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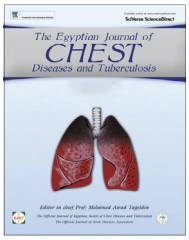

ORIGINAL ARTICLE

Role of Quantiferon TB gold assays in monitoring the efficacy of antituberculosis therapy

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KEYWORDS

Tuberculosis;
Quantiferon-TB gold;
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Acid-fast bacilli;
Sputum culture;
Cell mediated immunity

Abstract *Rationale:* The impact of antituberculous treatment on (IFN)-γ response to mycobacterial tuberculosis antigens have been widely investigated but the results have been controversial.

Aim of work: To evaluate the role of Quantiferon TB gold assays as one of the interferon-gamma release assays (IGRAs) for monitoring the efficacy of antituberculosis therapy in patients with active disease.

Subjects and methods: Thirty patients with active pulmonary TB were enrolled in this cross-sectional study where they were subjected to history taking, clinical examination, chest X-ray, direct smear examination of sputum samples for AFB using Ziehl–Neelson stain performed on three visits; up on enrollment, 2 and 6 months later. Lowenstein Jensen medium cultures of sputum samples were done for isolation of *Mycobacterium tuberculosis* on first visit. All patients in the study group were subjected to QuantiFERON-TB Gold estimation on the three visits.

Results: The mean sensitivity and specificity of QFT-G test was 85.9% and 62.6% respectively. Using χ^2 analysis, there was a statistically significant association between QFT-G results and culture results upon enrollment and Acid fast bacilli positivity on second and third visits. Studying the changes in QFT-G results throughout the whole study period revealed a statistically significant decrease in number of QFT-G positive cases from 24/29 patients (82.8%) at first visit to 4/25 patients (16%) at the third visit. All 21/25 patients (84%) who became QFT-G negative at the end of the study had a complete clinical and microbiological recovery of the TB disease.

Conclusion: The analysis of QFT-G assay results showed that in the majority of our TB patients there was a correlation between clinical treatment outcome and changes of IFN-γ response to *M. tuberculosis*-specific antigens.

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Introduction

The immune response in tuberculosis (TB) plays a fundamental role in the outcome of *Mycrobacterium* tuberculosis infection. It is clear that the immune system reacts efficiently in the vast majority of infections. This is particularly evident in the case of TB, where most people infected by the tubercle bacillus do not develop the disease throughout their lifetimes [1]. Therefore, although the host cell-mediated immunity is enough to control the progression of disease, it fails to exert sterile eradication and hence, those two billion infected persons suffer the latent form of TB [2].

As for other intracellular infections, the primary protective immune response is cell mediated rather than antibody mediated. *M. tuberculosis* resides inside the macrophage and is relatively resistant to microbicidal mechanisms that efficiently eliminate other phagocytosed bacteria. This is due in part to the ability of the tubercle bacilli to hinder macrophage activation by IFN-γ and IL-12. Several studies have confirmed the critical importance of these cytokines in both human and mice *M. tuberculosis* infection. In addition, deficiencies in IL-12 or IFN-γ, or their receptors, render the individual more susceptible to mycobacterial infections [3,4].

Attempts have been made to exploit the T-cell response for rapid diagnosis of *M. tuberculosis* infection, through the interferon (IFN)-γ assays. These assays are based on the fact that T-cells sensitized with tuberculous antigens will produce IFN-γ when they are re-exposed to mycobacterial antigens. A high amount of IFN-γ production is then presumed to correlate with TB infection [5].

The QuantiFERON®-TB Gold test (QFT-G) is a whole-blood test for use as an aid in diagnosing *M.* tuberculosis infection, including latent tuberculosis infection (LTBI) and tuberculosis (TB) disease. This test was approved by the US Food and Drug Administration (FDA) in 2005 [6].

