Immunological study among Tuberculosis patients in Baghdad .

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Abstract:

Background*: Mycobacterium tuberculosis (Mtb)* infection may cause overt disease or remain latent. Interferon gamma release assays (IGRAs) detect *Mtb* infection, both latent infection and infection manifesting as overt disease, by measuring whole blood interferon gamma (IFN-ɣ) responses to *Mtb* antigens such as ESAT-6, CFP-10 and TB7.7. Biomarkers are crucial to the development of new diagnostic tools for tuberculosis diagnosis and could be instrumental in reducing morbidity and mortality and curtailing spread of tuberculosis. In this study, cytokines such as Tumor necrosis factor –alpha (TNF-α) and Interleukine-10 (IL-10) in addition to IFN- ɣ were assessed as biomarkers of tuberculosis infection and reactivation; tuberculosis disease and tuberculosis cure.

Objective: This study was designed to evaluate the immune status of patients with respect to *Mycobacterium tuberculosis.* A prospective study was conducted to obtain more clarification about the impact of causative agent . The study included immunological diagnosis by Quantiferon –TB Gold-Test (QFT) and estimation the serum levels of IFN-ɣ, TNF-α and IL-10 in TB patients and control.

Methods: The present study included fifty TB patients attending the National Reference Laboratory of Tuberculosis /Baghdad during the period April to July 2014 were recruited for this study. For the purpose of comparison, 50 control samples (respiratory disease and healthy) matched by age and gender were also included. The patients and control groups were examined for the presence of TB by Quantiferon TB Gold test.

Results: Forty five samples of TB patients with significance difference ($p \leq 0.01$) were positively detected by Quantiferon-TB Gold Test. The present study revealed significant difference in IFN-ɣ, TNF-α and IL-10 levels between TB patients and control groups.

Conclusion: High levels of Interferon-gamma, Tumor necrosis factor-alpha and Interleukine-10 in tuberculosis patients serum indicate an important role of this cytokines in the pathogenesis of tuberculosis, so they could be considered as a good biomarkers for diagnosis and considered as a target for future therapy.

Keyword: Quantiferon–TB Gold test, Tuberculosis, Tumor necrosis factor-alpha ,Interleukine-10.

Introduction:

Tuberculosis (TB) is a disease caused by bacteria that are spread through the air from person to person. The TB bacteria are put into the air when a person with TB disease of the lungs or throat coughs, sneezes, speaks, or sings.. The TB bacteria usually attack the lungs, but can attack any part of the body such as the kidney, spine, and brain [1].(TB) is caused by a type of bacterium called ***Mycobacterium tuberculosis***[2] .Control of this disease depends largely on early detection and treatment of active cases. Smear microscopy and nucleic acid amplification test are the two currently available rapid confirmatory tests for active TB disease diagnosis, but both are limited by their poor sensitivity [3,4]. Another confirmatory test for active TB disease is culturing the mycobacterium, but it requires several weeks to obtain the results[3]. 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 Quanti FERON®-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), 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. 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].

TNF-*α* is crucial in containment of latent infection and protects the host from developing active TB[8] .

IL-10 deactivates macrophage function by down regulating IL-12 and TNF*α* expression, which in turn reduces production of IFN*γ* by T cells and thus aids in *Mtb* survival[9].

Materials and methods

Subjects:

The study was conducted at National Reference Laboratory of Tuberculosis /Baghdad from April to July 2014 . A total of 100 samples, including suspected cases and controls (including healthy and lower respiratory disease). A full history was taken from each

case,,, including the sex, age, residency, occupation, BCG vaccination, history of cough, night sweating, hemoptysis, fever and loss of weight. All patients were examined for the (QFT_GIT) ,TNF-α and IL-10. Six ml of blood was collected by venipuncture using a disposable syringe or vacuum tube needle for each patient enrolled in this study, One ml of whole blood was added to each of the three Quantaferon tubes: (Nil, TB Antigen and Mitogen) for ELISA usage.3 ml of blood were centrifuged and serum separated and transferred into clean test tube and stored at -20°C for further serological testing such as IL-10 and TNF-α.. IL-10,TNF-α and QFT-G testing were performed according to manufacturer's instructions .

