

Combined IFN- γ and IL-2 Release Assay for Detect Active Pulmonary Tuberculosis: A Prospective Multicentre Diagnostic Study in China

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Abstract

Background: We performed a prospective multicenter diagnostic study to evaluate combined interferon- γ (IFN- γ) and interleukin-2 (IL-2) release assays for detecting active pulmonary tuberculosis (TB) in China.

Methods: Adult patients presenting with symptoms suggestive of pulmonary TB were consecutively enrolled in three TB-specialized hospitals. Sputum specimens and blood samples were collected from each participant at enrollment. *In vitro* measurements of IFN- γ and IL-2 release from *Mycobacterium tuberculosis* (MTB)-specific antigen-stimulated patient peripheral blood mononuclear cells were conducted using enzyme-linked immunosorbent assays (ELISAs).

Results: Between July 2017 and December 2018, a total of 3,245 patients with symptoms suggestive of pulmonary TB were included in the final analysis. Of these patients, 2,536 were diagnosed with active TB, including 1,092 definite and 1,444 clinically diagnosed cases. Overall sensitivity and specificity rates of the IFN- γ release assay were 83.8% and 81.5%, respectively, as compared to IL-2 assay rates yielding greater specificity (94.3%) but lower sensitivity (72.6%). Notably, a parallel-type combination test for IFN- γ /IL-2 provided greatest overall sensitivity (87.9%) but relatively low specificity (79.8%). Meanwhile, a series-type combination test had an overall sensitivity rate of 68.5% that, when stratified by case subgroup, yielded sensitivity rates of 72.1% and 65.8% for definite and clinically diagnosed TB cases, respectively.

Conclusion: We developed a new immunological method to differentiate between active TB and other pulmonary diseases. Our data demonstrated that both series-type and parallel-type IFN- γ /IL-2 combination tests may improve diagnosis of active TB cases in different settings. Additionally, the diagnostic accuracy of the series-type combination assay correlated with disease severity in our patient cohort.

Background

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis* (MTB) complex, remains a major cause of mortality and morbidity worldwide, especially in developing countries [1, 2]. According to recent estimates, 10.0 million incident TB cases and 1.4 million TB deaths were reported in 2018, raising global public health concerns [2]. Early and accurate diagnosis of TB is essential for timely initiation of proper treatment and reduction of TB transmission within the community [3]. However, of the estimated 10.0 million new 2018 TB cases, only 7.2 million were actually diagnosed [2], due mainly to the lack of highly sensitive and accessible diagnostic tests and methods [4]. Recently, WHO recommended use of molecular diagnostic tests, such as GeneXpert MTB/RIF and TB-LAMP, for initial TB testing in order to increase case detection rates [5, 6]. Nevertheless, despite their efficacy in detecting culture-positive cases, such tests lack necessary sensitivity for achieving effective TB diagnosis of culture-negative patients, who account for half of the total TB burden [4]. In view of the paucibacillary nature of most culture-

negative cases, this rigorous challenge highlights the urgent need to develop novel immunological diagnostic tests of patient blood rather than direct detection of bacteria or nucleic acids in specimens [4].

Over the past decade, advances in our understanding of host immune mechanisms against tubercle bacilli have facilitated development of new immunological tests [7]. Such tests include interferon- γ (IFN- γ) release assays (IGRAs), which are widely used *in vitro* immune-based blood tests that measure T-cell responses to MTB-specific antigens as a means to detect MTB infection [8]. The high accuracy of these assays reflects the fact that they rely on detection of IFN- γ , considered the most important cytokine secreted by host type 1 (Th-1) T cells for controlling MTB infection [9]. Although IGRAs are currently considered the gold standard of testing for diagnosing latent tuberculosis infection (LTBI), they have not been formally approved for use in differentiating active TB from LTBI [10]. Recently, a new generation of IGRAs has been developed that measures immunological responses to MTB-specific secreted protein TB 7.7 in addition to previously utilized MTB antigens ESAT-6 and CFP-10; TB 7.7 was added to boost activation of the host cellular immune response, thereby achieving increased sensitivity for identifying LTBI [11]. However, use of a limited set of antigens has limited the usefulness of IGRA results for diagnosing active cases or for predicting patient risk of developing active TB disease. Therefore, discovery of other immunological biomarkers is needed before diagnostics can be developed that effectively discriminate between active TB and LTBI [3].