The first IFN-assays made use of purified protein derivative (PPD) as the stimulating antigen; more recent assays, use antigens that are specific to *M. tuberculosis,* such as the early secretory antigen target 6 (ESAT6), and the culture filtrate protein 10 (CFP10), protein antigens are coded by genes located in the region of difference l (RDl) of the *M. tuberculosis* genome and are much more specific than PPD, since they are not shared with *Mycrobacterium* bovis bacillus Calmette–Guerin (BCG) or most nontuberculous mycobacteria (NTM) with the exception of *Mycrobacterium marinum*, *Mycrobacterium* sulzgai and *Mycrobacterium kansassi*. Thus these tests provide an advantageous alternative to tuberculin test with its false- positive results especially in BCG-vaccinated persons and in areas of high incidence [5].

The QFT-Gold offers a number of advantages compared with the TST, including increased specificity in persons who have had a BCG vaccination, and elimination of the need for a second visit to read the TST. Providers and facilities providing TB testing should determine if QFT-Gold will replace the TST or will be used to confirm positive TST results. Considerations include cost, feasibility, TST reading rates, and availability of the test. Maximum benefit from QFT-Gold is likely to be realized in BCG vaccinated individuals, populations with poor adherence rates to TST reading, and in settings where quality assurance and training of skin testing is poor or lacking [7].

QFT-G, as with the TST, cannot differentiate infection associated with TB disease from LTBI. A diagnosis of LTBI requires that TB disease be excluded by medical evaluation, which should include checking for suggestive symptoms and signs, a chest radiograph, and, when indicated, examination of sputum or other clinical samples for the presence of *M. tuberculosis* [8].

As with a negative TST result, negative QFT-G results should not be used alone to exclude *M. tuberculosis* infection in persons with symptoms or signs suggestive of TB disease. The presence of symptoms or signs suggestive of TB disease increases the likelihood that *M. tuberculosis* infection is present, and these circumstances decrease the predictive value of a negative QFT-G or TST result. Medical evaluation of such persons should include a history and physical examination, chest radiograph, bacteriologic studies, serology for human immunodeficiency virus (HIV), and, when indicated, other tests or studies [9].

IGRAs are dynamic and both conversions and reversions occur when serial testing is done; this has been shown to occur among contacts as well as health care workers. There is no consensus on what the best definition for conversion is – different definitions appear to produce different rates of conversions. QFT reversions were defined as baseline IFN-$\gamma \geqslant 0.35$ and follow-up IFN-$\gamma < 0.35$ IU/ml [10].

Some reversions may reflect clearing of TB infection (spontaneous or due to treatment). Some reversions may merely be due to biological variations among IGRA positive individuals, and some reversions may be due to variability in laboratory and test procedures. Other studies suggested that IGRA responses are inherently transient and generally require continued exposure to TB antigens to maintain high frequencies. They argue that reversions may simply reflect the life cycle of *M. tuberculosis*, where the mycobacterium enters a dormant state in which it may not reliably secrete antigens such as early secreted antigen target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), but instead secrete other antigens (which are not used in currently available IGRAs). Alternatively, ESAT-6 and CFP-10 may be secreted intermittently over the life cycle of *M. tuberculosis*, which may partly explain the variations over time. These hypotheses are worthy of further study [11].

Aim of work

This study was planned to evaluate the role of Quantiferon TB gold assays as one of the interferon-gamma release assays (IGRAs) for monitoring the efficacy of antituberculosis therapy in active tuberculous patients.

Subjects and methods

Thirty patients with active pulmonary TB were enrolled in this cross-sectional study from August 2009 to February 2010, the enrollment was carried out in OMRANYA Chest hospital. Where active tuberculosis patients were admitted from the out-patients clinic.

The participants consisted of 18 males and 12 females.

Exclusion criteria

- Debilitating diseases: diabetes, renal or hepatic impairment or failure, acquired immune deficiency syndrome (AIDS).
- Extra-pulmonary tuberculosis.

– Pregnancy.
– Children.

All of the patients were subjected to

1- History including:
- Pulmonary questionnaire: where the patients were asked about the following:
- Persistent productive cough, with or without hemptysis
- Breathlessness
- Weight loss
- Loss of appetite
- Fever; 38 °C or above (night fever-night sweats
- Past history: of old TB infection.

