Original Article

Utility of an interferon-gamma release assay as a potential diagnostic aid for active pulmonary tuberculosis

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Abstract

Introduction**:** Sensitivity, specificity, early confirmation and obtaining an optimal specimen are challenging problems in active tuberculosis (TB) diagnosis. Interferon-gamma release assay (IGRA) is a good indicator for latent TB but can it be useful as a diagnostic tool for active TB? This study was designed to address these challenges and assess the potential of IGRA as a diagnostic indicator of active pulmonary TB by comparing it with other MT diagnostic conventional methods and molecular methods.

Methodology: The study was conducted on 91 patients with suspicion of pulmonary active TB. QuantiFERON-TB-Gold In-Tube, a commercial IFN-gamma assay, was compared with Ziehl Neelsen (ZN) smear, Lowenstein Jensen's (LJ) egg-based culture, and real-time polymerase chain reaction. The final clinical diagnosis was the standard comparator of the study.

Results: Active pulmonary TB was confirmed in 48/91 (52.7%) patients. Sensitivity, specificity, positive predicted value (PPV), and negative predicted value (NPV) were 72.9%, 100%, 100%, 76.78% for ZN smear, 77.1%, 97.67%, 97.36%, 79.24% for LJ culture, 89.9%, 67.4%, 75.4%, 85.3% for IGRA, and 66.6%, 95.3%, 94.1%, 71.9% for real-time PCR, respectively.

Conclusion: Albeit confounding in the case of latent TB infected patients presenting with non-TB pulmonary disease, IGRA was more sensitive than the other conventional and molecular methods**,** so it may improve diagnostic accuracy when used in combination with other standard methods. High NPV of IGRA for the diagnosis of active TB proposed an additional role of this test to exclude the infection with active TB.

Key words: Interferon gamma release assay; TB real time-PCR; TB culture; TB smear; active pulmonary TB

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Introduction

Tuberculosis (TB) is the most important single infectious cause of mortality and morbidity worldwide. In 2009, there were an estimated 9.4 million incident cases, 14 million prevalent cases, and approximately 1.7 million deaths because of TB [1]. Rapid and accurate diagnosis is critical to the care of tuberculosis (TB) patients and to arrest disease transmission [2]. Despite the considerable specificity of direct detection of the causative agent, active tuberculosis diagnosis still suffers technical obstacles such as results delay, difficulties in obtaining representative specimens, and dependence on personal skills and subjectiveness.

QuantiFERON-TB-Gold In-Tube (Cellestis Ltd, Victoria, Australia) is a commercial test which depends on the interferon gamma release assay (IGRA). It is based on the fact that T cells sensitized with tuberculous antigens will produce IFN-γ when they are re-exposed *ex vivo* to mycobacterial antigens; a high amount of IFN-γ production is then presumed to correlate with TB infection [3]. The current IFN-γ assay uses the early secretory antigen target 6 (ESAT-6), and the culture filtrate protein 10 (CFP-10); both proteins are coded by genes located in the region of difference 1 (RD1) of the *Mycobacterium tuberculosis* genome and are not shared with *M. bovis* BCG or most nontuberculous mycobacterias NTM, with the exception of *M. marinum, M. sulzgai and M. kansasii* [3]. In addition to these specific antigens, it uses TB7.7 encoded by a phage-inserted region (phiRv2) which is highly specific for *M. tuberculosis* [4].

Our study aimed to assess the potential of IGRA as a novel promising indicator for the diagnosis of active pulmonary tuberculosis, whether used alone or combined with other TB-detection diagnostic tests. The prospective nature of this study allowed a direct comparison between IGRA and microscopic

examination, culture, and real time PCR for TB suspected patients.

Methodology

Specimens

This study recruited 91 suspected active pulmonary TB patients who were not immunosuppressed by infection or drug therapy and did not have a previous active TB infection. Respiratory specimens (85 sputum, three bronchial wash, and three pleural fluid) and peripheral blood samples were collected between January and September 2010. Informed consent was obtained from all subjects.

Respiratory specimen preparation and processing

Respiratory specimens were digested and decontaminated using the BBL MycoPrep Specimen Digestion/Decontamination Kit (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer´s instructions. Aliquots of the resulting suspension were used to (i) prepare smears stained by the Ziehl-Neelsen (ZN) method for microscopic exam as previously described [5], (ii) inoculate onto Lowenstein Jensen's (LJ) slants (Biomerieux, Marcy l'Etoile, France) according to World Health Organization (WHO) guidelines [6], and (iii) extract *M. tuberculosis* DNA.