Quantiferon TB-Gold Test (QFT)

QuantiFERON®-TB Gold IT test is performed in two stages. First, whole blood, which collected into each of the QuantiFERON®-TB Gold blood collection tube which include a Nil Control tube, TB Antigen tube, and a Mitogen Control tube. 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 was measured by ELISA. Results were calculated and interpreted by the assay software as positive, negative or indeterminate, according to manufacturer instruction. Tests were interpreted as indeterminate if the mitogen minus Nil was <0.5,or the nil was> 8.0 , tests were interpreted as negative if the TB antigen minus nil was< 0.35 or if the TB antigenminusnil was ≥0.35 but was< 25% of the nil value; tests were interpreted as positive If the TB antigen minus Nil was ≥ 0.35 and was ≥ 25% of the nil value.

Tumor necrosis factor-α (TNF-α) (Bekman Coulter, Ltd, France)

This ELISA (Bekman Coulter, Ltd, France) is a one immunological step sandwich type assay. Samples and calibrators are incubated in the micro-titer plate coated with the first monoclonal antibody anti-TNFα, in presence of the second anti-TNF-α monoclonal antibody linked to alkaline phosphatase. After incubation, the wells are washed and the bound enzymatic activity is detected by addition of a chromogenic substrate. The intensity of the coloration is proportional to the TNFα concentration in the sample or calibrator. The sample results are calculated by interpolation from a calibrator curve that is performed in the same assay as that of the sample. Draw the curve, plotting on the horizontal axis the TNFα concentration of the calibrator and on the vertical axis the corresponding absorbance. Locate the absorbance for each sample on the vertical axis and read off the corresponding TNFα concentration on the horizontal axis. Using a computer, we recommend the use of a curve fit equation Y = A +Bx +Cx2 (quadratic mode).

Interleukin -1o (Ray biotech)

This assay employs an antibody specific for human IL-10 coated on a 96-well plate. Standards and samples are pipetted into the wells and IL-10 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human IL-10 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of IL-10 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

Results

Distribution of patients sample studied according to QFT.

In the present study, the number of patients which detected as positive TB by QFT was 45 patients ,while the number of patients which detected as non-TB by QFT was 5 patients. Chi-square showed that there was highly significant statistical difference between ve+ and ve- QFT (14.208) (P≤0.01), (Table 1).

Table (1): Number and percentage of distribution of patients sample studied according to QFT**.**

QFT level	No.	Percentage (%)
Ve+	**45**	90.00
Ve-	**5**	10.00
Chi-square value	**---**	14.208 **
** (P≤0.01).		

Serum levels of QFT,IL-10 and TNF-α between studied groups

In the present study, the comparison between studied groups in QFT,TNF-α and IL-10 levels was studied. The number of patients was (50) and the mean of QFT,IL-10 and TNF-α was(3.54±0.26), (53.02±8.37) and (74.34±8.49) respectively more than the number of respiratory disease patients (25) and the mean of QFT,IL-10 and TNF-α was (0.866±0.19),(22.24±3.92) and (49.12±4.56) respectively and compare to healthy control (25) and the mean of QFT, IL-10 and TNF-α was (0.556± 0.25), (7.51±0.53) and (27.80±15.03).The LSD value of QFT,IL-10 and TNF-α was (0.786), (22.153) and (29.658) respectively and there were significant statistical difference between patients , respiratory disease patients and healthy control in QFT,IL-10 and TNF-α and(P ≤0.05).(Table 2).

Table(2): Comparison between studied groups in QFT, IL- 10 and TNF-α levels (mean ± SE)

QFT level	No.	Percentage (%)
Ve+	**45**	90.00
Ve-	**5**	10.00
Chi-square value	---	14.208 **
** (P≤0.01).		