2- Clinical examination:
- General examination
- Local examination

3- Laboratory: ESR: Erythrocyte sedimentation rate
4- Chest roentgenogram
5- Microbiology:

(A) Direct smear examination for AFB by Ziehl–Neelsen stain:
At first visit: up on enrollment.
2nd visit: 2 months later.
3rd visit: 6 months later.

(B) Culture medium for isolation of Mycobacterium tuberculosis (Lowenstein–Jensen medium) *BBL™ MycoPrep™*

All patients in the study group were subjected to culture using Lowenstein–Jensen (L–J) medium at the time of enrollment.

1- The Whole Blood IFN-gamma test, QuantiFERON®-TB Gold

All patients in the study group were subjected to QuantiFERON-TB Gold estimation:

At first visit: up on enrollment.
2nd visit: 2 months later.
3rd visit: 6 months later.

The QuantiFERON-TB Gold In-Tube (IT) is an in vitro diagnostic test using a peptide cocktail simulating ESAT-6, CFP-10 and TB7.7 proteins to stimulate cells in heparinised whole blood. Detection of interferon-γ by Enzyme-Linked Immunosorbent Assay (ELISA) is used to identify in vitro responses to the peptide antigens that are associated with *M. tuberculosis* infection.

N.B: Individuals infected with *M. tuberculosis* complex organisms usually have lymphocytes in their blood that recognise mycobacterial antigens (existed on the tubes wall). This recognition process involves the generation and secretion of the cytokine, IFN-γ. The detection and subsequent quantification of IFN-γ forms the basis of this test.

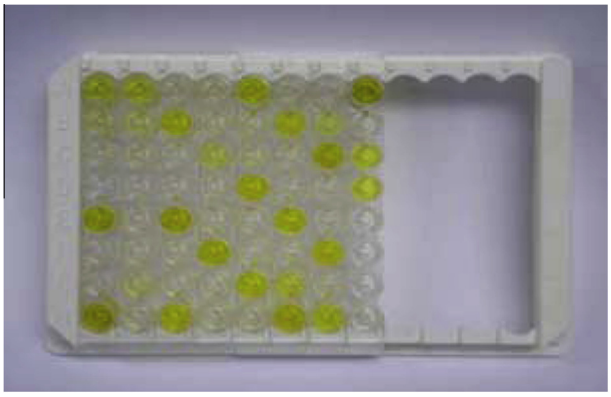
Contents of the Quantiferon TB-G-It packet: cellestis limited (Australia) and cellestis Inc., (package insert, 2006)

Tuberculosis and control antigen blood collection tubes

1. Nil control (Grey cap).
2. TB antigen (Red cap).
3. Mitogen control (Purple cap) – did not used in this study.

ELISA Components in the packet:

1. *Microplate strips:* Containing the ELISA wells.
2. *Human IFN-γ Standard, lyophilized:* For preparation of fresh dilutions of the kit standard for each ELISA session as a control before calculation of the results.
3. *Green diluent. (GD):* Share in the kit dilutions.
4. *Conjugate 100X Concentrate, lyophilized:* A conjugated monoclonal antibody to IFN-γ, this is an enzyme linked antibody that mixed with the infected plasma samples.
5. *Wash Buffer 20X Concentrate:* For removal of the excess non-mixing materials.
6. *Enzyme Substrate Solution:* When added to conjugate, it achieves color that indicates positivity.
7. *Enzyme stopping solution:* Finally, added to halt the reaction.



N.B: The non visible reaction between the plasma samples and the conjugate shows a colored visible reaction after adding the enzyme substrate that indicates INF-γ existence in the patient plasma sample

Calculations and test interpretation

The software performs a quality control assessment of the assay, generates a standard curve and provides a test result for each subject as negative or positive through:

1. These calculations can be performed using software packages available with microplate readers, and standard spreadsheet or statistical software (such as Microsoft Excel).
2. Construct a log(e)–log(e) standard curve by plotting the log(e) of the mean OD (y-axis) against the log(e) of the IFN-γ concentration of the standards in IU/mL (x-axis).
3. Use the standard curve to determine the IFN-γ concentration (IU/mL) for each of the test plasma samples, using the OD value of each sample.