Whole-Blood Interferon-γ Assay

Blood specimens were drawn, and the IGRA assay was performed using the QuantiFERON-TB Gold *In-Tube* (QFT-GIT) kit, and two QFT-GIT collection tubes for each patient (Nil Control and TB Antigen tubes) manufactured by (Cellestis Ltd, Victoria, Australia) according to the manufacturer′s instructions. The optical density was measured using a Sunrise Remote microplate reader (Tecan, Grödig, Austria) fitted with a 450 nm filter and a 690 nm reference filter. Results above the cutoff value of 0.35 IU/mL were considered positive.

DNA extraction and amplification

DNA extraction was performed using an INSTANT Mycobacteria DNA Kit (AJ Roboscreen, GmbH, Leipzig, Germany). The extracted specimens were stored at -80ºC until amplification was performed. Real-time PCR was carried out using the IS6110 DNA TripleHYB assay using a RoboGene *Mycobacterium tuberculosis* Qualitative Kit (AJ Roboscreen, GmbH, Leipzig, Germany) on a SmartCycler (Cepheid, Sunnyvale, CA, USA) according to the manufacturer´s instructions. The validity of extraction was evaluated by beta-globin DNA real-time PCR using a FastStart DNA Master Hybridization Probes Kit on the LightCycler (both from Roche Diagnostics, Germany). The final reaction mixture of 10 µl contained 3 mM $MgCl_2$, 0.5 µM primers each (forward primer: TAAgCCAgTgCCAgAAgAgCC, reverse primer: ATCATTCgTCTgTTTCCCATTCTAAAC), and 0.2 µM probes each (Fluorescein probe: CCCTTggACCCAgAggTTCTTTgAgTCCT-Fluorescein, LightCycler Red probe: LC-Red640-TggggATCTgTCCACTCCTgATgCTgTTATg-phosphate) manufactured by TIB Molbiol, Germany. The temperature profile was initiated at 95ºC for 10 minutes followed by 45 cycles of denaturation at 95ºC for 10 seconds, annealing at 59ºC for 10 seconds, with a final extension at 72ºC for 32 seconds.

Final diagnosis

The final diagnosis was obtained from the patient´s medical record. It was made on the basis of all clinical, radiological, microbiological, and pathological information gathered.

Statistical analysis

Sensitivity, specificity, positive predicted value (PPV), and negative predicted value (NPV) compared to the final diagnosis of active TB disease were calculated for each diagnostic test. Statistical calculations were conducted to investigate the potential improvement of sensitivity and specificity of the TB detection methods by combining the use of IGRA and each of the other methods applied on respiratory specimens. Ninety-five percent confidence intervals (CIs) were estimated according to the binomial distribution. Correlations between tests were analyzed nonparametrically with Kendall´s tau-b correlation coefficient. A p-value of < 0.05 was considered to be significant. Analyses were performed using PASW statistics 18 software (SPSS, Chicago, Illinois, US).

Table 1. Negativities, positivities, sensitivities, specificities, PPVs and NPVs of ZN smear, culture, real time PCR and IGRA tests in active TB suspected patients recruited in this study, n = 91[a]

	Negative	Positive	P-value[b]	Sensitivity, % 95% CI	Specificity, % 95% CI	PPV, % 95% CI	NPV, % 95% CI
ZN Smear [c]	56 (61.5%)	35 (38.5%)	0.0005	72.9 63.58-82.21	100 99.995-100.005	100 99.995-100.005	76.78 67.93-85.62
Culture [d]	53 (58.2%)	38 (41.8%)	0.0003	77.1 68.29-85.9	97.67 94.5-100.83	97.36 93.99-100.72	79.24 70.74-87.73
IGRA [e]	34 (37.4%)	57 (62.6%)	0.0007	89.9 83.58-96.21	67.4 57.57-77.2	75.43 66.4-84.45	85.29 77.86-92.71
Real-time PCR [f]	57 (62.6%)	34 (37.4%)	0.0006	66.6 56.71-76.48	95.3 90.86-99.73	94.11 89.17-99.04	71.92 62.5-81.33
Diagnosis [g]	43 (47.3%)	48 (52.7%)					

[a] PPV, Positive predictive value; NPV, Negative predictive value.
[b] Correlations between tests were analyzed using Kendall´s tau-b correlation coefficient; a P-value < 0.05 was considered statistically significant, with 95% confidence intervals.
[c] Smears of respiratory samples were stained by Ziehl neelsen method for the detection of AFB (mycobacteria species).
[d] Lowenstein Jensen's media was used for mycobacteria culture of respiratory samples.
[e] IGRA = Interferon gamma releasing assay in whole blood performed in suspected active TB patients to detect the exposure to MTB (results above the cutoff 0.35 IU/ml were considered as positive).
[f] Real-time PCR = Real-time polymerase chain reaction targeting both the multicopy target IS6110 insertion element and also a common genomic subsequence on respiratory samples.
[g] Final diagnosis taken from patient´s medical record.