Serum levels of QFT,IL-10 and TNF-α levels in patients

In the present study, LSD (Least Significant Difference) value of both QFT level and IL-10 level were (2.045) and (58.868) respectively, there were non-significant statistical difference between patient categories and the level of QFT, IL-10, while LSD – value of TNF-α was (53.825), there was significant statistical difference between patient categories and TNF-α (P≤0.05), (Table3)

Table (3):Effect of Patient categories in QFT, IL-10 and TNF-α (mean ± SE)

Patient categories	Number	Mean ± SE		
		QFT	IL-10	**TNF-α**
OLD	12	3.41 ± 0.44	49.75 ± 20.1	58.17 ± 3.87
MDR	6	3.73 ± 0.59	56.17 ± 31.5	156.67 ± 61.4
NEW	28	3.57 ± 0.41	47.39 ± 8.52	65.85 ± 4.15
Mono	4	3.38 ± 0.65	97.50 ± 45.2	58.75 ± 12.1
LSD value	---	2.045 NS	58.868 NS	53.825 *
* (P≤0.05)				

Discussion:

Distribution of patient samples studied according to QFT.

In the present study, the number of patients which detected as positive TB by QFT was 45 patients ,while the number of patients which detected as non-TB by QFT was 5 patients. Chi-square showed that there was highly significant statistical association between ve+ and ve- QFT (14.208) (P≤0.01), (Table 1).

Negative Quanti-FERON®-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 [10]

The results of present study were agreement with previous studies [11,12].

Serum levels of QFT,IL-10 and TNF-α between studied groups

In the present study, the comparison between studied groups in QFT,TNF-α and IL-10 levels was studied. The number of patients was (50) and the mean of QFT,IL-10 and TNF-α was(3.54±0.26), (53.02±8.37) and (74.34±8.49) respectively more than the number of respiratory disease patients (25)and the mean of QFT ,IL-10 and TNF-α was (0.866±0.19),

(22.24±3.92) and (49.12±4.56) respectively and compare to healthy control (25) and the mean of QFT,IL-10 and TNF-α was (0.556± 0.25), (7.51±0.53) and (27.80±15.03).The LSD value of QFT,IL-10 and TNF-α was (0.786), (22.153) and (29.658) respectively and there were significant statistical association between patients , respiratory disease patients and healthy control in QFT,IL-10 and TNF-α and(P ≤0.05).(Table 2).

Mean IFN-ɣ level of TB patients was higher than that of the control groups .,this may be due to elevated IFN-ɣ ESAT6 antigen which is the early secretory antigen target protein is released by *M. tuberculosis* and can activate *M .tuberculosis* specific IFN-ɣ producing CD4+ T cells[13].Clinical studies have shown an association between immune reactivity to ESAT6 with that of increased bacterial load and disease pathology in tuberculosis [14].This result is agreement with [15,16].

High levels of TNF-α were reported in chronic TB accompanied by the evaluated release of TNF-α receptors resulting in fever, necrosis and weight loss. This implies that TNF-α has both protective and immunopathologic effects [17]. The results of this study is agreement with [18].

With regard serum IL-10 levels, the production of such anti-inflammatory cytokine in response to *Mtb* may down regulate the immune response and limit tissue injury , but excessive production of such cytokines may result in failure to control the infection [19]. In contrast to the results of this study were agreement with [19,20] that cytokines levels were found to be equivalent to those of the control group at the end of therapy and in agreement with[21] reported that high initial production of IL-10 in patients with PTB remained unchanged with the treatment.

QFT,IL-10 and TNF-α levels in patients

In the present study, LSD value of both QFT and IL-10 was (2.045) and (58.868) respectively, there were non-significant statistical difference between patient categories and QFT, IL-10, while LSD –value of TNF-α was (53.825), there was significant statistical association between p. categories and TNF-α (P≤0.05), (Table 4-5-3).

IFN-ɣ plays a key role in regulating of mycobacterial clearance by its ability to coordinate appropriate defense mechanisms required for *Mtb* control[22].

The current study showed that the mean QFT were higher among MDR TB patients in comparison with other categories of patients. This finding was in agreement with study in Brazil [23] who reported that immune response is decreased in resistance TB, and[24].

The current study showed that the mean IL-10 were higher among drug resistance TB patients in comparison with other patient categories .This finding was agreement with study in Brazil[25] who demonstrated that increased IL-10 was observed in cultured MDR-TB cells following ESAT-6 stimulation ,than other patient categories, and in agreement with [26].