Besides IFN- γ , interleukin-2 (IL-2) is also produced by Th1 cells as a cytokine that stimulates both Th1 cells and cytotoxic T lymphocytes [9]. Results of numerous studies suggest that IL-2 plays important roles in protective immune responses against MTB infection, with detection of increased IL-2 levels in body fluids correlating with active TB disease [12]. However, discordant results regarding the utility of IL-2 for predicting active TB development from LTBI have been reported [13, 14], although conclusions of numerous studies were drawn based on statistically weak results obtained from studies with small sample sizes. Therefore, additional clinical data is needed to evaluate IL-2 as a marker for differentiating between active TB and LTBI or other respiratory diseases. In this work we addressed this concern by conducting a prospective multicenter diagnostic study to evaluate combined IFN- γ and IL-2 release assays for use in detection of active pulmonary tuberculosis in China.

Materials And Methods

Participants

We conducted a prospective multicenter study at three TB-specialized hospitals in China, including Guangzhou Chest Hospital, Hunan Chest Hospital, and Xinjiang Uyghur Chest Hospital. Adult patients presenting with symptoms suggestive of pulmonary TB were consecutively enrolled after exclusion of those aged < 18 years and those unwilling to provide informed consent. Accepted enrollees were first interviewed by clinical physicians at time of enrollment then a baseline blood sample was drawn and demographic data were collected via completion of a case report form. Patient follow up was maintained for 6 months thereafter to monitor treatment responses of patients receiving anti-TB treatment. For

patients with non-TB disease, a final diagnosis was made by hospital clinicians based on medical records. The study was approved by the Ethics Committee of Guangzhou Chest Hospital. All participants provided written informed consent before participating in the study.

Definitions

Diagnoses of all participants were categorized into four groups based on physical examination, laboratory results, and response to medications: i. definite tuberculosis: microbiological culture or positive MTB molecular diagnostic test result, clinical symptoms, and radiological findings suggestive of TB; ii. clinically diagnosed tuberculosis: clinical symptoms and radiological findings suggestive of TB plus appropriate response to anti-TB therapy; iii. latent tuberculosis infection: positive IGRA results and no clinical evidence of active TB; iv. Others: negative IGRA results and final diagnosis of a non-TB respiratory disease. Active TB cases included definite and clinically diagnosed TB cases, while non-TB cases included LTBI and other diseases.

Laboratory procedures

Sputum specimens and blood samples (8 mL) were collected from all participants at enrollment. Sputum was stained with auramine O and examined for acid-fast bacilli using fluorescent microscopy according to guidelines of the National Tuberculosis Program in China [15]. Specimens were processed immediately using a method based on N-acetyl-L-cysteine (NALC). After neutralization with PBS buffer, each suspension was centrifuged for 15 min at $3,000 \times g$ then each resuspended pellet was inoculated into a BACTEC MGIT tube (BD Microbiology Systems, USA) [16]. All positive cultures underwent species identification for MTB using the MPT64 antigen method, while positive cultures without MPT64 expression were further identified using other previously reported molecular diagnostic methods [16]. Additionally, 1.0 mL of each sputum specimen was digested with 2.0 mL of Xpert sample reagent. After incubation at room temperature for 15 min, 2.0 mL of inactivated mixture was pipetted into an Xpert MTB/RIF cartridge.

