (TB) is one of the most important infectious causes of FUO because of its high prevalence, poor prognosis as well as difficulty to reach a definite diagnosis [4]. Active TB (ATB) patients with FUO usually do not have typical clinical manifestations, including fever, cough, weight loss, and positive chest X-ray results, which makes it relatively difficult to be diagnosed by experienced clinicians. Compared with pulmonary TB (PTB), the manifestations of extrapulmonary TB (EPTB) tend to vary considerably and the symptoms of patients of EPTB are often atypical. Furthermore, the poor accessibility of the EPTB focus increases the difficulty for concrete diagnosis [5].

In routine clinical practice, the traditional methods of culture and

Abbreviations: ATB, active tuberculosis; BCG, Bacillus Calmette–Guérin; CFP-10, culture filtrate protein; CNS, central nervous system; EPTB, extrapulmonary tuberculosis; ESAT-6, early secreted antigenic target 6; FUO, fever of unknown origin; IFN-γ, interferon-γ; IGRAs, interferon-γ release assays; LTBI, latent tuberculosis infection; Mtb, Mycobacterium tuberculosis; PBMCs, peripheral blood mononuclear cells; PTB, pulmonary tuberculosis; QFT, QuantiFERON-TB Gold; TB, tuberculosis; ROC, receiver operating characteristic; SFC, spot-forming cells; T-SPOT, T-SPOT®.TB; TST, Tuberculin skin test.

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https://doi.org/10.1016/j.jctube.2021.100290

Available online 20 December 2021

microscopy are still the most commonly used methods and are considered gold standard for diagnosing ATB. However, they come with unsatisfactory positive predictive value and can be rather time-consuming. Tuberculin skin test (TST) is another widely used method for detecting TB infection, including ATB and latent TB infection (LTBI); however, the results can be influenced by age and immunological status and the test has cross reactions with nontuberculous mycobacterium infection and Bacillus Calmette-Guérin (BCG) vaccination [6,7]. The BCG vaccine was reported to significantly reduce the risk of TB by 50% on average [8], which is recommended and widely used in many countries such as India, Brazil, Russia and China [9]. Therefore, many patients are diagnosed with ATB according to their clinical presentation and their response to anti-TB therapy, and the time interval from onset of fever to diagnosis is relatively long [10]. Therefore, a fast and accurate method is needed for the diagnosis of TB in patients with FUO.

Interferon-γ release assays (IGRAs), represented by the QuantiFERON-TB Gold in Tube (QFT) based on whole blood and T-SPOT.TB (T-SPOT) based on peripheral blood mononuclear cells (PBMCs), have been widely used for the diagnosis of TB infection. IGRAs can detect the presence of TB by measuring interferon-γ (IFN-γ) secretion by lymphocytes responding to *Mycobacterium tuberculosis* (*Mtb*) specific antigens, such as early secreted antigenic target 6 (ESAT-6), culture filtrate protein (CFP-10) and TB7.7 [11]. Compared with TST, IGRAs have been shown to have higher specificity because they have low cross reaction with BCG vaccination and nontuberculous mycobacterium infection except for *Mycobacterium kansasii*, *Mycobacterium szulgai*, *Mycobacterium marinum* and *Mycobacterium riyadhense.* However, IGRAs still cannot effectively distinguish between ATB and LTBI [12,13]. Another critical problem is that the interpretation of the results of IGRAs needs to be optimized to meet the specific clinical needs, because the diagnostic performance of IGRAs is still unsatisfactory using the manufacturers' suggested cut-off values, which were set in areas where rates of TB are not as high [11]. Specifically, for patients with FUO, the application of IGRAs and the method for optimizing the results of its interpretation are still lacking sufficient evidence.

To evaluate the clinical utility of T-SPOT and QFT in detecting ATB among patients with FUO, we conducted a prospective cohort study in a general hospital in China, in a high TB endemic area. Investigation for adjusting the cut-off values of T-SPOT and QFT was also performed for higher diagnostic accuracy while diagnosing ATB in those patients with FUO in real-world clinical practice. Cut-offs were adjusted using statistical analysis in the cohort population and then validated in a second, independent cohort.

