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DETERMINATION OF IFN- γ IN SUSPECTED LATENT TB INFECTION (LTBI) PATIENTS AND ITS ASSESSMENT AS DIAGNOSTIC AND PROGNOSTIC MARKER AND ITS CORRELATION WITH THE ADA AND CRP

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ABSTRACT

Background: Latent tubercular infection is a condition in which a person is infected with Mycobacterium tuberculosis, but does not currently have active tuberculosis disease which should be detected for TB control and elimination because treatment of LTBI can prevent infected persons from developing TB disease and stop the further spread of TB. Therefore, in our present study we found the usefulness of Interferon-gamma (IFN-g) Release Assays (IGRAs) in the diagnosis of latent TB and correlation of ADA and CRP with the INF- γ in relation to our earlier research[13] done in the suspected latent TB infection (LTBI) patients.

Method: Whole blood is collected into each of the QFT blood collection tubes, which include a Nil Control tube, TB Antigen tube, and an optional mitogen tube. The tubes are incubated at 37°C as soon as possible within 16 hours of collection. Following a 16 to 24 hour incubation period, the tubes are centrifuged, the plasma is removed and the amount of IFN- γ (IU/mL) is measured.

Result: Serum IFN- γ is also estimated by TB Quantiferon GOLD test and compared with ANOVA test in all the three groups. We found IFN- γ as 0.46-+0.16mg/dl in Group A, 1.04-+0.28mg/dl in group B and 0.46-+0.15 in Group C.

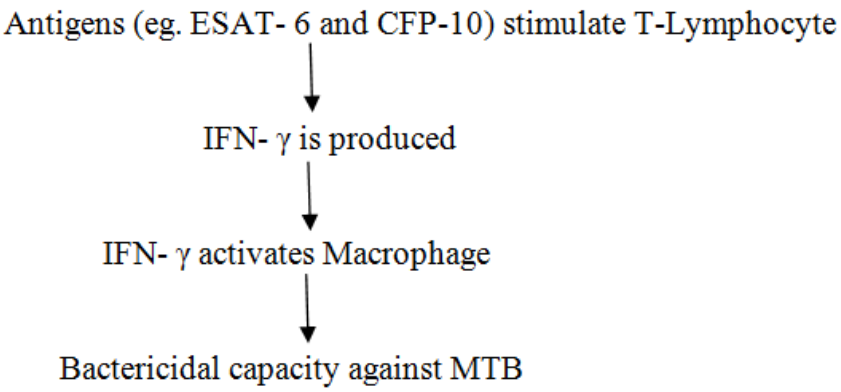
Conclusion: T-cell interferon-gamma release assays (IGRAs) are more specific and probably more sensitive than the tuberculin skin test (TST) that is unaffected by BCG vaccination for the diagnosis of latent tuberculosis infection (LTBI). The role of these new blood tests in this patient population is therefore of considerable interest. The two test of IGRAs i.e. ELISA has a similar sensitivity to the TST, whereas the ELIS pot is more sensitive.

KEYWORDS: Interferon, Tuberculin, Latent, Immunosuppression, Mycobacterial

INTRODUCTION

Tuberculosis (TB) is the single infectious cause of approximately 2 million death worldwide annually.[1] Advances in mycobacterial genomics and cellular research has resulted in the development of the interferon gamma release assays (IGRAs), which detect latent tuberculosis infection (LTBI) by measuring interferon (IFN-γ) release in response to antigens present in Mycobacterium tuberculosis, but not bacilli Calmette-Guerin (BCG) vaccine and most non tuberculous mycobacteria.[2, 3]. The Interferon-gamma (IFN-g) Release Assays (IGRA), is an alternative immunodiagnostic approach[4] offering better specificity[2, 5] and senstivity[1] in the diagnosis as a prognostic marker[6] of latent tuberculosis (TB) infection (LTBI) than the tuberculosis skin test (TST) for *M. tuberculosis* than PPD because they are not shared with any BCG

vaccine strains or most species of non tuberculous mycobacteria other than *M. marinum*, *M. kansasii*, *M. szulgai* and *M. flavescens*[7, 8].



The main risk with latent tuberculosis is that approximately 10% of these patients (5% in the first two years after infection and 0.1% per year thereafter) will go on to develop active tuberculosis having added risk, in particular situations such as medication that suppresses the immune system or advancing age[9].

