RESEARCH ARTICLE

Interferon Gamma Release Assay in response to PE35/PPE68 proteins: a promising diagnostic method for diagnosis of latent tuberculosis

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ABSTRACT. Tuberculosis control relies on the identification and preventive treatment of people who are latently infected with *Mycobacterium tuberculosis* (Mtb). PE/PPE proteins have been reported to elicit CD4 and/or CD8 responses either in the form of whole recombinant proteins or as individual peptides. Very few of the PE and PPE proteins have been previously tested for responses in patients with TB and healthy donors. This is the first study to evaluate the Interferon Gamma Release Assay (IGRA) after stimulation with PE35 and PPE68. The antigen-specific levels of IFN-γ following stimulation with QuantiFERON-TB gold in-tube (QFT-G-IT) antigens, and PE35 and PPE68 recombinant proteins were evaluated in 79 children and 102 adults, respectively. Using QFT-G-IT kit, latent tuberculosis infection (LTBI) was detected in 26 children (33%) and 41 adults (40%); IGRA following stimulation with PE35 and PPE68 recombinant proteins, was positive, respectively, in 36 (46%) and 32 (40.5%) children, respectively. In addition, 53 adults (52%) had positive results following stimulation with these two proteins. The sensitivity and specificity of IGRA following stimulation with recombinant PE35 in children were 76% and 80%, and following stimulation with recombinant PPE68 in this group, it was 73% and 75%, respectively. Meanwhile, there is no gold standard test for LTBI. Our designed tests using PE35 and PPE68 PE/PPE proteins, two PE/PPE proteins not present in BCG vaccins, which elicit CD4 and/or CD8 responses, might be helpful for rapid diagnosis of TB and improve the detection of LTBI. However, further validation studies to determine the advantage of IGRAs using these proteins, alone or combined, are highly recommended.

Key words: Interferon Gamma Release Assay, diagnostic method, PE35, PPE68

Worldwide, tuberculosis (TB) remains as one of the most deadly infectious diseases [1]. It is estimated that two billion people are latently infected with *Mycobacterium tuberculosis* (*Mtb*), and this huge reservoir might cause the pan/epidemic spread of the bacterium [2].

TB control relies on the identification and preventive treatment of people who are latently infected with *Mtb* [3]. Early diagnosis and treatment of latent tuberculosis infection (LTBI) are considered to be the most effective strategies for reducing the incidence of TB in the population. Children are particularly vulnerable to the development of severe disease and death following TB infection, and those with LTBI may serve as a reservoir for future transmission of TB disease [4].

There is no way to directly detect the presence of LTBI. The diagnosis of LTBI relies on the measurement of host immune responses to *Mtb* antigens. Until the early 2000s, the only test available for LTBI was the tuberculin skin test (TST). Then, two diagnostic tests for LTBI have been approved: QuantiFERON-TB gold in-tube (QFT-G-IT) (Cellestis, Chadstone, Australia) and the T-SPOT.TB test (Oxford Immunotec Inc., Marlborough, MA, USA). Recently, QuantiFERON-TB Plus (QFT-Plus, QIAGEN), a new generation QFT-G-IT test, was introduced as a key milestone in the development of diagnostic tests for LTBI [5]. It includes two tubes: TB1 tube contains ESAT-6-derived and CFP-10-derived peptides (TB 7.7, present in QFT-G-IT, has been removed), which elicit cell-mediated immune responses from CD4+ T-helper lymphocytes, and TB2 tube, which contains shorter peptides and stimulates IFN-γ production by both $CD4^+$ and $CD8^+$ T-cells [5].

In our previous study, we had reported the potential role of novel proteins of the PE/PPE family (*PE35/PPE68*) in the diagnosis of TB [6]. These genes code for conserved N-terminal proline–glutamate (PE) and proline–proline–glutamate (PPE) motifs [7]. PE/PPE proteins display numerous repetitive sequences and possess abundant immunogenic regions, representing a rich source of B- and T-cell epitopes [8]. At least 20 PE/PPE proteins have been reported to elicit CD4 and/or CD8 responses either in the form of whole recombinant proteins or as individual peptides [9].