2. Materials and methods

2.1. Study setting and population

This prospective study was conducted from March 2016 to May 2018 in Huashan Hospital affiliated with Fudan University in China. Adult patients (≥14 years old) admitted to the infectious disease ward and those who met the diagnostic criteria of classic FUO were recruited in the study. The inclusion criteria of FUO were defined as: (1) oral temperature > 38.3℃, recorded on at least twice ; (2) fever lasting for>3 weeks; and (3) no definite etiology diagnosis in spite of investigations after 7 days of medical observation [14–16]. Exclusion criteria were: (1) nosocomial FUO, which is defined as the hospitalized patient's temperature > 38.3 °C without infection being present or incubating on admission [14]; (2) patients known to have HIV infection; and (3) patients with known malignancy. To investigate the adjusted cut-off values of both the QFT and T-SPOT tests, we designed the following two independent cohorts, according to the objective of our study: derivation cohort and validation cohort. The study was approved by the Ethics Committee of Huashan Hospital affiliated with Fudan University. Written informed consent was obtained from all patients enrolled in this study.

2.2. Definition of the subjects

Patients underwent clinical examinations to confirm or exclude the diagnosis of ATB. All the participants were classified into the following four diagnostic categories, according to their respective clinical data, by two experienced clinicians independently:

(1) Confirmed ATB, which refers to patients with positive microbiological culture of *Mtb* and suggestive clinical symptoms and radiological findings;
(2) Clinically diagnosed ATB, whose clinical and radiological features highly suggest ATB and are unlikely to be caused by other disease. Appropriate response to anti-TB therapy, and histological supportive evidence if available were also necessary [17]. Tissue biopsy specimens (lung, pleural, pericardial, peritoneal, synovial, terminal ileum) allow for histopathologic examination, and those showing histopathological pattern containing (giant cells + granuloma + caseation) were considered as supportive evidence for ATB;
(3) Clinically indeterminate, which means final diagnosis of TB is neither highly probable nor reliably excluded;
(4) ATB excluded, which means sputum smear and culture for *Mtb* were negative and patient showed improvement in symptoms and radiological abnormalities after treatment with antibiotics that have no inhibiting effect on *Mtb* or found to have alternative diseases such as viral pneumonia or connective tissue disease, as a confirmed diagnosis.

2.3. Blood sample collection

The vacuum blood collection vessels for anticoagulation were prepared with heparin lithium and marked. Eight milliliters of venous whole blood were collected from the subjects, and then the anticoagulant was slowly reversed and mixed three times immediately after light injection. Hemolysis was avoided during collection. The whole blood samples were not immersed in ice or frozen to prevent the hemostatic cells from losing their activity.

2.4. T-SPOT and QFT tests

2.4.1. T-SPOt

The T-SPOT test was performed following the instructions in the assay kit (Oxford Immunote Ltd., Oxford, UK) [18]. Briefly, PBMCs were isolated from whole blood with the help of Ficoll-Hypaque density gradient centrifugation. Next, the cells were incubated with two antigens (ESAT-6 in panel A; CFP-10 in panel B). The procedure was performed in the plates pre-coated with anti-interferon-c antibodies at 37 °C for 16 to 20 h. After application of alkaline phosphatase-conjugated second antibody and chromogenic substrate, spots were scored using an automated ELISPOT plate reader (AID-Gmb-H, Germany). The result of T-SPOT.TB was considered positive if (1) Panel A or Panel B had six or more spots than the negative control when the spots of the negative control ≤ 5; (2) the number of spots in Panel A or B was at least two times higher than that of the negative control when the spots of the negative control > 5.

2.4.2. QFt

QFT tests were performed according to the manufacturer's instructions (Cellestis, Darmstadt, Germany) [19]. Briefly, 1 mL of whole blood was drawn into three QFT tubes and incubated at 37 °C within 4 h after collection. Following a 24-h incubation period, the tubes were centrifuged, and the plasma was harvested from each tube to determine the concentration of IFN-γ. The QFT results were calculated and interpreted using the manufacturer's QFT software. The result was considered positive if the value was ≥ 0.35 IU/mL and ≥ 25% more than the nil control with any value for mitogen tube, and negative if the

concentration of the positive control minus negative control is ≥ 0.5 IU/ml, with the calculated value ≥ 0.35 IU/mL but < 25 % more than the nil control value, or <0.35 IU/ml.