The software performs a quality control assessment of the assay, generates a standard curve and provides a test result for each subject as negative or positive.

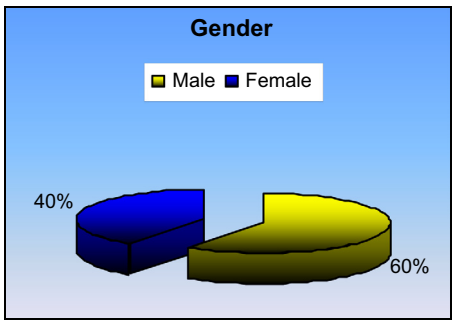


Figure 1 Pie chart representing gender distribution among the studied group.

Results

This study was conducted on 30 cases; 18 males and 12 females. Their ages ranged between 17 and 87 years (mean ± SD = 38 ± 20.2)

Statistical analysis

Qualitative data were presented as frequencies and percentages. χ^2 (x^2) test was used for studying the association between Quantiferon with AFB and Quantiferon with culture results.

Quantitative data were presented as minimum, maximum, means and standard deviation (SD) values.

Sensitivity, specificity and diagnostic accuracy of Quantiferon were calculated as follows:

$$\text{Sensitivity } (\%) = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \times 100$$

$$\text{Specificity } (\%) = \frac{\text{True negative}}{\text{False positive} + \text{True negative}} \times 100$$

$$\text{Diagnostic accuracy}(\%) = \frac{\text{True positive} + \text{True negative}}{\text{Total number}} \times 100$$

An increase in sensitivity means a decrease in false negative cases, while an increase in specificity means a decrease in false positive cases.

The significance level was set at $P \leqslant 0.05$. Statistical analysis was performed with SPSS 16.0® (Statistical Package for Scientific Studies) for Windows.

Quantiferon, ZN and culture results in first visit

Culture. At first visit, 1 case was excluded. Twenty-two subjects (75.9%) showed positive culture and 7 subjects (24.1%) showed negative culture.

Acid fast bacilli. At first visit, 1 case was excluded. All cases (29 cases) showed positive Z–N.

Quantiferon. At first visit, 1 case was excluded. Twenty-four subjects (82.8%) showed positive Quantiferon test and five subjects (17.2%) showed negative test.

There was a statistically significant association between Quantiferon and culture (*P*-value = 0.001). Twenty-one cases (95.5%) with positive culture showed positive Quantiferon. Four cases (57.1%) with negative culture showed negative Quantiferon.

Quantiferon and ZN results in second visit. There was a statistically significant association between Quantiferon and AFB (*P*-value < 0.001). Three cases (100%) with positive AFB showed positive Quantiferon. Twenty-three cases (95.8%) with negative AFB showed negative Quantiferon.

Quantiferon and ZN results in third visit. There was a statistically significant association between Quantiferon and AFB (*P*-value < 0.001). Three cases (75%) with positive AFB showed positive Quantiferon. Twenty cases (95.2%) with negative AFB showed negative Quantiferon (see Fig. 1).

Discussion

Monitoring the efficacy of anti-TB therapy is crucial for the better control of the spread of MTB infection; IGRAs are novel indirect blood tests for *M. tuberculosis* infection and a promising marker of mycobacterial burden and disease activity.

Our study shows that the pattern of changes in IFN-γ responses with treatment is highly significant; the results of QFT G IT test correlate with results of the microbiologic sputum smear, culture and clinical outcome. The progressive decline of (IFN)-γ response measured by QFT G IT test reflects the reduction of mycobacterial burden following a successful anti-tuberculous treatment.