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Although very few PE and PPE proteins have previously been tested for responses in patients with TB and healthy donors, this is the first study in which the Interferon Gamma Release Assay (IGRA) was evaluated after stimulation with PE35 and PPE68.

MATERIAL AND METHODS

Protein expression of recombinant PE35 and PPE68

The pET32a(+)-*PE35* and pET32a(+)-*PPE68* construct plasmids [6] were transformed into *E. coli BL21 DE3* (Novagen, Germany) expression host [10]. Proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Purification of recombinant PE35 and PPE68

Recombinant PE35 and PPE68 were purified by nickel-nitrilotriacetic acid (Ni_2^+-NTA) metal affinity chromatography according to the manufacturer's recommendations for purification of proteins under soluble conditions (Qiagen, USA) [10]. After purification, the pure fractions of protein were pooled together and were dialyzed against 10 mM Na_2HPO_4, pH 8.0. The dialyzed protein was aliquoted and kept at -20 °C. Endotoxins were removed using Triton X-114 according to the method previously described [11].

In this cross-sectional study, children who were referred to the Children Medical Center Hospital, an Iranian referral hospital, with their patents/guardians were enrolled. We excluded those who were immunodeficient. All participants had received the BCG (Bacillus Calmette-Guérin) vaccination either in infancy or during childhood. Written informed consent and completed questionnaires were obtained from the parents/guardians. The study received approval from the Ethical Committee of Tehran University of Medical Sciences (100676).

Whole blood stimulation and IFN-γ release assay

About 5 ml of whole blood was collected from all the study participants into heparinized tubes, and another 3 ml of blood was collected directly into QFT-G-IT.

Whole blood stimulation and measurement of IFN-γ levels were performed using QFT-G-IT as per the manufacturer' instructions.

The recombinant PE35 and PPE68 proteins were evaluated at a final concentration of 5 μg/mL by a 3-day whole blood assay [6]. The positive control was phytohemagglutinin (PHA) and was used at a final concentration of 5 μg/mL. Cultures were incubated at 37 °C with 5% CO_2. At all sites, supernatants from each well were harvested on day 3 and stored at -20 °C before testing using ELISA.

IFN-γ was measured in the supernatant using Human IFN-γ ELISA kit (Mabtech AB, Sweden) in plasma supernatants (kept at -20 °C) after stimulation with PE35 and PPE68 proteins. All the plasma specimens were tested in duplicate and expressed in pg/mL. QFT tests were regarded as positive if the antigen-stimulated response of IFN- (TB Ag minus nil) was $\geq$0.35 IU/mL and $\geq$25% of nil value (17.5 pg/mL) or negative if the mitogen-stimulated response (mitogen minus nil) was $\geq$0.5 IU/mL (25 pg/mL) and the antigenstimulated response was $<$0.35 IU/mL.

Table 1
The Interferon gamma release assay (IGRA) performance characteristics in children and adults.

		Children	Adults
		N (%)	N (%)
Sex	Male	59 (75)	29 (28)
	Female	20 (25)	73 (72)
Age (years)		8.3 ± 2.7	34.6 ± 5.9
Results of IGRA test using QFT-G-IT antigen	Positive	26 (33)	41 (40)
	Negative	54 (68)	61 (60)
IGRA using PE35 protein	Positive	36 (46)	53 (52)
	Negative	43 (54)	49 (48)
IGRA using PPE68 protein	Positive	32 (40.5)	53 (52)
	Negative	47 (59.5)	49 (48)

Statistical analysis

Receiver operating characteristic (ROC) curve analysis was conducted to determine the best IFN-γ result thresholds in the diagnosis of LTBI, relatively to specific *Mtb* antigens PE35 and PPE68, and the corresponding sensitivity and specificity were reported. The area under the ROC curve (AUC) and 95% confidence interval (CI) were also calculated. Cutoffs were estimated at various sensitivities and specificities and at the maximum Youden's index (YI), that is, sensitivity − specificity − 1 [12]. IFN-γ concentrations was determined using concentration of antigen-stimulated culture minus the concentration of the respective control plasma.