2.5. Statistical analysis

The specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to evaluate the diagnostic performance of T-SPOT and QFT. For sensitivity and specificity calculations, confirmed ATB and clinically diagnosed ATB were included as patients and ATB exclude patients as controls. The Pearson's Chi-square test was used to compare the positive proportions, and the Mann-Whitney test was used to compare the IFN-γ level in different groups. Test concordance was assessed using the kappa (κ) statistic. To assess diagnostic values of the two tests in detecting ATB infections, receiver operating characteristic (ROC) analysis was used as described in [19]. ROC curves allow tests to be compared over a variety of cut-off points, and sensitivity, specificity, PPV and NPV for each cut-off value were calculated for evaluation. Statistical analysis was performed using the statistical software GraphPad Prism (version 8.0; GraphPad Software, Inc.) and SPSS (version 19.0; IBM Corp, Chicago, IL, USA). $P < 0.05$ was regarded to be statistically significant.

3. Results

3.1. Clinical characteristics of patients

A total of 573 patients who were admitted to the infectious disease ward of Huashan Hospital and presented with FUO were recruited in our study as the derivation cohort. Among them, 32 were excluded due to loss to follow-up (n = 21), incomplete medical history (n = 8), or death (n = 3). Of the 541 patients who were finally analyzed, 29 (5.4%) were diagnosed as confirmed ATB, 73 (13.5%) as clinically diagnosed ATB, 24 (4.4%) as clinically indeterminate, and 415 (76.7%) were excluded from ATB (Fig. 1). The demographic and clinical characteristics of the recruited subjects are listed in Table 1. In the group of patients with confirmed ATB and clinically diagnosed ATB, the number of males was higher (62/102, 60.8%) than those in the ATB excluded group (46.5%, p = 0.011), and more patients (18/102, 17.6%) had previous history of ATB than those in the ATB excluded group (6.5%, p = 0.0012). Most patients (n = 474, 87.6%) had underlying disease other than TB related to fever, which included infection (n = 135, 28.5%), connective tissue disease (n = 174, 36.7%), malignancy (n = 85, 17.9%), and miscellaneous diseases (n = 80, 16.9%). The causes of infection included Epstein-Barr virus infections (n = 17), cytomegalovirus infections (n = 10), bartonellosis (n = 5), brucellosis (n = 4), occult abscesses (n = 19), salmonellosis (n = 9), urinary tract infections (n = 31), bone and joint infections (n = 16), endocarditis (n = 3), and others (n = 21).

The patients with confirmed ATB and clinically diagnosed ATB included PTB (n = 26) and EPTB (n = 76). The patients with EPTB were diagnosed as central nervous system (CNS) TB (n = 31), lymph node TB (n = 8), bone and joint TB (n = 12), intestinal TB (n = 6), and others (n = 19). The numbers of cases with various focus locations and the underlying disease of all the patients are listed in Table 1. Among the 415 cases excluded from ATB, 157 were finally diagnosed as connective tissue disease, 126 were diagnosed as infection other than TB, 78 were diagnosed as neoplasm and 54 were diagnosed as other disease. There were still 24 patients (4.44%) diagnosed as clinically indeterminate.

3.2. Diagnostic performance of the T-SPOT and QFT assays for ATB

All the participants in the derivation group were tested with both T-SPOT and QFT assays, and the positive numbers of the two tests in different subgroups are presented in Table 2. There was no significant difference in the sensitivity between the confirmed ATB cases and clinically diagnosed ATB cases for both T-SPOT (82.8% vs. 86.3%, p = 0.7577) and QFT (72.4% vs. 71.2%, p = 0.8957). According to the final diagnoses of these patients with FUO, the overall sensitivity and specificity of T-SPOT in detecting ATB was 85.3% (95% CI 77.0–91.0%) and 71.8% (95% CI 67.3–76.0%), respectively. For QFT, the sensitivity and specificity were 72.3% (95% CI 62.8–80.1%) and 77.0% (95% CI 72.7–80.8%), respectively. The NPV and PPV of T-SPOT for ATB was 95.2% (95% CI 92.1–97.1%) and 42.9% (95% CI 36.2–49.7%), and the NPV and PPV of QFT was 91.8% (95% CI 88.4–94.3%) and 43.7%