At the first visit, Fig. 2 shows the correlation between the culture and AFB results, one case was excluded (Deviations from the procedure described in the Package Insert). All cases were microbiologically AFB smear confirmed, however, 22 subjects (75.9%) showed positive culture while seven subjects

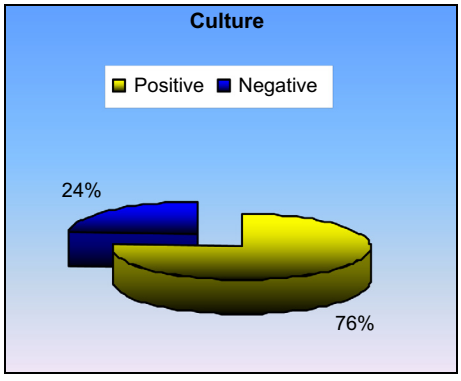


Figure 2 Pie chart representing culture results at first visit.

Table 1 Frequency and percentages of Quantiferon test results in the first visit.

Visit	Quantiferon	Freq.	%
First visit (on admission)	Positive	24	82.8
	Negative	5	17.2
	Total	29	100

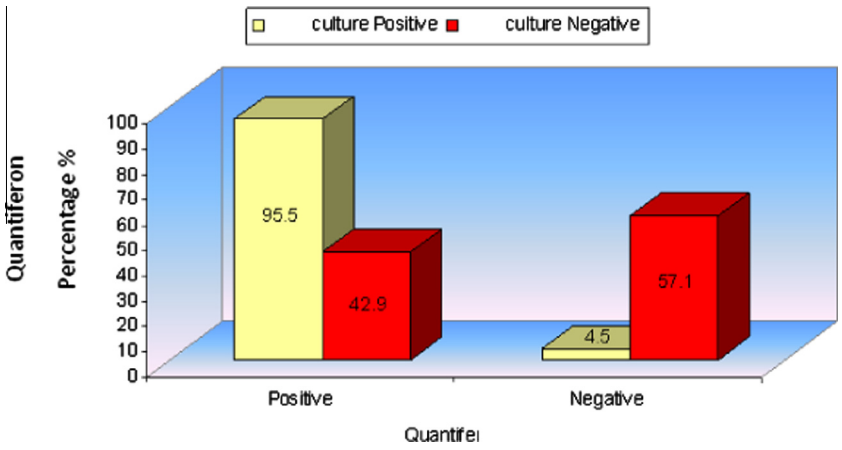


Figure 3 Bar chart representing the association between culture and Quantiferon test at first visit.

Table 2 Accuracy measures of Quantiferon compared to Z–N at first visit.

Z–N	Positive	Negative	Total
	Freq.	Freq.	
Quantiferon			
Positive	24	0	24
Negative	5	0	5
Total	29	0	29

Table 3 Statistical comparison between Quantiferon and culture results at first visit.

Culture	Positive (n = 22)		Negative (n = 7)		P-value
	Freq.	%	Freq.	%	
Quantiferon					
Positive (n = 24)	21	95.5	3	42.9	0.001*
Negative (n = 5)	1	4.5	4	57.1	

* Significant at $P \leqslant 0.05$.

Table 4 Statistical comparison between Quantiferon and AFB results at second visit using χ^2 test.

AFB	Positive (n = 3)		Negative (n = 24)		P-value
	Freq.	%	Freq.	%	
Quantiferon					
Positive (n = 4)	3	100	1	4.2	< 0.001*
Negative (n = 23)	0	0	23	95.8	

* Significant at $P \leqslant 0.05$.

(24.1%) showed negative culture, the later could be due to either harsh decontamination of the specimen or MOTT (mycobacteria other than tuberculosis). Clinically, most patients with MOTT infections are initially suspected to have *M. tuberculosis* and managed as such. In our study, we did not add NAD + or NADP + to L–J medium during culture preparations, thus we did not confirm presence of MOTT in cultures of our patients with sputum positive smear.