Statistical analysis was performed using the statistical software STATA 11 (StataCorp, College Station, TX, USA), and p value $<$0.05 was considered statistically significant.

RESULTS

In this study, recombinant PE35 and PPE68 were expressed successfully and used as *Mtb* stimulators. The antigen-specific levels of IFN-γ following stimulation with QFT-G-IT antigens and PE35 and PPE68 recombinant proteins were evaluated in 79 children and 102 adults. The mean age of the enrolled children and adults was 8.3 ± 2.7 and 34.6 ± 5.9 years, respectively (*table 1*).

According to the results obtained from GFT-G-IT tests, LTBI was detected in 26 children (33%) and 41 adults (40%); IGRA following stimulation with PE35 and PPE68 recombinant proteins was positive in 36 (46%) and 32 (40.5%) children, respectively. In addition, 53 (52%) adults had positive results following stimulation with these two proteins (*table 1*).

According to ROC curve analysis, the results suggest that PE35 (AUC = 0.72) and PPE68 (AUC = 0.74) are antigens that stimulate T-cells of children with LTBI (*figures 1 and 2*). The best cutoff points for these two proteins were 17.36 and 29.7 pg/mL, respectively (*table 2*).

The median level of IFN-γ following stimulation with QFT-G-IT antigens and PE35 and PPE68 recombinant proteins in different situations (QFT-G-IT positive/negative; IGRA with PE35 positive/negative and IGRA with PPE68 positive/negative) in children and adults based on the mentioned cutoff (*table 2*) are indicated in *table 3*.

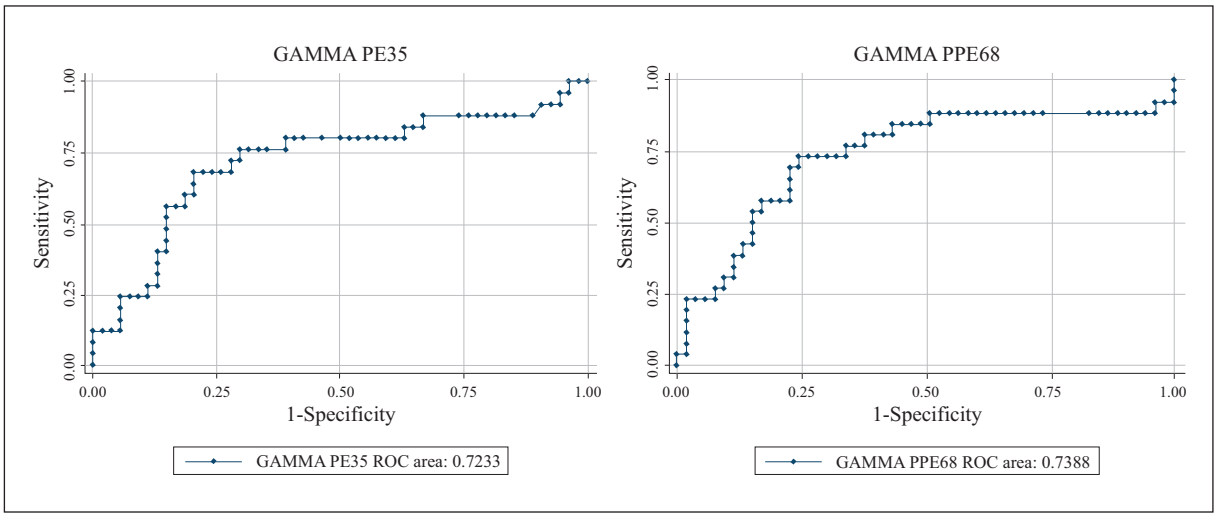

Figure 1
A receiver operator characteristic (ROC) plot illustrating sensitivity and specificity of IGRA using PE35 (left panel) and PPE68 proteins (right panel) for the detection of LTBI in children.