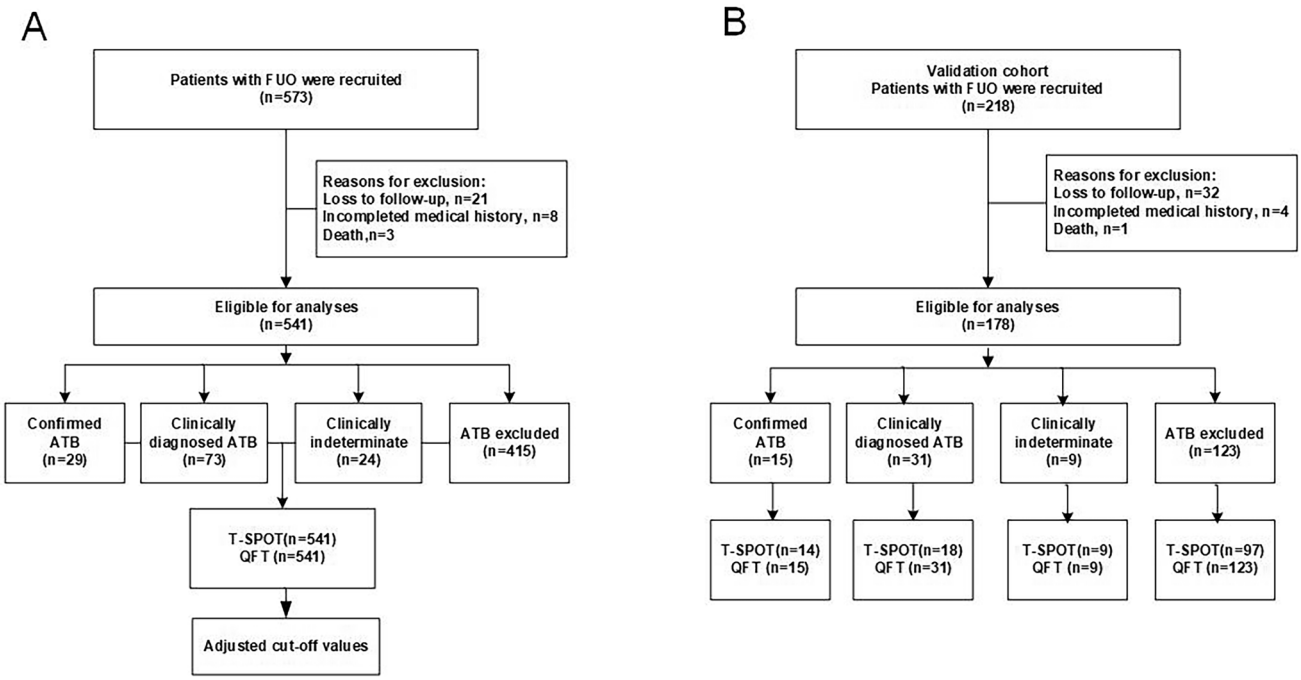

Fig. 1. Study flow diagram. (A) Derivation cohort. (B) Validation cohort. Abbreviations: ATB, active tuberculosis; FUO, fever of unknown origin; QFT; QuantiFERON-TB Gold in Tube; T-SPOT, T-SPOT®.TB test.

Table 1
Demographic and clinical characteristics of the subjects enrolled in derivation cohort.

	Confirmed ATB	Clinically diagnosed ATB	Clinically indeterminate	ATB excluded
Total, n (%)	29(5.4%)	73(13.5%)	24(4.4%)	415(76.7%)
Median age (IQR), year	45(35–67)	43(25–69)	48(25–78)	47(26–75)
Men, n (%)	18(62.1%)	44(60.3%)	11(45.8%)	193(46.5%)
BCG vaccination (based on presence of scar and vaccination records	21(72.4%)	61(83.6%)	16(66.7%)	324(78.1%)
Evidence of previous TB (%)	4(13.8%)	7(9.6%)	2(8.3%)	15(3.6%)
Contact history of pulmonary TB (%)	3(10.3%)	15(20.5%)	3(12.5%)	27(6.5%)
Duration of fever (days), (median, IQR)	70.5(48.0–121.5)	82.0(51.2–153.5)	92.5(47.0–163.5)	85.0(47.5–135.5)
Pulmonary TB	5	21	N/A	N/A
Extra-pulmonary TB	76	52	N/A	N/A
CNS TB	12	19	N/A	N/A
Lymph node TB	2	6	N/A	N/A
Bone and joint TB	3	9	N/A	N/A
Intestinal TB	2	4	N/A	N/A
Disseminated TB	1	3	N/A	N/A
Tuberculous serositis	2	4	N/A	N/A
No definite site	2	7	N/A	N/A
Underlying disease				
Connective tissue disease	3	11	3	157
Infection other than TB	2	3	4	126
Malignancy	1	2	4	78
Others	8	15	3	54