Regarding the Quantiferon results in the first visit Table 1, 24 subjects (82.8%) showed positive Quantiferon test and five subjects (17.2%) showed negative results. Todd [12] explained why negative QuantiFERON®-TB Gold IT result does not preclude the possibility of *M. tuberculosis* infection or tuberculosis disease; false-negative results could be due to stage of infection (e.g., specimen obtained prior to the development of cellular immune response), co-morbid conditions which affect immune functions, incorrect handling of the blood collection tubes following veni-puncture, incorrect performance of the assay, or other immunological variables (see Fig. 3 and Table 2).

Table 3 evaluated the association between Quantiferon and culture results at first visit, in which there was a statistically significant association between Quantiferon and culture (P-value = 0.001). The table showed one positive culture patient with negative Quantiferon result. Kobashi et al. [13] pointed out that this may be due to the decrease of interferon-gamma production due to advanced patient age or lymphocytopenia. The table also showed three subjects with negative culture who gave positive QFT-G-IT responses, these discordant results in culture reaction could be due to either too harsh decontamination or unequal distribution of the bacilli in the specimen during the culture procedures (see Table 4).

In Table and Fig. 4, there was a statistically significant association between Quantiferon and AFB (P-value < 0.001) in the second visit. Three cases (100%) with positive AFB showed positive Quantiferon. Twenty-three cases (95.8%) with negative AFB showed negative Quantiferon.

The results showed non conversion of positive sputum smears in three subjects at the end of two months of treatment. Kuaban et al. [14] studied the non conversion of sputum smears at 2 months of treatment and found that it is significantly associated with age above or equal to 40 years and the presence of numerous bacilli (3+) on pre-treatment sputum smears. Patients with these factors who do not smear convert after two months of treatment should be given a fully supervised treatment for the entire duration of therapy so as to prevent in particular treatment default.

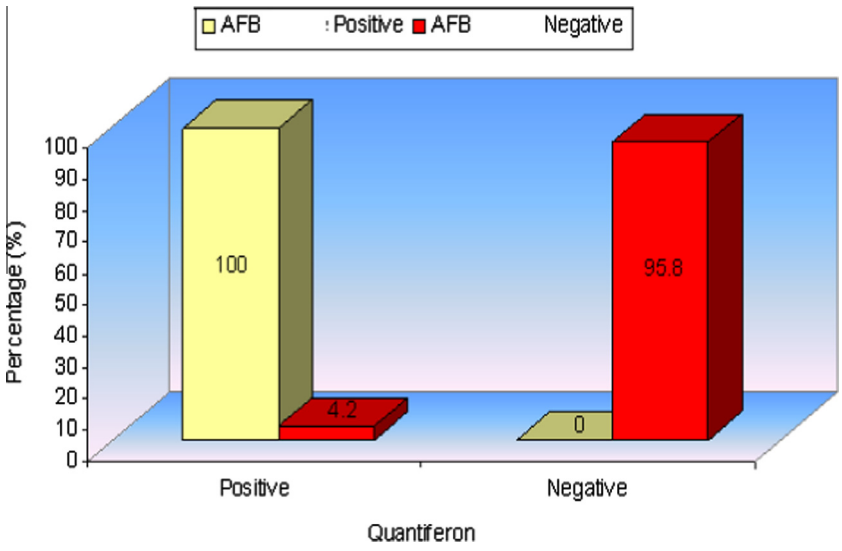


Figure 4 Bar chart representing the association between AFB and Quantiferon test at second visit.

Table 5 Accuracy measures of Quantiferon compared to AFB at second visit.

AFB	Positive	Negative	Total
	Freq.	Freq.	
Quantiferon			
Positive	3	1	4
Negative	0	23	23
Total	3	24	27

Sensitivity (%) = 100%, specificity (%) = 95.8.
Diagnostic accuracy (%) = 96.3%.

Table 6 Statistical comparison between Quantiferon and AFB results at third visit using χ^2 test.

AFB	Positive (n = 4)		Negative (n = 21)		P-value
	Freq.	%	Freq.	%	
Quantiferon					
Positive (n = 4)	3	75	1	4.8	$<0.001^*$
Negative (n = 21)	1	25	20	95.2	

* Significant at $P \leqslant 0.05$.

