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ROLE OF THE QUANTIFERON-TB TEST IN RULING OUT PLEURAL TUBERCULOSIS: A MULTI-CENTRE STUDY

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Diagnosing pleural tuberculosis (plTB) might be difficult due to limited sensitivity of conventional microbiology tools. As *M. tuberculosis* (MTB)-specific T cells are recruited into pleural space in plTB, their detection may provide useful clinical information. To this aim, in addition to standard diagnostic tests, we used the QuantiFERON-TB Gold In-Tube (QFT-IT) test in blood and pleural effusion (PE) samples from 48 patients with clinical suspicion of plTB, 18 (37.5%) of whom had confirmed plTB. Four of them (22.2%) tested positive with a nucleic acid amplification test for MTB. The tuberculin skin test was positive in most confirmed plTB cases (88.9%). Positive QFT-IT tests were significantly more frequent in patients with confirmed plTB, as compared to patients with an alternative diagnosis, both in blood (77.7 *vs* 36.6%, p=0.006) and in PE samples (83.3% *vs* 46.6%, p=0.02). In addition, both blood and PE MTB-stimulated IFN-γ levels were significantly higher in plTB patients (p=0.03 and p=0.0049 *vs* non-plTB, respectively). In blood samples, QFT-IT had 77.8% sensitivity and 63.3% specificity, resulting in 56.0% positive (PPV) and 82.6% negative (NPV) predictive values. On PE, QFT-IT sensitivity was 83.3% and specificity 53.3% (PPV 51.7% and NPV 84.2%). The optimal AUC-derived cut-off for MTB-stimulated pleural IFN-γ level was 3.01 IU/mL (77.8% sensitivity, 80% specificity, PPV 68.4% and NPV 82.8%). These data suggest that QFT-IT might have a role in ruling out plTB in clinical practice.

Pleural tuberculosis (plTB) is an extra-pulmonary manifestation of active tuberculosis (TB), representing about 4% of all TB cases (1). Despite the characteristic finding of increased count of lymphocytes in pleural effusion (PE), confirmation of plTB diagnosis based on PE analysis is problematic

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due to the low rates of positive microscopic and culture tests from PE samples (2). Diagnostic accuracy of culture for *Mycobacterium tuberculosis* (MTB) and nucleic acid amplification techniques (NAAT) in PE ranges from 12 to 70% (2-3) and from 30 to 100% (2, 4), respectively. Biomarkers related to pleural T cell activation, like adenosine deaminase (ADA) and unstimulated interferon-gamma (IFN-γ), also have suboptimal performance, with sensitivity values varying in different studies from 50% to 100% (5-7). Specificity of these biomarkers is also variable due to the increased levels of both ADA and IFN-γ in para-pneumonic effusions and lung malignancies. As such, pleural biopsy is still regarded as the diagnostic "gold standard" for the diagnosis of plTB, allowing the identification of typical caseating granulomas (2); however, the invasiveness of the procedure limits its widespread use in routine clinical practice.

IFN-γ release assays (IGRAs) for diagnosis of latent TB infection (LTBI) are based on the detection and quantification of IFN-γ released by sensitized peripheral blood T lymphocytes after *in vitro* stimulation with MTB-specific antigens (8). There are three commercially available assays, the ELISpot-based T-SPOT.*TB*™ test (Oxford Immunotec Inc., Abingdon, UK) and the two ELISA-based assays, QuantiFERON-TB Gold and QuantiFERON-TB Gold In-Tube (QFT-IT) (Cellestis Ltd., Carnegie, Victoria, Australia). The latter test represents an improvement of the ELISA-based platform as whole blood is drawn directly into "vacutainer" tubes pre-coated with antigens and ready for incubation. IGRAs rely on the use of two MTB-specific antigens, the Early Secretory Antigen Target (ESAT)-6 and the Culture Filtrate Protein (CFP)-10: QTF-IT also includes the recombinant protein TB7.7. These antigens are absent from most environmental non-tuberculous mycobacteria and from *M. bovis* BCG vaccine strains (9-10), thus providing IGRA with excellent specificity (11). Despite the fact that IGRA do not discriminate LTBI from active TB, pooled sensitivity of blood assays has recently been estimated to be higher than 80% in detecting active TB disease as a surrogate of TB infection, at least in developed countries (11- 12). Some reports indicate that IGRAs might be helpful in selected cases of pulmonary TB cases (13-17) and in patients with extra-pulmonary TB, including plTB and TB meningitis, by detecting the release of IFN-γ by antigen-specific T cells directly at the infection site (18-25).