TB: tuberculosis; IQR: interquartile range; BCG: Bacillus Calmette-Guérin; CNS: central nervous system.

(36.4–51.3%), respectively.

The head-to-head comparison and concordance between T-SPOT and QFT in different groups are shown in Table 3. In total, there was a moderate agreement of T-SPOT and QFT in confirmed ATB cases ($\kappa = 0.316$), clinically diagnosed ATB ($\kappa = 0.370$), and patients excluded from ATB ($\kappa = 0.573$). The agreement was 75.9% (95% CI 57.6–88.1%), 78.1% (95% CI 67.2–86.1%) and 83.1% (95% CI 79.2–86.4%), respectively.

3.3. Investigation of different cut-off values of T-SPOT and QFT for detecting ATB

According to our results, the diagnostic performance of both T-SPOT and QFT were not satisfactorily accurate for diagnosing ATB in patients with FUO. The PPV was relatively low for both T-SPOT (42.9%) and QFT (43.7%), which could cause false positive results for ATB diagnosis. In routine clinical practice, if diagnostic anti-TB treatment is given to these kinds of patients presenting as FUO according to the current IGRAs results, it could create a burden (e.g., wastage of time, costs and adverse effects of drugs, anxiety provoked by testing) that exceeds such benefit. Therefore, since the risk of anti-TB treatment is high, clinicians want to be extremely sure of the diagnosis and might recommend treatment only when the probability of ATB is very high (specificity > 90%) for better clinical decision-marking.

Therefore, in order to reduce the false positive rate of IGRAs, we first performed receiver operating characteristic (ROC) analysis to evaluate the diagnostic potential of the T-SPOT and QFT assays in differentiating ATB from other diseases in these patients with FUO. The area under curve was 0.83 (95% CI 0.67–0.89) for T-SPOT and 0.85 (95% CI 0.63–0.89) for QFT, with no significant difference ($p = 0.4315$). According to the results of ROC analysis, we then selected the cut-off values corresponding to a specificity of>90%, and a threshold value of 125 SFCs/2.5 $\times$ 10^5 cells for T-SPOT and 4 IU/mL for QFT was used to differentiate between patients with ATB and others, which resulted in a PPV of 42.9% and 43.7%, respectively. The diagnostic performance of different cut-off values is shown in Table 4.

3.4. Validation of the new cut-off

To validate the adjusted cut-off values for the T-SPOT and QFT assays, we prospectively recruited another cohort of 218 patients with FUO as a validation cohort. Of these, 37 were excluded due to loss of follow-up (n = 32), incomplete medical history (n = 4), and death (n = 1). Of the 178 patients who were finally enrolled, 15 were finally diagnosed as confirmed ATB, 31 were diagnosed as clinically diagnosed ATB, 9 were clinically indeterminate and 123 were excluded from ATB (Fig. 1).

All the patients in the validation cohort were tested by QFT (n = 178), while some were tested with T-SPOT (n = 138). The sensitivity and specificity calculated according to the manufacturer-suggested cut-off

Table 2
Diagnostic performance of T-SPOT and QFT for detecting ATB in patients with FUO.