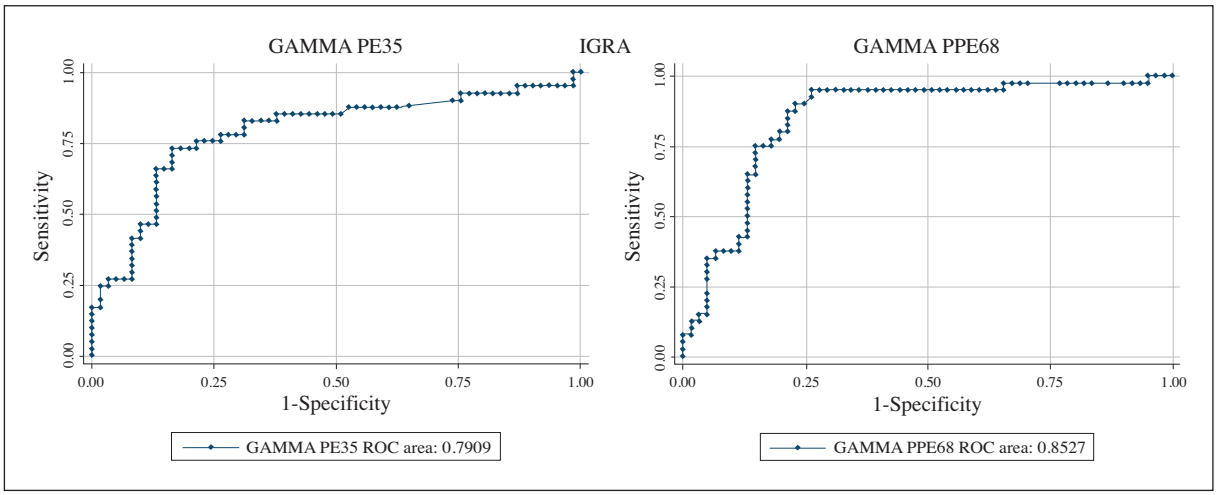

Figure 2
A receiver operator characteristic (ROC) plot illustrating sensitivity and specificity of IGRA using PE35 (left panel) and PPE68 proteins (right panel) for the detection of LTBI in adults.

The sensitivity and specificity of IGRA following stimulation with recombinant PE35 in children were 76% and 80%, respectively. In addition, the sensitivity and specificity following stimulation with recombinant PPE68 in this group were 73% and 75%, respectively. In adults, PE35 and PPE68 antigens presented the AUC values of 0.79 and 0.85, respectively. The highest sensitivity and specificity were obtained at cutoff 11.7 pg/mL for PE35 and 23.7 pg/mL for PPE68 antigen, respectively (sensitivity 83% and specificity 69% for PE35 and sensitivity 92.5% and specificity 74% for PPE68) (*table 3*).

Using PE35 and PPE68 proteins, six (6/25, 24%) and seven (27%) samples of children yielded negative results with IGRA, respectively, whereas the results of these samples using the conventional QFT-G-IT were positive. On the other hand, 17 patients (17/54, 31.5%) and 13 (13/53, 24.5%) children had positive results with IGRA using PE35 and PPE68 proteins, respectively,whereas their results using the conventional QFT-G-IT were negative (*table 4*).

In adults, three patients (3/40, 7.5%) had positive result with the conventional QFT-G-IT, whereas IGRA using PPE68 protein showed negative result. Moreover, seven (7/41, 17%) patients were negative for QFT-G-IT test but had positive results following stimulation with PE35 (*table 4*).

DISCUSSION

The immunology of TB is complex, and understanding of LTBI is even more challenging; therefore, investigations of a rapid and specific diagnosis method for LTBI are highly recommended. IGRA tests are mainly recommended for increasing the test specificity using the TST in children who have received a BCG vaccine, and for increasing sensitivity for diagnosing LTBI in patients at high risk of developing progression from LTBI to active TB disease [13, 14].In the last decade, development of *in vitro* immunodiagnostic tests helped to overcome the deficiencies of the TST [15-17]. Because IGRAs identify the cellular immune response to *Mtb* by quantifying the release of IFN-γ after *in vitro* stimulation of T-cells with *Mtb*-specific antigens [18], the identification of regions of the *Mtb* genome that

are not present in BCG provides a unique opportunity to develop new highly specific diagnostic reagents [6, 19].

