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QuantiFERON-TB performance enhanced by novel *Mycobacterium tuberculosis*-specific antigens

To the Editor:

One of the most significant developments in the diagnosis of tuberculosis (TB) infection has been the introduction of whole-blood based interferon-γ release assays (IGRAs) [1–3]. IGRAs, commercially available as QuantiFERON-TB Gold In-Tube test (QFT) (QIAGEN, Germantown, MD, USA) and T-SPOT.TB (Oxford Immunotec Ltd, Abingdon, UK), are based on the detection of a T-cell immune response towards RD1 antigens (ESAT-6 and CFP-10), with the addition of the TB7.7 antigen for the QFT only [1]. IGRAs are endowed with great specificity, as the antigens used are almost exclusively expressed by the *Mycobacterium tuberculosis* (MTB) complex, but not *Mycobacterium bovis* bacille Calmette–Guerin (BCG) [1–3]. However, the diagnostic sensitivity of IGRAs can be improved (75–85% in HIV-negative active TB patients) especially in countries with a high TB burden [3].

To improve the performance of the IGRAs, addition of MTB antigens TB7.7, Rv3425 and EsxV to the current antigen cocktail has already been attempted [4, 5]. Following a similar strategy, we evaluated the addition of six novel peptides to the QFT antigen cocktail to improve the diagnostic performance in terms of accuracy and dynamic range of interferon (IFN)-γ level in active TB patients.

To this end, a multicentre study was performed under different clinical settings. Study group characteristics are reported in table 1. Active TB patients (n=85) were enrolled in three different clinical centres at the time of admission for being suspected of having TB and diagnosed according to Centers for Disease Control and Prevention/American Thoracic Society criteria [6]. Control subjects (n=290) without any risk factors for latent TB infection were enrolled in two TB low-risk countries (Australia and Italy)

TABLE 1 Demographics and results of the QFT and QFT plus novel peptides in the different study groups

Group and country of origin	Subjects	Age years	Sex M/F	Notes	QFT		QFT-Six	IFN-γ[#] IU·mL^{-1}	
					Indeterminate	Positive[#]	Positive[#]	QFT	QFT-Six
Healthy controls									
Italy	34	24±5	14/20	Low exposure risk	0 (0)	0 (0)	0 (0)	0.00 (0–0.01)	0.00 (0–0.01)
Australia	206	38±14	90/116	Low exposure risk	0 (0)	4 (1.9)	3 (1.4)	0.00 (0–0.01)	0.00 (0–0.01)
Bulgaria	50	37±8	19/31	Medium exposure risk; BCG vaccinated	0 (0)	2 (4)	2 (4)	0.06 (0.02–0.11)	0.09 (0.05–0.16)
Active TB, HIV negative								3.88 (0.88–10)	5.01 (1.20–10)[¶]
Italy	31	39±15	14/17	Low-incidence country	0 (0)	27 (87)	30 (97)		
South Africa	12	42±13	7/5	Medium/high-incidence country; BCG vaccinated	0 (0)	9 (75)	9 (75)		
India	22	39±10	18/3	Medium/high-incidence country; BCG vaccinated	3 (13)	16 (84)	16 (84)		
Active TB, HIV infected								0.82 (0.22–2.10)	0.99 (0.39–2.26)[¶]
South Africa	4	36±7	1/3	Medium/high-incidence country; BCG vaccinated	1 (25)	3 (100)	3 (100)		
India	16	38±10	12/4	Medium/high-incidence country; BCG vaccinated	4 (25)	8 (67)	9 (75)		

Data are presented as n, mean±SD, n (%) or median (interquartile range). QFT: QuantiFERON-TB Gold In-Tube test (QIAGEN, Germantown, MD, USA); M: male; F: female; QFT-Six: QFT plus the six novel peptides; IFN: interferon; TB: tuberculosis; BCG: bacille Calmette–Guerin. [#]: percentages and IFN-γ levels were evaluated on valid tests not including indeterminate results; [¶]: $p<0.05$ *versus* QFT alone (Wilcoxon matched paired test).

and in a TB medium-risk country (Bulgaria). The study was approved by the institutional review boards of all participating clinical institutions. Informed consent was obtained from each study subject before blood sample collection.