Test results	Confirmed ATB	Clinically diagnosed ATB	Clinically indeterminate	ATB excluded	Total
T-SPOT					
Positive	24	63	14	116	217
Negative	5	10	9	296	320
Borderline	0	0	1	3	4
Total	29	73	24	415	541
SFCs (ESAT-6)	34(5–136)	42(6–234)	11(2–71)	2(0–11)	
SFCs (CFP-10)	33.5(6–216.5)	37(5–219)	12(5–48)	2(0–9)	
QFT					
Positive	21	52	11	94	178
Negative	8	20	12	315	355
Indeterminate	0	1	1	6	8
Total	29	73	24	415	541
IFN-gamma levels	3.1(0.45–9.33)	4.1(0.45–7.31)	1.53(0.73–4.21)	0.16(0.02–0.79)	

SFCs: spot-forming cells; ESAT-6: early secreted antigenic target of 6 kDa; CFP-10: culture filtrate protein 10 kDa; QFT-GIT: QuantiFERON-TB Gold in Tube; IFN-γ: interferon-gamma.

Table 3
Comparison of responder numbers and agreement among the T-SPOT and QFT test in different groups.

		QFT				
		Positive	Negative	Indeterminate	Total	Agreement, Kappa
T-SPOT	Confirmed ATB	Positive	19	5	0	24	75.9%, 0.316
		Negative	2	3	0	5	
		Indeterminate	0	0	0	0	
		Total	21	8	0	29	
	Clinically diagnosed ATB	Positive	50	13	0	63	78.1%,0.370
		Negative	2	7	1	10	
		Indeterminate	0	0	0	0	
		Total	52	20	1	73	
	ATB excluded	Positive	71	44	1	116	83.1%, 0.573
		Negative	23	271	2	296	
		Indeterminate	0	0	3	3	
		Total	94	315	6	415	

Table 4
The diagnostic performance of different cut-off values.

	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	NPV (95% CI)	PPV (95% CI)
T-SPOT (SFCs/2.5*10^5)					
Manufacturer's suggested	5	85.3% (76.59%-91.26%)	71.8% (67.19%-76.08%)	95.2% (92.00%-97.18%)	42.9% (36.01%-49.98%)
High specificity	125.0	43.1% (33.49%-53.31%)	90.3% (86.91%-92.89%)	86.5% (82.83%-89.52%)	52.4% (41.26%-63.28%)
High sensitivity	4.0	90.2% (82.30%-94.94%)	42.0% (37.20%-46.93%)	94.5% (89.90%-97.20%)	27.8% (23.10%–33.01%)
Maximum (Sensitivity + Specificity –1)	8.0	89.2% (81.13%-94.23%)	70.6% (65.93%-74.94%)	96.4% (93.39%-98.07%)	42.9% (36.22%-49.89%)
QFT (IU/ml)					
Manufacturer's suggested	0.4	72.3% (62.33%-80.50%)	77.0% (72.57%-80.95%)	91.8% (88.29%-94.41%)	43.7% (36.13%-51.59%)
High specificity	4.0	41.6% (31.99%-51.82%)	94.1% (91.27%-96.12%)	86.7% (83.11%-89.66%)	63.6% (50.82%-74.86%)
High sensitivity	0.1	90.1% (82.13%-94.89%)	36.7% (32.03%-41.57%)	93.8% (88.49%-96.79%)	26.0% (21.55%-30.99%)
Maximum (Sensitivity + Specificity –1)	1.1	67.3% (57.18%-76.13%)	85.1% (81.17%-88.32%)	91.3% (87.94%-93.88%)	52.7% (43.76%-61.50%)

and optimized values are listed respectively in Table 5. The specificity of T-SPOT was 92.8% by the cut-off of 125 SFCs/2.5 × 10^5 cells, which was significantly higher to that calculated by the default value (81.4%, $p = 0.0184$). The specificity of QFT was 94.3% by the cut-off of 4 IU/ml, which was also significantly higher than that by the default value (74.8%, $p < 0.0001$). Details of sensitivities and specificities at different thresholds are shown in Table 5. The NPV and PPV of adjusted T-SPOT for ATB were 95.2% (95% CI 92.1–97.1%) and 42.9% (95% CI 36.2–49.7%), and the NPV and PPV of adjusted QFT were 91.8% (95% CI 88.4–94.3%) and 43.7% (36.4–51.3%), respectively. These data suggest that the performance of adjusted values in the validation cohort was similar to that in the derivation cohort.