In the same table, one patient showed persistent positive Quantiferon result in spite of the conversion of the sputum from positive to negative.

Katiyar et al. [15] attributed the reasons of why immune responses to even specific antigens (ESAT-6 and CFP-10) may not have dropped below the pre-defined level of a positive test after anti-tuberculosis treatment to exposure to environmental mycobacteria commonly encountered in the tropics, some of which share the ESAT-6 and CFP-10 genes. Sallakci et al. [16] also suggested that may be there is inter-individual variation in the strength of IFN-γ response that can be partly explained by genetic polymorphisms in the host.

When we evaluated patients' sensitivity and specificity during treatment in the second visit as shown in Table 5, the specificity of QFN-G-IT was 95% and the sensitivity was 100%.

We set out to compare the association between Quantiferon and AFB results at third visit as shown in Table 6 and Fig. 5 and found a statistically significant association between Quantiferon and AFB (P-value < 0.001). Three cases (75%) with positive AFB showed positive Quantiferon. Twenty cases (95.2%) with negative AFB showed negative Quantiferon. The table pointed out to two cases; one of them showed positive Quantiferon with negative AFB result and was considered to be a False positive result of Quantiferon test. The other case, showed positive AFB and negative Quantiferon highlighting the fact that not all positive AFB smears are *M. tuberculosis* but could also be MOTT and again emphasizing the role of cultures to accurately diagnose pulmonary TB and differentiate Typical from Atypical Tuberculous infection.

Regarding the QFT-G assay sensitivity and specificity in the third visit as shown in Table 7, the specificity QFN-G-IT decreased from 100% to 75%, and the sensitivity almost constant (95% and 95.2%) respectively.

In Table 8 we discussed the change by time in Quantiferon results over the three visits. There was a statistically significant decrease in number of positive cases at second visit and comparing the results of the first visit to third visit after 6 months (end of the study period), there was a statistically significant decrease in number of QFT-G positive cases from 24/29 patients (82.8%) to 4/25 patients (16%) respectively. All 21/25 patients (84%) who became QFT-G negative at the end of the study had a complete clinical and microbiological recovery of the TB disease.

According to Hill et al. [11], T cell responses, especially weakly positive responses, tend to fluctuate over time, even in the absence of specific treatment.

Carrara et al. [17], showed that an in vitro immune diagnostic assay based on the response to ESAT-6-multiepitopic peptides can be detected in patients who do not respond to therapy. A possible explanation is that secretion of *M. tubercu-*

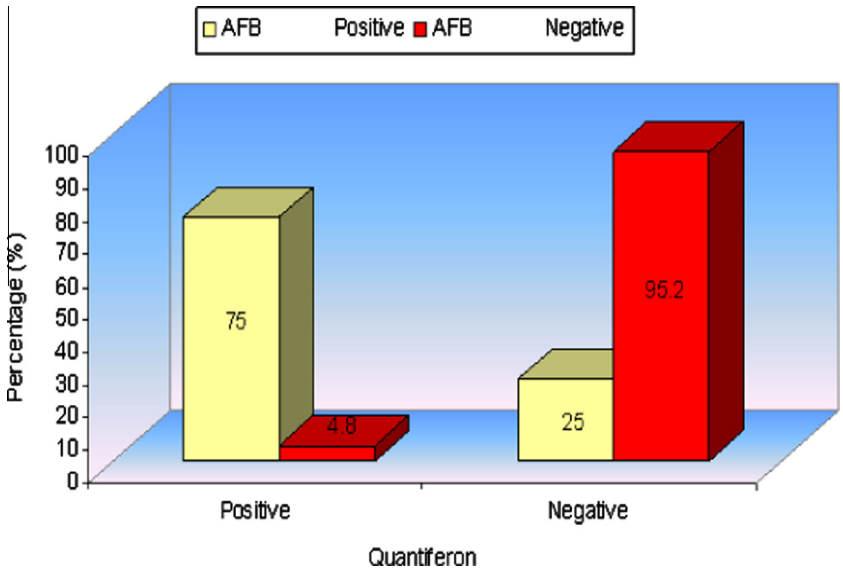


Figure 5 Bar chart representing the association between AFB and Quantiferon test at third visit.