For blood samples, 3 mL was tested to detect MTB using the QuantiFERON-TB Gold method according to the manufacturer's instructions. In addition, peripheral blood mononuclear cells (PBMCs) were isolated from the remaining 4 mL of blood using lymphocyte cell separation media (TBD, Tianjin, China) within 4 h of blood withdrawal. Next, PBMCs at a density of 2.5×10^6 cells/mL were stimulated with an ESAT-6-CFP-10-Rv1985c fusion protein (T), positive control phytohemagglutinin (PHA) (P), or negative control AIM-V medium (N) at 37 °C for 16–20 h. Cell culture supernatants were collected after 16–20 h of incubation then were stored at -20 °C until cytokine determination assays were performed. PBMC supernatant levels reflecting IFN- γ and IL-2 release during MTB-specific antigenic stimulation were determined using an enzyme-linked immunosorbent assay (ELISA). Values of released MTB-specific antigen-stimulated cytokines were calculated by subtracting negative control (N) values from TB antigen (T) values.

Statistical analysis

The original data were entered into a computer database using a double data entry method and the EpiData Entry data entry program (<http://www.epidata.dk/>). Data within the database were analyzed using SPSS 20.0 (SPSS, Chicago, IL, USA). Chi-square analysis was performed to investigate distributions of TB cases among various patient subpopulations stratified according to demographic characteristics across experimental groups. Student's t-test was used to analyze continuous demographic variables and cut-off values were determined using receiver operating characteristic (ROC) curve analysis. *P* values less than 0.05 were interpreted as statistically significant. All statistical calculations were conducted using SPSS version 15.0 (SPSS).

Results

Participants

Between July 2017 and December 2018 a total of 3,547 patients with symptoms suggestive of pulmonary TB were enrolled in the study. Of these cases, 293 (8.3%) were excluded from analysis based on exclusion criteria, including 85 with a history of TB diagnosis, 152 lost to follow-up, 12 who withdrew consent, 3 deaths, and 48 without available laboratory results. Ultimately 3,245 patients were included in the final analysis (Fig. 1) for which demographic and clinical characteristics of the study population are summarized in Table 1. The median age of the cohort was 52.0 years (IQR 33.0–65.0), and 61.1% of patients were male. Of 3,245 patients, 2,536 were diagnosed with active TB, including 1,092 definite TB and 1,444 clinically diagnosed TB cases. The remaining 718 non-TB cases were subclassified based on final diagnosis, with pneumonia and lung cancer the most frequently observed diseases that together accounted for 84.8% of non-TB cases. In addition, 223 (31.1%) of the 718 cases were classified as LTBI.

Table 1
Demographic and clinical characteristics of participants enrolled in this study

| Characteristic ^a | No. of participants (%) (N= 3254) |
|---|--------------------------------------|
| Median age (IQR)- yr | 52.0(33.0–65.0) |
| Male sex – no. (%) | 1989(61.1%) |
| Region | |
| Guangzhou | 901(27.7) |
| Hunan | 1044(32.1) |
| Xinjiang | 1309(40.2) |
| Classification | |
| Active TB | 2536(77.9) |
| Definite TB | 1055(33.6) |
| Clinically diagnosed TB | 1481(44.4) |
| Non-TB | 718(22.1) |
| Pneumonia | 382(11.7) |
| Lung cancer | 227(7.0) |
| Bronchiectasis | 82(2.5) |
| NTM | 27(0.8) |
| ^a IQR, inter quartile range; TB, tuberculosis; NTM, nontuberculous mycobacteria. | |

Diagnostic utility of IFN- γ and IL-2 release assays

ROC curves for IFN- γ and IL-2 release assays conducted on samples obtained from active TB cases are shown in Fig. 2. The area under the ROC curve for the IFN- γ assay was 0.859 [95% confidence interval (95% CI): 0.842–0.875] and for the IL-2 assay was 0.865 (95% CI: 0.851–0.879). Optimal cut-off values for IFN- γ and IL-2 results were determined to be 7.38 ng/L and 20.19 ng/L, respectively. Based on these cutoff values, rates were calculated for assay sensitivity, specificity, positive predictive value, and negative predictive value and are summarized in Table 2. Overall sensitivity and specificity rates of the IFN- γ assay were 83.8% (95% CI: 82.2–85.2) and 81.5% (95% CI: 78.4–84.2), respectively. As compared to IFN- γ assay rates, the IL-2 assay specificity rate was higher (94.3%; 95% CI: 92.3–95.8) while the sensitivity rate was lower (72.6%; 95% CI: 70.8–74.3).