4. Discussion

The diagnosis and treatment of FUO remains a challenge in real-world clinical practice. The objectives of our study were to find better ways to diagnose ATB presenting as FUO with IGRAs, and to determine the method of adjusting the interpretation of the results of IGRAs when applying them to those patients. In this study, 791 subjects with FUO were recruited as the derivation cohort (n = 573) and the validation cohort (n = 218), and the diagnostic performance of T-SPOT and QFT assays were evaluated. We then defined the cut-off value of T-SPOT as 125 SFCs/2.5 × 10^5 cells and cut-off value of QFT as 4 IU/mL rather than 5 SFCs/2.5 × 10^5 cells and 0.4 IU given by manufacturers, which helped in obtaining a high PPV for accurate diagnosis of ATB in patients with FUO.

Our study has several limitations. First, some of the TB patients were clinically diagnosed rather than culture or histology confirmed. Infection other than TB may be misdiagnosed, such as NTM infection and other bacterial infections that would respond to RIPE regimen (e.g., *Legionella*, *Listeria*, *Neisseria*, and *Staphylococcus aureus*), which could lead to underestimation of the diagnostic potential of the two assays. Longitudinal cohort studies will be required with careful clinical characterization of the patients into confirmed TB infection and disease groups to validate the accuracies and the new cut-off values. Second, the new cut-off values were not evaluated with an emphasis on immunocompromised or immunosuppressed patients including those receiving immunosuppressive therapy, those with HIV infection, and children. Finally, this study was performed in a single clinical center. Hence, multi-site, longitudinal cohort studies with a larger sample size and broader range of disease are warranted in future.

FUO may be caused by many diseases, which can vary depending on the region and time duration [20]. In China, a country with a high TB burden, ATB is one of the differential diagnoses regularly considered

Table 5
Diagnostic performance of the adjusted cut-off values for T-SPOT and QFT in the validation cohort.

	Cut-off	Confirmed ATB	Clinically diagnosed ATB	Clinically indeterminate	ATB excluded	Sensitivity (95% CI)	Specificity (95% CI)
T-SPOT (SFCs/2.5*10^5)	5	78.6% (11/14)	72.2% (13/18)	77.8% (7/9)	18.6% (18/97)	75.0% (56.2%-87.9%)	81.4% (72.0%-88.3%)
	125	35.7% (5/14)	55.6% (10/18)	22.2% (2/9)	7.2% (7/97)	46.9% (29.5%-65.0%)	92.8% (85.2%-96.8%)
QFT (IU/ml)	0.4	66.7% (10/15)	74.2% (23/31)	77.8% (7/9)	25.2% (31/123)	71.7% (56.3%-83.5%)	74.8% (66.0%-82.0%)
	4	46.7% (7/15)	45.2% (14/31)	33.3% (3/9)	5.7% (7/123)	45.7% (31.2%-60.8%)	94.3% (88.2%-97.5%)

during the evaluation of FUO. A retrospective analysis of 1,641 cases of class FUO in China demonstrated that 19.5% of the cases were caused by ATB [21]. Another study of 997 FUO cases showed that the TB infection was the leading etiology of FUO for 21.8% of the FUO cases [4]. In our cohort, ATB accounted for 17.8% (102/573) of the FUO cases and 44.7% (102/228) of the infectious disease cases of FUO, which was similar to those reported in previous studies. There are several possible reasons why ATB has become the main cause of FUO in China. The frequency of ATB in FUO is associated with the high incidence rate of TB infection in China, and TB can affect people of all ages with various clinical manifestations. At the end of 2015, the *Mtb* infection rate was estimated to be 36.7% in mainland China [22], and the predicted ATB prevalence was 139 to 221 per 100,000 population in Shanghai [23]. Moreover, most ATB patients with FUO cannot be diagnosed by traditional diagnostic methods, such as AFB smear or culture [24], and EPTB frequently involves anatomical sites that are not easily accessible and require invasive procedures for diagnostic confirmation. In real-life clinical practice, a considerable proportion of ATB suspected cases presenting as FUO were negative for routine diagnostic methods, and diagnostic anti-TB treatment was then performed. This kind of treatment strategy has very limited clinical applications, because the efficacy of anti-TB treatment is usually seen after 1–2 weeks. Therefore, a wrong diagnosis of TB infection could lead to serious delay of the right treatment [25].