IGRA positivity was associated with higher CD4 cell counts, which suggests that the test may be compromised by immunosuppression[10]. Two commercial interferon-gamma release assays (IGRAs) have been developed; The Quanti FERON-TB Gold (QFT-G, Cellestis, Australia) and the newer generation QuantiFERON-TB Gold In-Tube (QFT-GIT, Cellestis, Australia). These are whole-blood based enzyme-linked immunosorbent assays (ELISA) measuring the amount of IFN- γ produced in response to three *M. tuberculosis* antigens (QFT-G: ESAT-6 and CFP-10; QFT-GIT: ESAT-6, CFP-10 and TB7.7). In contrast, the enzyme-linked immunospot (ELISPOT)-based T-SPOT.*TB* (Oxford Immunotec) measures the number of peripheral mononuclear cells that produce INF- γ after stimulation with ESAT-6 and CFP-10[11]. Targeted testing is an essential TB prevention and control strategy that is used to identify, evaluate, and treat persons who are at high risk for latent tuberculosis infection (LTBI) or at high risk for developing TB disease once infected with *M. tuberculosis*. Identifying persons with LTBI is important to the goal of TB control and elimination because treatment of LTBI can prevent infected persons from developing TB disease and stop the further spread of TB.

Table 1: Differences between Tuberculin Skin Test (TST), QuantiFERON – TB Gold in-Tube Method and T-Spot TB Assay

	TST	QuantiFERON – TB Gold in Tube	T-Spot. TB
Intended use	Latent TB diagnosis	Latent TB diagnosis	Latent TB diagnosis
Studies	in-vivo	ex-vivo	ex-vivo
Technique	Skin prick test	ELISA	ELISPOT
Antigen (s) used	PPD	ESAT-6,CFP10,TB7.7(p4)	ESAT-6,CFP-10
Result reported as	Skin induration in mm.	IFN-gamma concentration	Spot-forming number
Result interpretation	Subjective	Objective	Objective
Result availability	48-72 hours	24 hours	24 hours
Patients visit required	Two	One	One
Influence by prior BCG vaccination	Yes	No	No
Cross-reactions with non-TB bacteria	Yes	Rare(M.fortuitum)*	No

Side effects	Yes(rare)	No	No
Booster effect	Yes(possible)	No	no

*Dyrhol-Riise, 2010[12]

Method

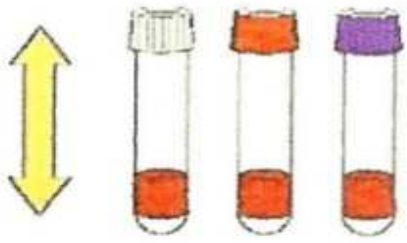
The present study was carried out in Department of Pathology of **SAAII College of Medical Science & Technology, Kanpur.**

The patients diagnosed as suffering from pulmonary TB as well as TB of various organs of body are the subject of study. Patients were drawn from indoor wards and out- patients department of L.L.R Hospital and Associated Hospital, Kanpur and T.B Hospital Azad Nagar, Kanpur. 100 authentic pulmonary TB patients were chosen for study in group B, while group C consisting of 100 cases of non-tubercular diseases with and group A consisting of 100 normal healthy age and sex matched controls. The clinical features and detailed history of each case is recorded in a standard format including exposure to infection, physical examination and chest radiography. Informed written consent was obtained from all subjects. Ethical clearance was obtained from institutional ethical Committee.

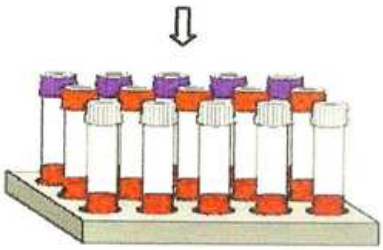
Procedure

The QFT G system uses specialized blood collection tubes, which are used to collect whole blood. Incubation of the blood occurs in the tubes for 16 to 24 hours, after which, plasma is harvested and tested for the presence of IFN- γ produced in response to the peptide antigens. The QFT test is performed in two stages. First, whole blood is collected into each of the QFT blood collection tubes, which include a Nil Control tube, TB Antigen tube, and an optional mitogen tube. The mitogen tube can be used with the QFT test as a positive control. This may be especially warranted where there is doubt as to the individual's immune status. The mitogen tube also serves as a control for correct blood handling and incubation. The tubes should be incubated at 37°C as soon as possible, and within 16 hours of collection. Following a 16 to 24 hour incubation period, the tubes are centrifuged, the plasma is removed and the amount of IFN- γ (IU/mL) measured by ELISA. A test is considered positive for an IFN- γ response to the TB antigen tube that is significantly above the Nil IFN- γ IU/ml value. If used, the mitogen-stimulated plasma sample serves as an IFN- γ positive control for each specimen tested. A low response to mitogen (<0.5 IU/mL) indicates an indeterminate result when a blood sample also has a negative response to the TB antigens. This pattern may occur with insufficient lymphocytes, reduced lymphocyte activity due to improper specimen handling, incorrect filling/mixing of the mitogen tube, or inability of the patient's lymphocytes to generate IFN-γ. The Nil samples adjust for background, heterophile antibody effects, or nonspecific IFN-γ in blood samples. The IFN- γ level of the Nil tube is subtracted from the IFN-γ level for the TB Antigen tube and mitogen tube.