Table 2

Diagnostic accuracy of the MTB antigen-stimulated IFN- γ and IL-2 for diagnosis of active tuberculosis

| Cytokine | AUC (95% CI) | Cut-off value (pg/mL) | Sensitivity (%, 95% CI) | Specificity (%, 95% CI) | PPV (%, 95% CI) | NPV (%, 95% CI) |
|---------------|------------------------|--------------------------|----------------------------|----------------------------|---------------------|---------------------|
| IFN- γ | 0.859(0.842– 0.875) | 7.38 | 83.8(82.2– 85.2) | 81.5(78.4– 84.2) | 94.1(93.0– 95.0) | 58.7(55.5– 61.7) |
| IL-2 | 0.865(0.851– 0.879) | 20.19 | 72.6(70.8– 74.3) | 94.3(92.3– 95.8) | 97.8(97.0– 98.4) | 49.3(46.6– 52.0) |

Abbreviation: AUC, area under curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Diagnostic utility of combination assays to detect both IFN- γ and IL-2 release

We further assessed the diagnostic value of combining detection of IFN- γ and IL-2 into a single assay for use in diagnosing active TB cases. As shown in Table 3, the highest sensitivity rate was obtained for a parallel-type combination test that detected both IFN- γ and IL-2 release, with a sensitivity rate of 87.9% (95% CI: 86.5–89.1) and overall specificity rate of 79.8% (95% CI: 76.6–82.6). Meanwhile, use of a series-type combination assay for IFN- γ and IL-2 correctly identified 689 of 718 non-TB cases, for a specificity rate of 96.0% (95% CI: 94.2–97.2), while providing an overall sensitivity rate of 68.5% (95% CI: 66.6–70.3). Notably, the sensitivity rate of the series-type combination assay for testing of definite TB cases (72.1%, 95% CI: 69.3–74.8) was significantly greater than the corresponding rate for clinically diagnosed TB cases (65.8%, 95% CI: 63.3–68.2; $P = 0.001$). Finally, of the 1,055 definite TB patients, 579 (54.9%) had smear-negative results. Sensitivity rates for combination assays for this population were: 73.1% (95% CI: 68.8–77.0) for series-type and 90.1% (95% CI: 87.0–92.6) for parallel-type combination assays.

Table 3

Diagnostic accuracy of the combined INF- γ and IL-2 for diagnosis of active tuberculosis cases

| Classification | Series combination of INF- γ and IL-2 | | Parallel combination of INF- γ and IL-2 | |
|---|---|----------------------|---|----------------------|
| | n/N | Estimate (95% CI) | n/N | Estimate (95% CI) |
| Sensitivity for active tuberculosis | | | | |
| All active tuberculosis | 1736/2536 | 68.5(66.6–70.3) | 2228/2536 | 87.9(86.5–89.1) |
| Definite tuberculosis | 761/1055 | 72.1(69.3–74.8) | 942/1055 | 89.3(87.2–91.1) |
| Smear-positive tuberculosis | 413/579 | 71.3(67.4–74.9) | 513/579 | 88.6(85.7–91.0) |
| Smear-negative tuberculosis | 348/476 | 73.1(68.8–77.0) | 429/476 | 90.1(87.0–92.6) |
| Clinically diagnosed tuberculosis | 975/1481 | 65.8(63.3–68.2) | 1286/1481 | 86.8(85.0–88.5) |
| Specificity for active tuberculosis | | | | |
| Active tuberculosis excluded | 689/718 | 96.0(94.2–97.2) | 573/718 | 79.8(76.6–82.6) |
| Active tuberculosis and LTBI excluded | 495/495 | 100.0(99.0–100.0) | 470/495 | 94.9(92.5–96.6) |
| Predictive values for all tuberculosis | | | | |
| Positive predictive value | 1736/1765 | 98.4(97.6–98.9) | 2228/2373 | 93.9(92.8–94.8) |
| Negative predictive value | 689/1489 | 46.3(43.7–48.8) | 573/881 | 65.0(61.8–68.2) |