MATERIALS AND METHODS

Patients and study design

Fifty-six consecutive HIV-negative patients with suspected plTB were prospectively enrolled at the Department of Respiratory Medicine of the University of Modena, the Department of Clinical and Experimental Medicine of the Federico II University of Naples, and the TB Division of the S. Camillo-Forlanini Hospital in Rome, all in Italy. Patients provided informed consent and the study was approved by the local Ethics Committees. PE samples drawn in the context of the routine clinical management were sent for conventional microbiology [acid-fast bacilli (AFB) staining and culture for mycobacteria] and TB-specific DNA amplification (Amplified MTD, Gen-Probe, San Diego, CA, USA): diagnostic work-up also included tuberculin skin test (TST) (5 IU PPD, Biocine Test PPD, Chiron, Siena Italy), interpreted according to current guidelines (26). To date, the TST threshold used was 10 mm. A cut off of 5 mm was chosen for cases with recent TB contact (n=3) or with fibrotic changes on chest X-ray consistent with old TB (n=2). QFT-IT was performed in all cases on peripheral blood and PE samples, according to the manufacturer's instructions. Briefly, 1 mL of blood or 1 mL of PE was drawn directly into each of the three collection tubes pre-coated with saline solution as negative control (Nil), with phytohaemagglutinin (mitogen, PHA) as positive control, and with ESAT-6, CFP-10 and TB 7.7: tubes were shacked and incubated at 37°C in a humidified atmosphere overnight. The following morning, after centrifugation, plasma and PE supernatants were harvested from each tube and stored at 4°C. ELISA-based levels of IFN-γ (IU/mL) were measured with an Automated System ELISA Reader (Dinex Technologies, West Sussex, UK). Levels of IFN-γ in the negative control tube (Nil) were subtracted from those recorded in the PHA and TB-specific tubes before calculating the final amount of the cytokine in each sample. Results were classified as follows: positive, if the level of IFN-γ was equal or greater than 0.35 IU/mL in the TB-specific tube, irrespective of the response to the mitogen; negative, if the concentration of IFN-γ was less than 0.35 IU/mL and equal or greater than 0.50 IU/mL in the positive control. An indeterminate result was recorded when the level of PHA-induced IFN-γ was less than 0.50 IU/mL and less than 0.35 IU/mL in response to TB antigens. According to the manufacturer's instructions, if IFN-γ level in the negative control tube was ≥8.0 IU/mL, the result was discharged as technical fault and excluded

from the statistical analysis.

Statistical analysis

Categorical and continuous variables were compared using parametric and non-parametric tests. Statistical values were expressed as median ± standard deviation (SD) for non-normally distributed variables. The Mann-Whitney test was used to compare IFN-γ levels across different groups. Results were considered statistically significant if the p value was <0.05. The optimal cut-off values, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the IFN-γ assay were assessed with receiver operating curve (ROC) analysis. Analyses were performed using the Stata software package (Stata Corp, Stata 10.0 software, TX USA).