Stage One- Blood Incubation and Harvesting

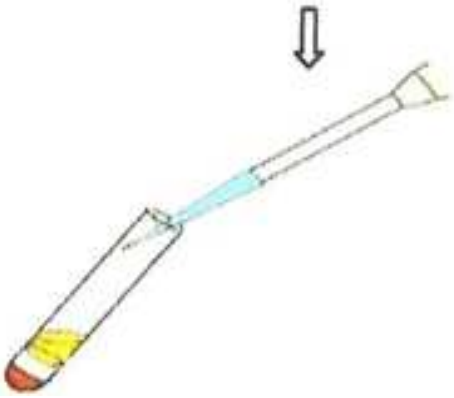


After blood collection Quanti FERON TB Gold tubes vigorous shaking was done for proper mixing.



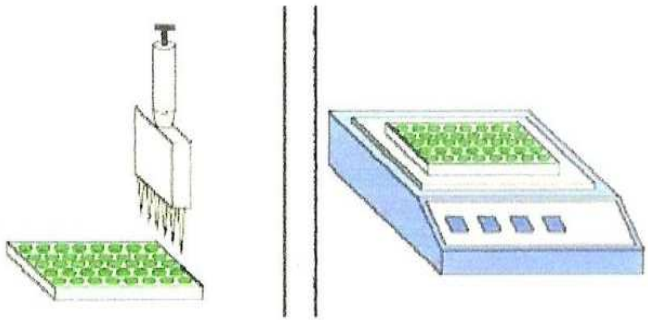
Within 15 hrs. of collection the tube were incubated in upright position for 16-24 hrs.

The tubes were then centrifuged at 2000-3000g for 15 minutes.

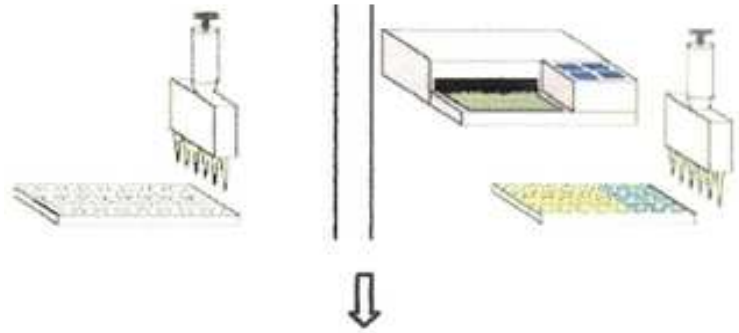


200pi plasma was harvested in uncoated microplates.

Stage Two - Human IFN- Γ: ELISA



50 pi of conjugate solution was added to each well. also 50 pi of plasma/ standard was added	shaking of covered plates were done for 120 minutes at room temperature



Plates were washed 8 times.100pi of substrate was added and incubated for 30min at room temperature	50 pi of stop solution was added and absorbance was read at 450nm within 5 min.

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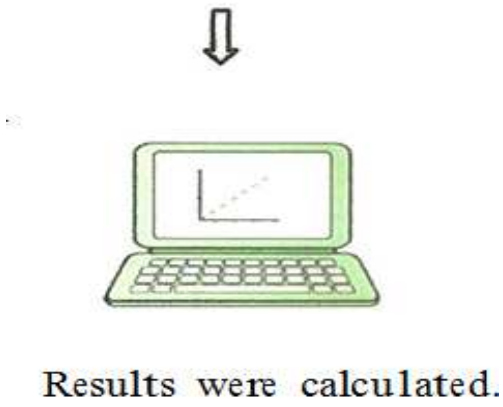
Substrate was added and incubated

For 30min at room temperature 50 pi of stop solution was added and absorbance was read at 450nm within 5 min.



RESULTS

Table 1: Serum TB IFN- Levels in Group A, B and C (Mean+S. D)

	Group A	Group B	Group C	P
	(n=100)	(n=100)	(n=100)	**Value**
IFN- γ (IU/L)	0.46+0.16	1.04-+0.28	0.46-+0.15	<0.05

Serum IFN- γ was also estimated by TB Quantiferon GOLD test were compared with ANOVA test in all the three groups and was found as 0.46-+0.16mg/dl in Group A, 1.04-+0.28mg/dl in group B and 0.46-+0.15 in Group C.

The upper 95% confidence interval for all the three groups are 0.49, 1.09 and 0.49 IU/Lm for Group A, B and C and for the lower 95% confidence intervals are 0.43, 0.98 and 0.43 IU/L, respectively. From this it is concluded that, there was significant difference between the serum IFN- γ by QFT test levels of Group A and B, Group B and C as the confidence interval are non-overlapping while there was no significant difference between the Group A and C as the confidence interval here are overlapping.