Current IGRAs, including T-SPOT and QFT, due to the strength of *Mtb*-specific antigens, perform better than the traditional tuberculin skin test in terms of their ability to discriminate *Mtb* infections from BCG vaccination or non-tuberculous mycobacterial infections [13]. However, one major limitation of IGRAs is that they fail to distinguish between ATB and LTBI, which could greatly hamper the early treatment and control of ATB [26]. Using the cutoff values given by the manufacturers, the two assays showed relatively low specificity for detecting ATB in the present study. These results were similar with those of previous studies in China, and high false positive rates of the two assays were also found in other studies for detecting ATB in FUO patients [28,29]. The reason of these results could be explained by the high latent TB endemic rate found in China [18,27]. Moreover, FUO is often accompanied by symptoms similar to ATB infection, such as fever, night sweats, cough and so on. Therefore, research and development of effective and accurate diagnostic tests for ATB, especially with high specificity or true positive rate, are urgently required in these patients.

The cut-off value and definition of conversion in the T-cell assays is a matter of debate and research both in high- and low-burden settings [30]. The single cut-off value given in the manufacturer's instruction is uniformly applied to the diagnosis of ATB, including both active and latent TB infection. However, because the IGRAs measures antigen-stimulated IFN-γ release in whole blood, the results of these assays are inherently continuous variables. Adjusting of the test results using different cut-off values could be necessary because of nonspecific variability and reproducibility of the continuous data [31]. Several other studies have also adjusted the cut-off values of T-SPOT or QFT to improve the diagnostic accuracy of these two assays [32–34]. Moreover, in countries or regions with different TB infection rates, the best cut-off value could be different from the recommended values by the manufacturers, and it is necessary to find out the best cut-off value suitable in such situations.

In this study, we focused on patients with FUO who have unique characteristics compared with other TB suspected patients. TB patients representing as FUO usually cannot be diagnosed by traditional methods, such as AFB smear or GeneXpert MTB/RIF, and the proportion of extrapulmonary TB is very high [24]. Moreover, in most of these patients, the diagnosis is complicated with other inflammatory or tumor diseases [35]. In such cases, it is necessary to investigate the best cut-off value in these cohorts, especially for real-life clinical applications. Our study proposed that the cut-off value could be raised to 125 SFCs/2.5 × 10^5 cells for T-SPOT and 4 IU/mL for QFT for FUO cases, which could allow for high specificity (PPV) but likely at some cost in sensitivity. For FUO patients, the diagnosis of ATB is particularly difficult with no specific clinical symptoms and absence of positive results from routine diagnostic tests. Therefore, in the real-world clinical application, rather than screening test, it is more important to obtain a higher PPV for the IGRAs to provide accurate evidence of ATB, so as to avoid taking anti-TB treatment by mistake. In addition, the tests with adjusted cut-off values could still be combined with other tests or clinical indicators, such as erythrocyte sedimentation rate and C-reactive protein, to increase the sensitivity.

5. Conclusions

TB infection continues to be an important consideration in the evaluation of patients with FUO in China. The diagnostic performance of T-SPOT and QFT are not satisfactory in detecting ATB in these patients with low PPV rate. Our results demonstrated that the adjusted cut-off value of 125 SFCs/2.5 × 10^5 cells for T-SPOT test and 4.0 IU/mL for QFT test could improve the diagnostic specificity when applied to FUO patients in high TB burden countries, which could provide more valuable reference for ATB diagnosis in real-life clinical applications and making better treatment decisions.

Funding

This work was supported by the Shanghai National Base Cultivation Project [grant numbers 20dz2210403].

Ethical statement

The study was approved by the Ethics Committee of Huashan Hospital affiliated with Fudan University. Written informed consent was obtained from all patients enrolled in this study.

Author contributions

Yaojie Shen performed the experiments. Xiao Qi contributed to analysis and manuscript preparation. Jing Wu performed the data analyses and wrote the manuscript. Yan Gao helped perform the analysis with constructive discussions. Lingyun Shao designed the project. Wenhong Zhang acquired the financial support for the project leading to this publication. Sen Wang contributed to analysis and the conception of the study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The study investigators gratefully acknowledge all study participants.

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