RESULTS

Demographic and clinical characteristics of the study population according to final diagnosis are reported in Table I. Eight patients were excluded from the data analysis as QFT-IT scored an indeterminate result: in 4 cases in blood due to a low response to PHA and in a further 4 patients on PE, two of them due to high IFN-γ levels in the negative control well. Patients with QFT-IT indeterminate results included 7 Italians and 1 foreign-born (Philippines) with a median age of 70 ±14 yrs and a Male:Female ratio of 6:1. Active TB was excluded in 7 cases; TST scored a positive result in one case affected by non-TB pleuritis and was missing in additional two non-TB patients. Interpretable QFT-IT results from both blood and PE samples were therefore available for 48 (85.7%) patients, classified according to their final diagnosis as follows: (i) definite plTB when culture was positive for MTB on PE or sputum and/or TB-NAAT was positive on PE or sputum; (ii) probable plTB when the diagnosis was based on consistent clinical and radiological findings and in the presence of a positive response to anti-TB treatment; (iii) non-plTB when an alternative diagnosis other than active TB was made. A diagnosis of either definite or probable plTB was obtained in 18 (37.5%) patients (15 of whom were definite plTB). Diagnoses other than plTB included para-pneumonic effusion (39.5%), malignancy (16.6%), and other causes (6.2%). TST was performed on 40 (83.3%) patients, 18 with plTB and 22 with another diagnosis: 16 (88.9%) patients with plTB and 6 (27.3%) non-TB patients were TST-positive. Results for NAAT were available for all patients: 7 out of 18 patients with plTB tested positive with TB-specific NAAT (4 cases on PE and 3 on sputum), corresponding to a sensitivity and specificity when applied to PE samples of 26.6% and 100%, respectively. Overall, QFT-IT was positive in 14 (77.7%) and 15 (83.3%) of the 18 plTB cases when performed on blood or PE samples, respectively. QFT-IT was positive on pleural fluid of all 15 patients with confirmed plTB, while 13 of them also had a positive QFT-IT on blood. Of the 3 patients with a diagnosis of probable plTB, QFT-IT was positive in 1 case in blood and in none in pleural fluid. In patients without plTB, QFT-IT was positive in 11 (36.6%) and 14 (46.6%) in blood and PE samples, respectively. Rates of positive QFT-IT were significantly higher in patients with plTB both in blood (p=0.006) and PE (p=0.02). MTB-specific IFN-γ levels were significantly higher in PE

Table I. *Demographic and clinical characteristics of the study population according to final diagnosis.*

	plTB patients	non TB patients
n, (%)	18 (37.5)	30 (62.5)
Median age* ± SD, yrs (range)	32 ± 16 (20-72)	71.5 ±15 (32-94)
Males, n (%)	10 (55.5)	22 (73.3)
Foreign-born§, n (%)	7 (38.8)	3 (10)
BCG-vaccinated, n (%)	8 (44.4)	2 (11.1)
TST positivity, n (%)	6 (88.9)	6 (27.3)
TB-specific NAAT positivity, n (%)	7 (46.6)	0 (0)

* *plTB vs non-TB patients, p< 0.0001; §Countries of origin included Romania, India, Ghana, Egypt, Russia, Somalia, Pakistan, and Poland.*

Table II. *QFT-IT based interferon-gamma levels in blood and PE samples according to patient final diagnosis.*

	25% percentile	median	75% percentile	SD	range
Blood QFT-IT IFN-γ*					
plTB patients	0.36	0.805	2.98	6.44	0.02-25.98
non TB patients	0.03	0.205	1.1	8.98	0.00-41.47
PE QFT-IT IFN-γ					
plTB patients	3.01	13.78	40.38	79.45	0.01-255.04
non TB patients	0.03	0.26	1.1	28.32	0.00-151.03

**IFN-γ levels are expressed as IU/mL*

Table III. *Performance of pleural and blood QFT-IT according to the cut-off level in patients with clinical suspicion of plTB.*