There are no large-scale published data regarding the interferon-γ release assay for PE/PPE antigens. Although they are of great interest as future candidate molecules for specific T-cell-based diagnosis of *Mtb* infection, they have not been evaluated for this purpose. In this study, we evaluated the diagnostic performance of IGRAs following stimulation with PE35 and PPE68.

According to our results, the sensitivity of IGRA following stimulation with PE35 and PPE68 was higher in adults than children (sensitivity 83% vs. 76% with PE35 and 92.5% vs. 73% with PPE68, respectively). However, the specificity of IGRA following stimulation with PPE68 was the same in both adults and children, and the specificity of this test decreases in response to PE35 in adults (80% in children and 69% in adults). In addition, the positive predictive value (PPV) of the test in response to both proteins was higher in adults (PPV 70% and 64% in adults vs. PPV 53% and 59% in children). The negative predictive value (NPV) of both IGRAs in response to PE35 and PPE68 was more than 85% in both children and adults.

Meanwhile, there is no gold standard test for LTBI; all of the available tests might have limitations. In this study, we compared our results with those of QFT-G-IT; therefore, there is possibility of invalid results with this kit as well.

Recently, discrepancies between the conventional QFT-G-IT and the new-generation QFT-Plus were reported [5, 20]. In the study of Hoffmann et al., 67 (67/163, 41.1%) and 70 (42.9%) samples yielded positive results with the conventional QFT-G-IT and the new QFT-Plus, respectively, and QFT-G-IT gave invalid results in two patients (1.2%). These two tests showed discrepant results for nine patients, three (3/9; 33.3%) of those having been positive for QFT-G-IT but negative for QFT-Plus and six (6/9; 66.7%) having been negative for QFT-G-IT but positive for QFT-Plus [20]. QFT-Plus produced 87.9% (95% CI 0.81-0.93) true-positive results [20], which revealed increased sensitivity compared to the estimates of 80% (95% CI 0.75-0.84) for QFT-G-IT, which was reported in the recent meta-analysis [4, 6]. The possible reason for this discrepancy might be the stimulation of *Mtb*-specific $CD8^+$ T-cells [5]. QFT-Plus contains two tubes: TB1 tube of this kit elicits a $CD4^+$ T-cell response, whereas the TB2 tube elicits both $CD4^+$ and $CD8^+$ T-cell responses.

In another report, comparison of the QF-T-GIT and the QFT-Plus demonstrated agreement in 68 results among 73 cases. Four patients were positive only with QFT-Plus (three of them scored positive for the TB_2 antigens only), and one patient was QFT-G-IT positive but QFT-Plus indeterminate [5].

In the study of Barcellini *et al.*, a significant difference in IFN-γ response between the two tubes of QFT-Plus test was found [5]. In our study, we observed high level of IFN-γ following stimulation with PE35 and PPE68. This difference might be due to the additional $CD8^+$ T-cell stimulation. Our designed tests using PE35 and PPE68 PE/PPE proteins elicit CD4 and/or CD8 responses and might be advantageous for improving the assay's accuracy in patients with low $CD4^+$ T-cell counts [5].

In conclusion, this study demonstrated that PE35 and PPE68 can provide a diagnostic method for diagnosis of LTBI in adults and children. However, further validation studies to determine the advantage of IGRAs using these proteins, alone or combined, to classify *Mtb* infection, are highly recommended. A cocktail of a number of such defined T-cell antigens could form the basis of new T-cell-based *in vitro* blood tests for *Mtb* infection that does not cross-react with BCG. Such tests could help with the rapid diagnosis of TB and might improve the detection of LTBI.

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