Discussion

Current tuberculosis diagnostic testing remains challenging despite years of development [3]. Poor sensitivity of tests based on conventional culture and molecular diagnostic methods highlights the need for novel diagnostics geared toward diagnosing active tuberculosis patients, especially those with paucibacterial infections [17]. In this study, we developed a new immunological diagnostic method that can differentiate between active TB and other pulmonary diseases based on cytokine release responses when patient PBMCs were exposed *in vitro* to MTB antigen. The sensitivity rate of the parallel-type combination assay for IFN- γ and IL-2 release was 87.9% as compared to the rate of 83.8% obtained using

an assay based only on IFN- γ release. The greater sensitivity rate of the combination assay over that of the single release assay mainly stemmed from the addition of testing for IL-2 release as a parallel diagnostic marker. Importantly, this disparity also reflects the fact that approximately 16% of active TB cases had negative IFN- γ results, a quarter of which yielded positive IL-2-release results. Similarly, a recent systematic review provided an estimated pooled sensitivity rate for QFT-GIT results of 81% for active TB cases [8] and aligned with previous studies attributing low sensitivity and false-negative IGRA test results to immunosuppression [8]. By contrast, based on positive control results (PBMCs stimulated with phytohemagglutinin) as an indicator of a patient's immune response, we did not identify immunosuppression as a risk factor for false-negative results obtained for active cases. Nevertheless, a potential explanation for negative IGRA results may relate to the spatial compartmentalization of T cells in the body during infection [18]. Considering that peripheral blood T cells are isolated from blood in order to measure IFN- γ levels via *in vitro* IGRA assays, such assays may miss TB antigen-specific T cells previously recruited to infection sites from the blood during the initial course of TB disease; this loss of blood antigen-specific T cells may thus explain the negative IGRA results we obtained for early TB cases.

Of note, we failed to detect IFN- γ release in a small number of active cases that harbored a small proportion of MTB-specific T cells that only secreted IL-2. In line with this observation, an experimental study on the dynamic relationship between IFN- γ and IL-2 profiles during the natural course of human tuberculosis demonstrated that newly detectable CD4 + T cells secreting only IL-2 were present during and after treatment [19]. This finding was potentially valuable as it could be used for differentiating between active TB and LTBI. In this work, we conducted a detailed analysis of MTB-specific cytokine excretion and confirmed that IL-2 release was more specifically associated with active TB cases than was IFN- γ release. During the course of chronic TB infection, MTB-specific IL-2-secreting T cells exhibiting high expression of PD-1 have been shown to emerge during the late phase of the disease that do not secrete IFN- γ [20]. The emergence of such cells may serve as a plausible explanation for the higher specificity observed for IL-2 versus IFN- γ release for assays used for detecting active TB. In such cases, series-type combination testing for both IFN- γ and IL-2 release provided increased specificity (96.0%) that was comparable to that obtained using molecular diagnostic testing of culture-positive cases. Thus, the series-type combination assay was more effective than other cytokine release tests evaluated here for diagnosing culture-negative cases.

In view of the diagnostic utility of combining IFN- γ and IL-2 release assays, we modeled false-positive and false-negative results obtained for active TB cases against the variable prevalence of active TB (Fig. 3) then we proposed two diagnostic algorithms suitable for different hospital settings. For general hospitals in China which are in charge of screening suspected tuberculosis cases, approximately 10% of patients with symptoms suggestive of TB are ultimately diagnosed with active TB [21]. In this type of clinical setting, use of parallel-type combination release assays with higher sensitivity would help clinicians identify more patients at high risk of active TB than would conventional smear microscopy. Meanwhile, in TB-specialized hospital settings the proportion of active TB cases of the total TB suspected cases approaches 50% [22], justifying use of the series-type combination assay to achieve

earlier diagnosis of active TB patients and prevent administration of toxic treatments with limited clinical efficacy.