Table 7 Accuracy measures of Quantiferon compared to AFB at third visit.

AFB	Positive	Negative	Total
	Freq.	Freq.	
Quantiferon			
Positive	3	1	4
Negative	1	20	21
Total	4	21	25

Sensitivity (%) = 75% specificity (%) = 95.2%.
Diagnostic accuracy (%) = 92%.

Table 8 Statistical analysis showing the changes in Quantiferon test by time using McNemar's test.

Visit	Quantiferon	Freq.	%
First visit (on admission)	Positive	24	82.8
	Negative	5	17.2
	Total	29	100
Second visit (2 months)	Positive	4	14.8
	Negative	23	85.2
	Total	27	100
Third visit (6 months)	Positive	4	16
	Negative	21	84
	Total	25	100
First visit vs. second visit	*P*-value	$<0.001^*$	
Second visit vs. third visit	*P*-value	0.857	
First visit vs. third visit	*P*-value	$<0.001^*$	

* Significant at $P \leqslant 0.05$.

losis-specific proteins, such as ESAT-6, requires metabolically active and viable bacilli and, thus, IFN-g-producing T cells specific for ESAT-6 selected epitopes are present at high

Table 9 The mean Quantiferon sensitivity and specificity of the three visits.

	1st visit	2nd visit	3rd visit	Mean
Sensitivity	82.8	100	75	85.9
Specificity	Not computed	95.8	92	62.6

frequency during the active phase of bacterial replication. It has been shown that these T cells are pre-activated in vivo and capable of rapid effector function. However, after receipt of efficacious therapy, *M. tuberculosis* stops replicating actively and the frequency of the T cells specific for these epitopes dramatically decreases and, thus, is not detected by this assay. However, the memory response to the whole ESAT-6 protein remains.

It has been suggested by Leyten et al. [18] that the short period of incubation (16–24 h), commonly used in IGRAs, detects responses of activated effector T cells that rapidly release IFN-γ when stimulated in vitro with antigen. Kaech et al. [19] confirmed that long-lived central memory T cells that may be less likely to release IFN-γ during the short period of exposure to antigen in the IGRA assay, could be detected after a longer period of in vitro stimulation. Since the effector response is driven by the antigen load, it was thought by Lalvani [20] that the presence of active mycobacterial replication (active untreated TB) is associated with increasing of the numbers of effector T cells. In the context of a low antigenic load, as in successfully treated patients, the number of effector cells specific to *M. tuberculosis* antigen could fall below the cut-off level.

According to Table 9 the mean sensitivity of QFT-TB test in our study was 85.9% while the mean specificity was 62.6%. This data are in agreement with previous studies that reported an excellent degree of sensitivity and specificity of the QFT-TB test for diagnosis of active TB. Kobashi et al. [13] concluded that the QFT-TB test had a mean sensitivity of 86% and a mean specificity of 94%. They showed that

Quantiferon test results concerning patients with active TB transiently, decreased during treatment involving anti-tuberculous drugs. The rate of positive QFT-TB test results was 86% at the initiation of treatment, 48% six months later, and 33% 12 months later. Kang et al. [21] reported 81% sensitivity among 54 patients, and Mori et al. [6] reported 89% sensitivity among 118 patients.

In conclusion,

the results analysis of QFT-G assay showed that in the majority of our TB patients there was a correlation between clinical treatment outcome and changes of IFN-γ response to *M. tuberculosis*-specific antigens. Indeed, for all patients who had a complete resolution of clinical disease and whose microbiological results were negative for *M. tuberculosis* at 6-month treatment completion, a progressive decline of IFN-γ release was seen and the baseline positive QFT-G result had turned negative.

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