Table 2: Cut- off Points for Various Biochemical Parameters for Diagnosis of TB

Parameter	Cut- Off Level
Serum ADA	>15.00 IU/L
Serum CRP	>1.5 mg/dl
Serum IFN- γ	>0.5 IU/L
Pleural fluid ADA/Sr. ADA ratio	>1.0
Pericardial fluid ADA/Sr. ADA ratio	>1.0
Peritoneal fluid ADA/Sr. ADA ratio	>1.0
CSF ADA/Sr. ADA ratio	>1.0

Considering the above cut- off values the sensitivity and specificity of each of the above parameter can be state. From this table, it can be reliably said that the patients having serum ADA levels more than 15 IU/L are tuberculous and can be categorized into Group B and those with level less than 15 IU/L are non-tuberculous. Similarly those having serum CRP and serum IFN- γ levels more than 1.5 mg/dl and 0.5 IU/L respectively are tuberculous and rest are non-tuberculous. Also the patients having body fluid ADA to serum ADA ratio more than 1.0 are tuberculous and can be categorized in Group B and those with ratio less than 1.0 are non-tuberculous.

Table 3: Sensitivity and Specificity of the Parameters Studied for the Diagnosis of Tubercular Infection

Parameter	Sensitivity	Specificity
Serum ADA	98%	100%
Serum CRP	95%	100%
Serum IFN- γ	98%	100%
Pleural fluid ADA/sr. ADA	100%	96%
Pericardial fluid ADA/sr. ADA	100%	100%
Peritoneal fluid ADA/sr. ADA	100%	100%
CSF ADA/sr. ADA	100%	100%

**ADA- Adenosine Deaminase,*

**CRP- C-reactive protein*

**Sr.-Serum*

**CSF- Cerebrospinal Fluid*

(Note: The values of ADA and CRP of extra pulmonary TB patients and pulmonary TB patients shown in Tables 2,3,4,5 were taken from our earlier research done with respect to the reference no.13.)

Table 4: Correlation between Serum QFT Test Results with Serum CRP in Subjects with Tubercular Infection

Parameter	R	'P' Value
IFN- γ v/s CRP	0.53	p<0.05

A significant positive correlation is found between serum IFN- by QFT TB test and serum CRP with Pearson's coefficient of correlation (r) 0.53.

Table 5: Correlation between Body Fluids ADA Level with Body Fluids CRP Levels in Subject with Tubercular Infection

Parameter	R	'P' Value
Pleural fluid ADA v/s CRP	0.38	p<0.05

A significant positive correlation is found between pleural fluids ADA with pleural CRP fluid CRP levels.

Pearson's coefficient of correlation (r) in pleural fluid is 0.38.

DISCUSSIONS

In our earlier research[13] we found the usefulness of ADA and CRP levels in blood as well as in various body fluids for the diagnosis of TB.

In present study, the results of IFN- γ levels were compared using one way ANOVA in all the three study groups of tuberculous patients, non- tuberculous patients and normal healthy controls. IFN- γ level were compared with ANOVA test in all the three groups and was found as 0.46+0.16mg/dl in A, 0.91+0.31mg/dl in group B and 0.45+0.14 in Group c and A statistically significant difference was found in all three groups i.e. a significant difference between the serum IFN- γ levels in non-tuberculous patients when compared with control healthy subjects and in tuberculous verses non-tuberculous patients with the non-tuberculous patients.

The result of ADA and CRP calculated in earlier research is correlated with IFN- γ where ADA and IFN- γ sensitivity and specificity observed is 98% & 100% and 98% & 100% respectively. The results obtained combined for the ADA and IFN- γ are useful for the definitive diagnosis of the tubercular infection.

In present study, a positive correlation between serum IFN- γ and serum CRP levels is also found. The Pearson's coefficient of correlation is 0.38 and the p value is showing that there is a significant positive correlation between the serum IFN- γ and serum CRP levels.

CONCLUSIONS

IFN- γ plays a pivotal role in protective immunity against Mycobacterium TB. Elevations of IFN- γ have been found in the affected lung and blood stream of patients with pulmonary TB.

It is also concluded that a correlation exists between body fluid ADA levels with body fluid CRP levels. The correlation is positive type and is statistically significant. Thus, by combining all these parameters i.e. ADA, CRP and IFN- γ the diagnosis of the tuberculous infection becomes more easy, accurate and fast emphasizing the need of their continued use and to differentiate pulmonary and extra pulmonary tubercular infection as well as helpful in finding out the LTBI concluding that the combined estimation of parameters such as ADA, CRP and IFN- γ in serum as well as body fluids is of great value in diagnosis and prognosis of these lesions.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent

Informed consent was obtained from all individual participants included in the study

Competing Interests

Authors have declared that no competing interests exist.

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