Another interesting finding of our study is the association between diagnostic accuracy and severity of disease in the population. The sensitivity rate of the series-type combination assay when used for testing of definite TB cases was significantly higher than the rate obtained for clinically diagnosed cases; this result aligns with results of a report by Chee and colleagues indicating that a quantitative T cell response is positively associated with mycobacterial burden and disease activity [23]. However, conflicting results reported for another experimental study ruled out any clear correlation between antigen burden and T cell response [24]. Nevertheless, although our combination assay results for active TB patients revealed enhanced *in vitro* IFN- γ /IL-2 release with increasing MTB-specific antigen dose, further research is needed to investigate the dynamics of MTB-specific IFN- γ /IL-2 secretion during anti-TB treatment.

This study had several obvious limitations. First, despite strict diagnostic criteria, some patients afflicted with respiratory illness may have been improperly diagnosed as active TB cases due to lack of laboratory support. Such a diagnostic error may have negatively impacted the reliability of our study conclusions. Second, although comorbidities such as diabetes and immunosuppression status are considered risk factors for negative immune responses, comorbidity data were not collected here and thus were not considered in our evaluation of the clinical performance of our immunological assays. Third, we only evaluated the diagnostic utility of combination assays for pulmonary TB cases and should therefore confirm whether these combination tests would be effective for diagnosing extrapulmonary TB as well.

Conclusions And Potential Impact

In conclusion, here we developed a new immunological diagnostic method to differentiate between active TB and other pulmonary diseases. Our data demonstrated that various IFN- γ /IL-2 combination assays hold promise as effective alternatives to current tests used to diagnose active TB cases in different settings. In addition, the diagnostic accuracy of a series-type combination assay correlated with disease severity in our patient cohort. Further research is needed to investigate the dynamics of MTB-specific IFN- γ /IL-2 release during anti-TB treatment.

Abbreviations

CI: confidence interval; ELISA: Enzyme-linked immunosorbent assay; IFN- γ : interferon- γ ; IL-2: interleukin-2; IGRAs: interferon- γ release assays; LTBI: latent tuberculosis infection; MTB: Mycobacterium tuberculosis; OR: odds ratio; PHA: phytohemagglutinin; PBMCs: peripheral blood mononuclear cells; ROC: receiver operating characteristic; TB: tuberculosis; Th-1: type 1;

Declarations

Consent for publication

Not applicable.

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Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contributions

All listed authors meet the requirements for authorship. JL and YP conceived and designed the study. PG, ZC, QL and YY collected clinical information and samples. HK, QW, ZY and DA analysed and interpreted the data. YT, YT, JL and PH wrote the manuscript. All authors critically reviewed and approved the final manuscript.

Ethics approval and consent to participate

This research complies with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Guangzhou Chest Hospital. Written informed consent was obtained from all individuals prior to enrollment.

Conflict of interest

All authors declare they have no conflicts of interest.