The current study showed that the mean of TNF-α were significantly higher among MDR TB patients in comparison to other patient categories. These results indicate that the serum TNF alpha level may be a good marker to predict the TB patient's clinical evolution. This finding was agreement with [27] who showed that the MDR-TB cases presented severe pulmonary lesions with increased tissue destruction. In this group, the serum TNF alpha level ranged from a slight elevation to a very high level .when serum TNF alpha levels decreased during clinical evolution, a clinical improvement was always noted. In contrast, when serum TNF alpha levels did not decrease during the evolution, a bad clinical course was noted . These results were in agreement with [23] who demonstrated that the overall immune response is decreased in resistant TB and the major role inflammatory cytokines may play in perpetuating pulmonary tissue damage ,and in agreement with [28].

Conclusions:

1-Quantiferon-TB Gold test has incorporated in the diagnosis of LTBI, especially in low incidence and high-resource settings.

2-QFT-TB Gold test, has excellent sensitivity and specificity unaffected by BCG vaccination.3-QFT-TB Gold test clinical potential, not only in the diagnosis of TB, but also in the evaluation of the efficacy of anti-TB treatments.

4--There was significant increase in IL-10 and TNF-α in TB patients compared with control groups, therefore it could be potential biomarker for the diagnosis of tuberculosis.

Recommendations:

*Use Immunological tests such as T-Spot test will be more useful for tuberculosis diagnosis.

*Use other parameters, e.g. Toll like receptors and other cytokines may be useful to give a clearer picture about the immune changes that occur in TB.

* Large sample may yield a good statistical results and interpretations.

*Since no single biomarker can be used as a diagnostic biomarker, so it is important to use a group of biomarkers to accomplish this issue

References

1-CDC. Learn the Signs and Symptoms of TB Disease.2014

2-NHS Choice. Tuberculosis causes.2012

3-Brodie D, Schluger NW .The diagnosis of tuberculosis. Clin Chest Med 2005 26: 247–271.

4-Pai M, Kalantri S, Dheda K. New tools and emerging technologies for the diagnosis of tuberculosis: part II. Active tuberculosis and drug resistance. Expert Rev Mol-Diagn 2006 6: 423–432.

5-P. Andersen. Specific immune-based diagnosis of tuberculosis.2000

6- T. Mori, M. Sakatani, F. Yamagishi, *et al*. Specific detection of tuberculosis infection: an interferon-g-based assay using new antigens. Am. J. Respir. Crit. Care Med., 2004 .170 , pp. 59–64.

7-A.F. Luetkemeyer, E.D. Charlebois, L.L. Flores, *et al* Comparison tuberculin skin testing in HIV infected individuals .Am. J. Respir. Crit. Care Med.,2007. 175 , pp. 737–742.

8-Mohan, V.P.; Scanga ,C.A. and YuK . Effect of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis :possible role for limiting pathology. Infection and immunity. 2001 Vol69 ,no(3), pp:1847-1855.

9-Fujiwara,N. and Kobayashi, K .Macrophages in inflammation .Current Drug Targets.2005. Vol4 , no(3) , pp:281–286.

10-Todd, B. The Quantiferon –TB Gold Test: a new blood assay offers a promising alternative in tuberculosis testing " Am J. Nurs., 2006;106:33-4.

11-Al-Jebouri, M.M. and Wahid, N.M. An Evaluation of QuantiFERON-TB Gold in Tube and Immunological Tests for TB .2014.vol 4 , no(13) ,pp: 2546-2554

12-Helmy N; Abdel latif,S; Kamel,M; Ashour, W . Role of Quantiferon TB gold assays in monitoring the efficacy of anti-tuberculosis therapy..Egyptian Journal of Chest Diseases and Tuberculosis 2012 .vol61 ,iss (4) ,pp 329-336 .

13-Qian, F. , Weihong, Q., Yiqun ,S. ,Jiangfang, H. , Dongli ,L. , Jing, Z. , Liching ,D. Evaluationtion of a new tuberculosis –related interferon gamma release assay for tuberculosis infection diagnosis in Huzhou, easternchina. Indian Journal of Pathology and Microbiology . 2013. vol.56,no.2, pp.125-128.