	% sensitivity	% specificity	%PPV	%NPV	LR+	LR-	%accuracy	AUC
PE QFT-IT*								0.78
≥ 0.35	83.3	53.3	51.7	84.2	1.78	0.31	64.6	
≥ 0.91	83.3	70.0	62.5	87.5	2.78	0.24	75.0	
≥ 3.01	77.8	80.0	68.4	82.8	3.89	0.28	79.2	
Blood QFT-IT*								0.68
≥ 0.35	77.8	63.3	56.0	82.6	2.12	0.35	68.7	
≥ 2.73	33.3	90.0	66.7	69.2	3.33	0.74	68.7	

* *referred cut-off points are expressed as IU/mL*

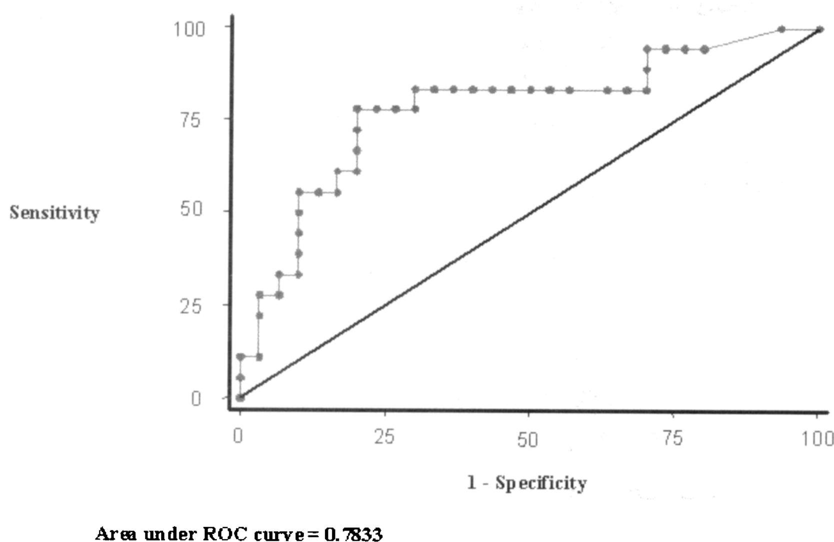

Fig. 1. *The Receiver Operating Curve (ROC) for TB vs non-TB pleural effusion, indicating that the optimal IFN-γ value is 3.01 IU/mL (77.8% sensitivity, 80% specificity).*

than in blood for both plTB (p=0.005) and non-plTB patients (p=0.04) (Table II): however, MTB-specific IFN-γ levels in both blood and PE were significantly higher in plTB compared to non-plTB cases (p=0.03 and p=0.001, respectively) (Table II).

Using the approved QFT-IT positive cut-off at

0.35 IFN-γ IU/mL, sensitivity and specificity of blood QFT-IT for plTB were respectively 77.8% and 63.3% (PPV 56.0%, NPV 82.6%). With the same cut-off value, sensitivity and specificity of QFT-IT on PE samples were respectively 83.3% and 53.3% (PPV 51.7%, NPV 84.2%). Based on a ROC curve analysis, we estimated the optimal cut-off point for MTB-specific IFN-γ levels in the QFT-IT assay (Fig. 1): optimal AUC-derived cut-off in PE was 3.01 IU/mL, providing 77.8% sensitivity and 80.0% specificity (PPV 68.4%, NPV of 82.8%): diagnostic accuracy increased from 64.6% to 79.2%. Conversely, diagnostic accuracy of QFT-IT remained unchanged with increased cut-off values in blood samples (Table III). Finally, the combined use of TST and PE QFT-IT (with the optimal cut-off point at 3.01 IU/mL) did not provide additional advantage, while being associated with increased PPV (77.8%).

DISCUSSION

Standard non-invasive diagnostic tools for plTB showed suboptimal performance in routine clinical practice. Some initial evidence suggested that *in vitro* assays measuring the release of IFN-γ by effector T cells stimulated with MTB-specific antigens might help to diagnose extra-pulmonary active TB cases, in particular if applied to biological samples representative of the infection site (18-25). It has been estimated that the frequency of clonally expanded antigen-specific effector T cells is over 10-fold higher at the site of active plTB and is theoretically null in patients without TB (27).