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Figures

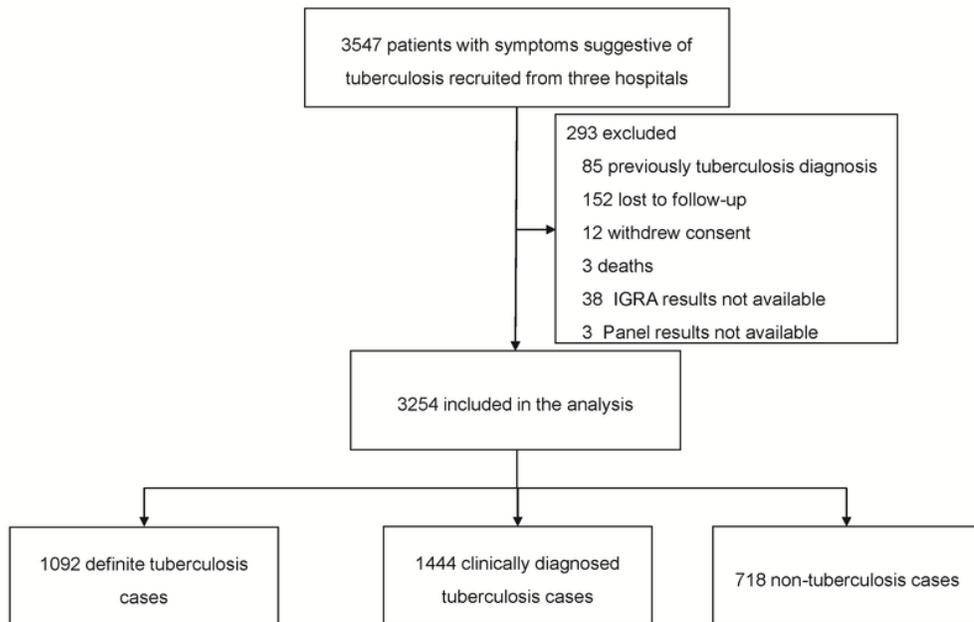


Figure 1

Enrolment of patients with symptoms suggestive of pulmonary tuberculosis

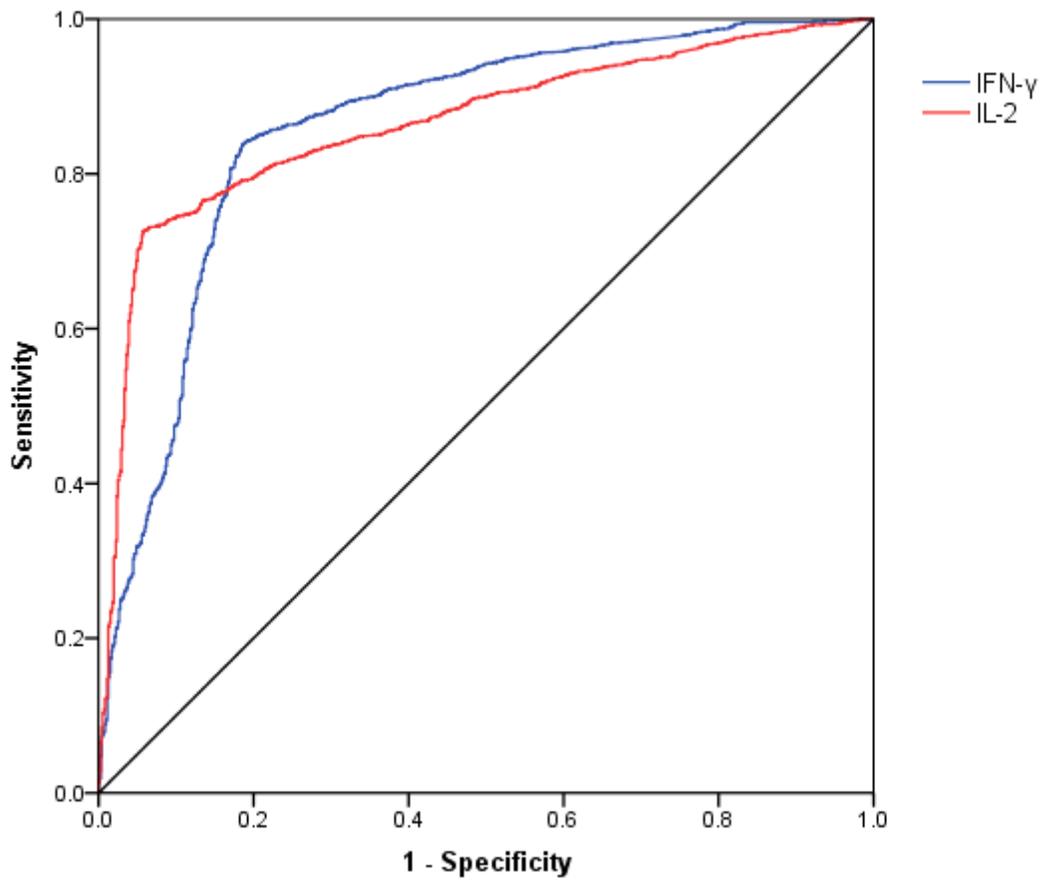
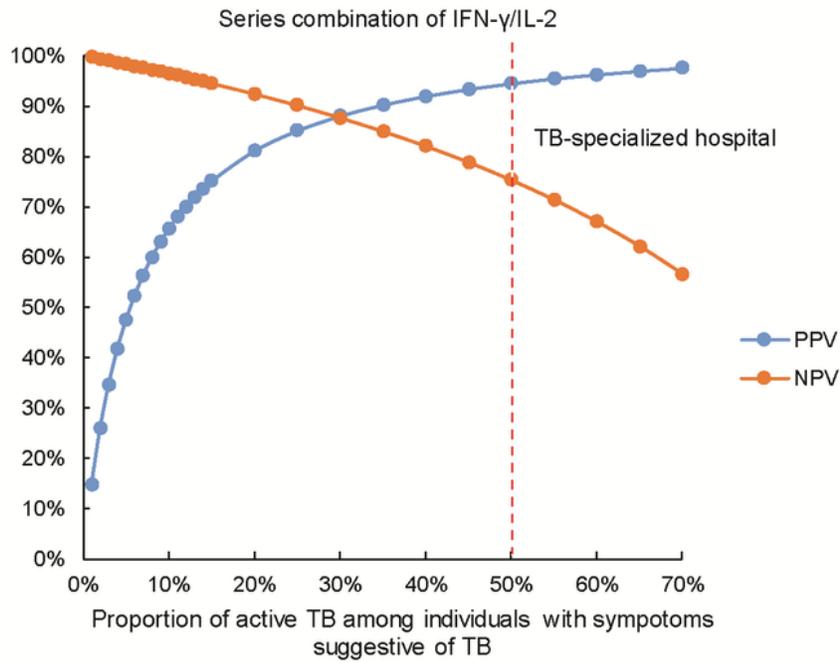