Results

Active TB was the final diagnosis in 48/91 (52.7%) patients, while the remaining 43/91 (47.3%) were finally diagnosed as non-TB patients as follows: respiratory infections (12/43), asthma (3/43), chronic obstructive pulmonary disease (COPD) (5/43), COPD with chronic infections (2/43), malignancy (5/43), malignancy with pneumonia (1/43), bronchiectasis (1/43), respiratory failure (1/43), silicosis (1/43), deep vein thrombosis-pulmonary embolism (1/43), pulmonary fibrosis (5/43), pneumonia (3/43), brucellosis (1/43), aspergillosis (1/43), and heart failure (1/43).

When the diagnostic tests were applied on the samples of the study group, the results were distributed (Table 1) as follows: acid-fast bacilli appeared in 35/91 samples (38.5%) and were not detected in 56/91 samples (61.5%). The sensitivity and specificity of the ZN smears were 72.9% and 100%, respectively. In addition, the ZN smear had a PPV of 100% and NPV of 76.78%. Culture was positive in 35/91 (41.8%) samples, and its sensitivity and specificity were 77.1% and 97.67%, respectively, whereas the PPV and NPV of culture were 97.63% and 79.24%, respectively. When IGRA was performed, test results were positive in 57/91 patients (62.6%) and negative in 34/91 (37.4%), resulting in a sensitivity of 89.9% and a specificity of (67.4%). PPV and NPV of the IGRA were 75.43% and 85.29%, respectively. Meanwhile, real-time PCR detected MTB DNA in 34/91 samples (37.4%), whereas 57/91 samples showed absence of DNA amplification product, giving a sensitivity of (66.6%) and specificity of (95.3%). PPV of the real-time PCR was 94.11%, and the NPV was (71.92%). All four diagnostic tests seemed to be correlated to the final diagnosis with a significant p-value < 0.05.

Forty-eight cases were diagnosed as active TB, of which 43 were positive and five were negative by IGRA, 35 were positive and 13 were negative by ZN smear, 37 were positive and 11 were negative by LJ culture, and 32 were positive and 16 were negative by real-time PCR. Meanwhile, 14 out of 43 participants diagnosed as non-TB patients were IGRA positive and 2/43 were real-time PCR positive (Table 2).

ZN smear detected 35/48 TB patients and missed 13/48 TB patients, whereas LJ culture detected 37/48 TB patients and missed 11/48 TB patients. Combining both methods together gave a sensitivity of 83.3% and a specificity of 97.6%. When comparing IGRA with ZN smear, IGRA was positive in 11/48 TB cases (22.9%) which were negative by ZN smear. At the same time, ZN smear was positive in 3/48 TB cases (6.25%) that were negative by IGRA. The IGRA method detected 11/48 (22.9%) extra cases that were not detected by LJ culture. On the other hand, culture was positive in 5/48 TB cases (10.41%) that were negative by IGRA. The positive results of IGRA and culture conflicted with the final diagnosis of one patient, for whom the final diagnosis was determined as non TB based on the negative ZN smear result only. The IGRA method detected 13/48 TB cases (27.08%), while MTB DNA was not detected by real-time PCR in their respiratory samples. MTB DNA was detected by real-time PCR

Table 2. Comparison of ZN smear, LJ culture, and real-time PCR in respiratory samples with IGRA in whole blood of non-active TB patients, n = 43.

Method	Results	Interferon gamma releasing assay (IGRA)	
		Positive	Negative
Ziehl-Neelsen Smear	Positive	-	-
	Negative	14 (32.5%)	29 (67.4%)
Lowenstein Jensen's Culture	Positive	1 (2.3%)	-
	Negative	13 (30.2%)	29 (67.4%)
Real-time Polymerase chain reaction	Positive	-	2 (4.6%)
	Negative	14 (32.5%)	27 (62.8%)

Table 3. Comparison of ZN smear, LJ culture, and real-time PCR in respiratory samples with IGRA in whole blood of diagnosed patients with active TB, n = 48

Method	Results	Interferon gamma releasing assay (IGRA)		p-value*
		Positive	Negative	
Ziehl neelsen Smear	Positive	32 (66.6%)	3 (6.25%)	0.497
	Negative	11 (22.9%)	2 (4.16%)	
Lowenstein Jensen's Culture	Positive	32 (66.6%)	5 (10.41%)	0.202
	Negative	11 (22.9%)	-	
Real-time Polymerase chain reaction	Positive	30 (62.5%)	2 (4.16%)	0.186
	Negative	13 (27.08%)	3 (6.25%)	

* Correlations between tests were analyzed using Kendall´s tau-b correlation coefficient; a p-value < 0.05 was considered statistically

Table 4. Calculated sensitivities and specificities when each of the detection methods in respiratory specimens was used in combination with IGRA in the study group, n = 91

Methods Combination	*Sensitivity (%), (CI)	*Specificity (%), (CI)
IGRA° + ZN Smear°	95.83, (91.63-100.022)	67.44, (57.62-77.25)
IGRA + LJ Culture°	100, (99.995-100.005)	67.44, (57.62-77.25)
IGRA + real-time PCR°	93.75, (88.67-98.82)	62.79, (52.66-72.91)

*Calculated against final diagnosis.
°ZN, Ziehl neelsen; LJ, Lowenstein Jensen's; IGRA, Interferon gamma release assay; PCR, Polymerase chain reaction

in 2/48 TB cases (4.1%) which were negative by IGRA. As shown in Table 3, no statistically significant correlation was found among IGRA and the other three diagnostic methods (p-values > 0.05).