14- Arend; Geluk , A.; Meijgaarden, K.E.; van Dissel J.T.; Theisen M.; Anderson , P. ; Ottenhoff, T.H. M. Antigenic equivalence of human T-cell responses to Mycobacterium tuberculosis-specific RD1 - encoded protein antigen s ESAT-6 and culture filtrate protein 10 and to mixture of synthetic peptides. Infect Immune .2000 .vol 68 , no (6) , pp 3314-3321.

15- Ribeiro, S.; Dooley ,K. ; Hackman, J.; Loredo ,C. ; Efron A.; Chaisson, R.E.; Conde, M.B.; Boechat, N. and Dorman, S.E. TSPOT.TB responses during treatment of pulmonary Tuberculosis. BMC Infect Dis.2009; 9:23-5.

16-Masood, KI; ME; Salahuddin, N. Expression of *M. tuberculosis* -induced suppressor of cytokine signaling (SOCS) 1, SOCS3, FoxP3 and secretion of IL-6 associates with differing clinical severity of tuberculosis .BMC Infect Dis. 2013 .vol 13.

17- Hussain, S.; Afzal, N; Javaid K; Ullah, M.I. ; Ahmad,T. and Saleem-Uz-Zaman. Level of Interferron –gamma in the blood of tuberculosis patients. Iranian Journal of Immunology, . 2014. vol 7,no(4),pp :240-6.

18-Bekker, L.G. ;Maartens, G. ; Steyn, L. ; Kaplan, G. ;Selective. Increase in plasma tumor necrosis factor-alpha and concomitant clinical deterioration after initiating therapy in patients with severe tuberculosis. J Infect Dis 2000; 1178 (2) 580-4

19- Thanoon, I. A Tumor necrosis factor-alpha, Interleukine-10 and Oxidant/antioxidant status in patients with pulmonary tuberculosis : Effect therapy, a follow up study."QMJ, 2012; 8(14)1-14.

20-Sharma ,S. and Bose, M. Role of cytokines in immune response to pulmonary tuberculosis. Ascian Prac J Allergy Immunol 2001; 19(3): 213-9.

21-Sahiratmadja,E. ; Alisjahbana, B. ; Boer, T. ; Adnan ,I. ; Maya, A. and Danusantoso,H. Dynamic changes in pro and anti-inflammatry cytokine profiles and gamma interferon receptor signaling integrity correlate with tuberculosis disease activity and response to curative treatment. Infect Immun. 2007 vol 75,no(2), pp 820-829.

22- Mora,E.P.; Toledo, V.P. ; Oliveira, M.H. ; Spindolade- Miranda , S . ; Andrade, H.M. andGuimaraes , T.M. .Pulmonary tuberculosis: evaluation of interferon – gamma levels as an immunological healing worker based on the response to Bascillus-calmette Guerin". Med Inst Oswaldo Cruz . 2004; 99(3):283-7.

23-Cooper, AM. . Cell mediated immune response in tuberculosis .Annual review of Immunology. 2009 .vol.27,pp. 393-422.

24-Fortes, A.; Pereira,K.; Ants, P.R.Z; Franken, C.L.M ; Dalcolombo, M. Ribeiro-Carvalho,K.S. ; Cunha, A; Geluk, A; Kristiki, A.; Kolk,P; Klaster, E.N; Sarno; Ottenhoff, T.H.M. and Sampaio, E.P. Detection of *in vitro* interferon-γ and serum tumor necrosis factor-α in multidrug-resistant tuberculosis patients .NCBI. 2005; 141 , (3):541-8.

25- Pinheiro, R.O. ; Oliveira, E.B. and Sampaio, E.P. . Different immunosuppressive mechanisms in multi-drug-resistant tuberculosis and non-tuberculous mycobacteria patients. ClinExpImmun .2013; 171(2):210-9.

26- Deveci, F., H. Handan, H.A. & Hamdi, M Changes in Serum Cytokine Levels in Active Tuberculosis With Treatment .Mediator Inflamm 2005; 25(5): 256-62.

27-Andrade ,D.R. ; Santos S.A. and Castro I.D. Correlation between serum tumor necrosis factor alpha levels and clinical severity of tuberculosis. Braz J Infect Dis.. 2008; 12 (3):226-33.

28-Lee,J.S; Song CH, Lim J.H ", Inflam Res, 2003; 52:297-304.