Figure 2

ROC curve of IFN- γ /IL-2 for differentiating active TB from non-TB

A



B

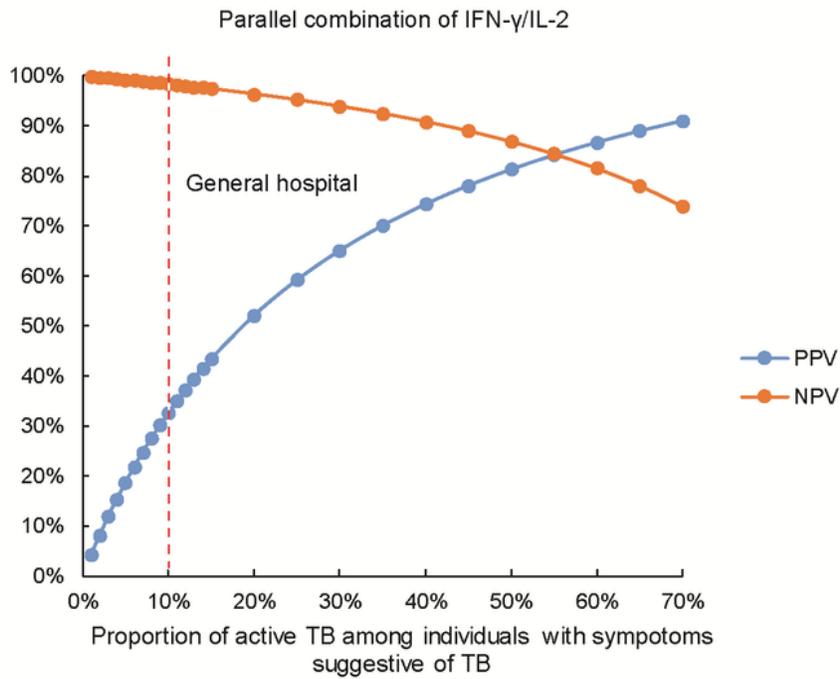


Figure 3

Positive predictive value and negative predictive value for TB detection using parallel and series combination of IFN- γ /IL-2 according to varying proportions of active TB among individuals with symptoms suggestive of TB