When combining ZN smear and IGRA, the sensitivity and specificity were 95.83% and 67.44%, respectively. When IGRA was paired with culture, sensitivity of 100% and specificity of 67.44% were observed, whereas pairing IGRA and PCR gave a sensitivity of 93.75% and specificity of 62.79% (Table 4).

Discussion

The specificity of the ZN smear reflected the presence of AFB in general, since the ZN staining method cannot distinguish between MTB and other mycobacterial species [7]. The relatively low sensitivity of the ZN smear method is due to its inability to detect less than 5,000-10,000 AFB per ml of specimen [7]. Accordingly, patients with infectious TB might not be diagnosed, resulting in ongoing transmission of disease in the community and eventually more severe disease in the individual [5].

False negativity obtained by culture might be caused by LJ culture contamination, which often involves the total surface of the medium and consequently the culture is usually lost [6]; furthermore, culture has been estimated to detect as many as 10-1,000 viable mycobacteria per mL of specimen [7]. Five TB cases 5/48 (10.41%) were positive by culture and negative by IGRA. Negative IGRA and real-time PCR in three out of these five cases may be due to other mycobacterial species which require additional identification tests on LJ isolates [8].

Real-time PCR was negative in 16/48 TB cases (33.3%); this reduction of the sensitivity might be due to the uneven distribution of bacilli in the sample [9]. The suboptimal extraction of nucleic acids was addressed in our study by the detection of human beta-globin DNA in all extraction products. From non-TB patients, real-time PCR was positive in 2/43 cases (4.6%), and this false positivity may due to laboratory contamination [8].

IGRA showed the highest sensitivity among the diagnostic tests used in this study. The false negative IGRA results were in two TB patients with positive results by the other tests. These two patients suffered from severe TB which indicates that more advanced disease may be associated with weak T-cell response [10] or it may be because of a genetic deficiency, such as the inability to produce sufficient IFN-γ, in the patient's immune system. In this situation, the additional mitogen tube optionally supplied in the IGRA assay kit might be used as a control to evaluate the immune response and IFN-γ production.

The specificity of IGRA in our study was low for diagnosis of active pulmonary TB compared with previously reported data [11,12,13]. This lower specificity could be explained by the fact that IGRA has not been shown to be able to discriminate between active and latent TB infection [14]. The IGRA positive results in 14 non-active TB cases could imply latent TB infection in these cases, all of whom had one or more of risk factors for TB infection and also lived in Syria, which is considered to be a high TB incidence area [15]. The diagnostic validity of our study tends to be low, and this might be a result of the random selection of patients. Meanwhile, other studies enrolled healthy people as negative controls and patients with confirmed active TB as positive controls [16]. However, patients with positive IGRA results may be at risk of developing active TB, so they need more follow-up monitoring.

Immune suppression induced by infections and use of biological agents such as anti-tumor necrosis factor alpha antibodies are increasing the reactivation of latent TB infection (LTBI), which is considered one of the most feared sequela. Screening for LTBI with the IGRA test may turn out to be extremely useful in this situation [10], as well as in patients with HIV infection with CD4 T cell counts greater than 250/mm^3 [17]. Infections by other mycobacteria such as *M. kansasii, M. szulgai and M. marinum* could also lead to positive results [18].

The IGRA method detected 7/48 extra TB cases (14.9%) that were not detected by ZN smear, LJ culture, or real-time PCR. Obtaining a proper specimen is crucial in laboratory practice; therefore, this negativity might be because of the poor quality of respiratory specimens as reported by the WHO [5] and observed in our study. In addition, when combining culture with IGRA, the overall sensitivity increased to 100%, suggesting that IGRA may play an important role in the diagnosis of active TB.

In conclusion, IGRA was more sensitive than other conventional methods and real-time PCR, but its low PPV confirmed the necessity to use it in combination with other methods for the diagnosis of active TB. It could be much more useful in areas with low prevalence of LTBI. High NPV of IGRA for the diagnosis of active TB proposed an additional role of this test to exclude the infection with active TB.

However, this test can be helpful in cases when it is difficult to obtain the proper specimen.

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