The novel peptides were derived from MTB-specific genes overexpressed in an *in vitro* model of macrophage infection and validated *ex vivo* in the lung of active TB patients [7–9]. Four MTB proteins (Rv0724A, Rv1251c, Rv1478 and Rv3479) were selected for further investigation upon expression profiles, antigenicity and cross-reactivity. Multi-epitope, human leukocyte antigen (HLA) class II promiscuous peptides were identified on the protein sequences by peptide binding motif analysis [8]. Six peptides (Rv0724A: GEIIFISGRLNGAA; Rv1251c: ELMARAAVLGSAH and AVIVRSELLTQYL; Rv1478: TAWITAVVPGLMV; Rv3479: RPVRRVLLFVVPSSGPAP and GSVRQLPSVLKPPLITLRTLTLSG) were designed, synthesised by Fmoc chemistry (American Peptide Company, Inc., Sunnyvale, CA, USA) and obtained >90% purity.

The QFT assay was performed according to the manufacturer's instructions. In parallel, a QFT plus the six novel peptides (hereafter referred to as QFT-Six) was performed by adding to one QFT antigen tube the pooled novel peptides at a final concentration of 1 $\mu g \cdot mL^{-1}$ each. The QFT-Six tube was then treated the same as any of the QFT tube assays. IFN-γ levels were determined using the QFT ELISA and results were interpreted according to the manufacturer's instructions.

The intracellular expression of IFN-γ in $CD4^+$ and $CD8^+$ T-lymphocyte subsets upon stimulation with the pool of the six novel peptides was evaluated using flow cytometry as previously described [10]. Data are expressed as median (interquartile range) or percentages. Comparison between groups was made using a paired or unpaired nonparametric test or Fisher's test, as appropriate. Variation in the QFT MTB antigen test after addition of the novel peptides was calculated by comparison with the normal QFT MTB antigen test. Deviation from the normal coefficient of variation of the QFT was determined according to the QFT reproducibility data [11]. All analyses were performed using the GraphPad Prism 5.0 software (Graphpad, San Diego, CA, USA).

Table 1 shows the results obtained in the different study populations with the QFT and the QFT-Six tests. As expected, the QFT showed high sensitivity (range: HIV-negative 75–87%; HIV-infected 67–100%) and specificity (range: 96–100%) in BCG-vaccinated and nonvaccinated control populations. The addition of the six novel peptide antigens to the QFT did not alter the specificity of the test in either BCG-vaccinated or normal control populations (table 1), although the antigens were derived from MTB proteins present in the BCG strain genomes. The addition of the six novel peptide antigens to the QFT did not determine a significant increase in test sensitivity, probably due to the already high performance of QFT in this study population [3]. However, with the limitation of the small numbers, the addition of the six novel peptides demonstrated a conversion to a positive result in three (30%) out of 10 HIV-negative and one (25%) out of four HIV-infected active TB patients with a negative result in QFT alone.

The addition of the six novel antigen peptides to QFT determined overall a significant increase of IFN-γ release with respect to the QFT alone, both in HIV-negative (QFT: 3.88 (0.88–10) IFN-γ $IU \cdot mL^{-1}$; QFT-Six: 5.01 (1.20–10) IFN-γ $IU \cdot mL^{-1}$; $p<0.01$ Wilcoxon paired test) and HIV-infected (QFT: 0.82 (0.22–2.10) IFN-γ $IU \cdot mL^{-1}$; QFT-Six: 0.99 (0.39–2.26) IFN-γ $IU \cdot mL^{-1}$; $p<0.03$ Wilcoxon paired test) active TB patients.

This observation was confirmed by the analysis of the coefficient of variation of the IFN-γ release after addition of the six novel peptide antigens to QFT. Considering the IFN-γ release inter-test reproducibility of QFT (14% [11]), a significant number of both HIV-negative (24 (51%) out of 47) and HIV-infected (five (56%) out of nine) active TB patients presented an increase of IFN-γ release greater than the normal range of variability of the IFN-γ release for QFT ($p<0.001$, all comparisons).

To corroborate this data, the contribution of the $CD4^+$ and $CD8^+$ antigen-specific responses to the six novel peptides in the pool were determined by flow cytometry in a subgroup of 12 active TB patients and nine QFT-negative controls. Active TB patients present, compared with controls, a significantly higher frequency of peptide-specific IFN-γ^+/$CD69^+$/$CD4^+$ (active TB: 0.08% (0.02–0.14%); controls: 0.01% (0–0.02%); $p<0.02$ Mann–Whitney test) and IFN-γ^+/$CD69^+$/$CD8^+$ (active TB: 0.07% (0.02–0.12%); controls: 0.01% (0–0.02%); $p<0.02$) T-lymphocytes. Therefore, although designed to present HLA class II binding capabilities, the novel peptides also contain HLA class I epitopes, as assessed by Immune Epitope Database analysis [12], supporting both $CD4^+$ and $CD8^+$ responses.