The findings of this multi-centre study indicate that the QFT-IT blood test detects plTB with 77.8% sensitivity and 63.3% specificity, while the same test applied to PE samples has 83.3% sensitivity and 53.3% specificity. The combined use of blood and pleural QFT-IT led to a slight increase of sensitivity (88.9%), while specificity was not modified. As predictable, pleural MTB-specific QFT-IT IFN-γ concentrations were significantly higher than those in blood of patients with plTB, likely reflecting active recruitment of MTB-specific IFN-γ-producing T cells to the site of disease. In addition, local immune-diagnosis by means of pleural QFT-IT was also performing better compared to TB-specific NAAT. Conversely, TST diagnostic performance was higher than expected, which in part may be due to high probability of TB infection in our study population, which included foreign-born cases from high TB prevalence countries and a high number of BCG-vaccinated individuals among plTB-patients. However, this was, in part, not surprising as pooled sensitivity of TST in studies has been shown to be no different from that of IGRA, at least in low prevalence countries (12). Both blood and PE QFT-IT positive results were obtained in a sizeable proportion of patients without plTB; nonetheless, MTB-specific IFN-γ levels were significantly higher in plTB cases. This finding likely reflects LTBI rates among patients without plTB. As a consequence, the inclusion of IGRAs in the diagnostic workout of active plTB cannot be apart from the clinical and epidemiological setting.

A recent meta-analysis addressing the usefulness of measurement of total non-specific IFN-γ in PE samples of patients with suspected plTB suggests this biomarker as a promising diagnostic tool with an estimated pooled sensitivity and specificity of 89% and 97%, respectively (28). To date, Chegou and co-workers have shown that *ex vivo* pleural fluid IFN-γ levels accurately identified TB in all patients in a high burden area, suggesting IGRA to have a poor additional diagnostic value (20). Detection of whole IFN-γ was superior to QFT-IT using blood and pleural fluid (73% and 57% sensitivity, with 71% and 87% specificity, respectively) and to QFT-G (the predecessor of QFT-IT) applied to isolated pleural fluid cells (100% sensitivity and 67% specificity). Similarly, Dheda and co-workers suggested that total IFN-γ is the most accurate test to distinguishing TB from non-TB pleural effusions in a cohort of 74 patients in South Africa. Clinical usefulness of both ELISA- and ELISpot-based tests measuring TB-specific IFN-γ was limited in their setting by the low accuracy and inability to isolate sufficient mononuclear cells to correctly perform the assays (7).

In our study population, although limited in size, being representative of routine clinical practice, the interpretation of QFT-IT on PE with a different cut-off value for positive results improved assay performance by increasing specificity, PPV and NPV, while sensitivity was almost unchanged. These findings, although interesting, have been derived

from a post-hoc ROC analysis and need to be validated in prospective large cohorts of patients.

In a previous report, we found that sensitivity and specificity of the ELISpot-based assay T-SPOT.*TB* applied to PE samples for plTB were 95% and 76%, respectively (19). Similar results have been obtained in a prospective study performed in Taiwan, reporting high sensitivity and specificity for T-SPOT.*TB* (22): furthermore, the performance of the assay was not reduced by low lymphocyte counts, as MTB-specific IFN-γ T cells were concentrated 8- to 10-fold in pleural fluid, compared to peripheral blood (22). Although these findings are limited by the small sample sizes and by study design, nonetheless, the two IGRAs may have differential performances when used with samples other than blood.

Our results confirm that QFT-IT is feasible in PE samples and may play a role as a rule-out test to exclude plTB in patients presenting with pleural effusions of unknown cause. Moreover, the data suggest that in order to maximize the information derived from QFT-IT performed on PE, the cut-off for positive results should be considerably increased: although this represents an intuitive and potentially important finding, it will need to be validated in larger prospective studies.

All Authors declare that no conflict of interest exists.

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