The development of rapid and sensitive diagnostic methods for active TB, latent TB infection and recent MTB infection is a key aspect of the TB control strategy [1–3]. The addition of the novel peptides to the QFT resulted in a significant increase in the IFN-γ release in active TB, without altering specificity, and also in the BCG-vaccinated control population. This is consistent with previous data in the literature

indicating that the addition of other non-RD1 proteins to QFT can result in an increase of the test performance [4, 5], as also observed in the changes implemented in the QFT test versions [1].

The significant increase of IFN-γ release after adding the novel peptide antigens to QFT in HIV-infected TB patients is of particular importance as poorer performance of IGRAs in immunocompromised patients has been reported [2]. This increase is likely to be due to the contribution of the $CD8^+$ T-cell response directed against the novel peptides, better preserved in HIV-infected subjects than $CD4^+$ responses [13, 14].

Furthermore, the significant increase in IFN-γ release in active TB, and thus dynamic range, after addition of the novel peptide antigens to the QFT assay might help in interpreting mild fluctuations in IFN-γ responses observed during serial testing with the QFT [15] and in reducing the variability of IGRA response often obtained under different settings and/or host backgrounds. This is consistent with the homogeneous performance obtained across the different settings in this study with the use of QFT and the addition of the novel peptide antigens.

The addition of other antigens to QFT may be perceived to have a potential negative impact on assay specificity, particularly when these antigens are present in the genome of BCG strains. However, the use of antigens preferentially expressed by MTB during its active replication phase [7–9], as in the present study, did not alter the specificity of the QFT assay, including in BCG-vaccinated controls, supporting the utility of this antigen selection strategy.

In conclusion, although there is a need for larger confirmatory studies, in particular in HIV-infected subjects, this study suggests that the addition of novel antigens to the QFT assay can strongly help in the development of a new generation of IGRAs, circumventing some of the challenges of the current assay in certain populations and maintaining the high specificity of the current QFT assay compared with the tuberculin skin test.

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Novel *Mycobacterium tuberculosis* antigens from macrophage infection model improve performance of QuantiFERON-TB http://ow.ly/TaqTO

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Tuberculosis elimination, patients' lives and rational use of new drugs: revisited

To the Editor:

The World Health Organization (WHO) has recently published a framework on tuberculosis (TB) elimination opening debates on elimination and multidrug-resistant (MDR)-TB [1, 2]. The document highlights MDR-TB as a core area, emphasising the moral duty of preventing the selection of MDR-TB (by treating susceptible cases correctly with first-line drugs) and the necessary efforts to treat it when diagnosed. Unfortunately, treating MDR- and extensively drug-resistant (XDR)-TB is much more onerous, lengthy and terribly expensive; treatment outcomes remain suboptimal, adverse events being frequent and severe [3–6]. The availability of new drugs has offered new possibilities for saving patients who were not previously treatable and posed new challenges related to their rational use in order to prevent selection of resistant strains of *Mycobacterium tuberculosis* [7–10].

The aim of this report is to describe a patient's 22-year ordeal with TB. He was treated for two decades unsuccessfully until the advent of new active drugs. We hope that this case will serve as a scenario to emphasise the importance and moral obligations of physicians not to foster resistance, as well as to conserve new drugs for subsequent generations.

In the 1990s, the approach to the treatment of resistant TB differed greatly and was based on the second-line TB drugs available at the time, in an era preceding the development of specific guidelines [11], and before the availability of new "off-label" drugs (linezolid and imipenem).

The patient in question is a HIV-negative Italian man, who was 32 years of age at the time of MDR-TB diagnosis (March 1991). He had been previously treated for pulmonary nodular TB between 1985 and 1991. He had no known risk factors or contacts for TB. He was transferred to the MDR-TB reference hospital in Sondalo, Italy, in March 1991 when his condition deteriorated following retreatment with first-line drugs and ciprofloxacin (table 1), a single active drug added to a failing regimen.

On admission he was sputum smear positive (grade 4+) and culture positive (20 colonies in solid medium); he was resistant to all first-line drugs, *para*-aminosalicylic acid (PAS) and terizidone, with susceptibility to ofloxacin and capreomycin. The disease had progressed, with bilateral nodular infiltrates with cavities in the upper left lobe. The initial regimen included amikacin, clofazimine, ofloxacin and PAS; he also underwent bisegmentectomy of the left upper lobe in November 1991.