Development of an Improved Whole Blood Assay for Diagnosis of Latent and Active Tuberculosis Cases

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Authors’ contributions
This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Latent TB infection (LTBI) is an infection where the presence of disease causing organism M. tuberculosis is there without any sign and symptoms of the disease hence mostly remains undiagnosed, though Tuberculin skin test (TST) and Interferon Gamma Release Assay (IGRA) were used to diagnose the LTBI. They have their limitations, TST gives major cross-reactivity with BCG vaccine and gives inaccurate results in individuals who have taken BCG and IGRA are very costly and variable sensitivity is repeated in various populations hence the modifications are needed in the IGRA for proper diagnosis of LTBI.

Objectives: In the proposed study we aimed to develop an improved whole blood assay towards a diagnosis of latent and active TB infection as an alternative to the Quantiferon QFT assay

Methodology: Synthetic antigenic peptides against latency specific antigens will be designed and synthesized. Designed peptides will be screened for LTBI specific cytokine by in-vitro experiments. Development & production of Whole assay using selected peptides evaluation of developed assay through ELISA in clinical samples.

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1. INTRODUCTION

Latent TB Infection (LTBI) is an infection where the presence of disease-causing organism Mycobacterium tuberculosis is there without any sign and symptoms of the disease [1,2]. Tuberculin skin test (TST) is a standard diagnostic test for LTBI [3]. In this test, an intradermal injection of purified protein derivative (PPD) is given to the patient & hypersensitivity response in the form of cutaneous induration was measured at the site of injection at 48-72 h. However, it was reported to give a false positive result among BCG vaccinated individuals [4-6].

Similarly, Interferon-Gamma Release Assay (IGRAs) is an advanced immunological tube assay, which measures the Interferon-Gamma level in the blood samples of the patient in response to challenge with tuberculosis antigens [7-9]. In earlier studies the prevalence of LTBI observed according to QFT-G is of around 48% in India [10,11]. However variable sensitivity of IGRA is reported for diagnosis of LTBI [12,13] so, there is also need of some improved tests for diagnosis of TB infection [14,15].

The main objective of the present study is to develop an improved blood assay for the diagnosis of LTBI infection. In brief, first will design and synthesis the synthetic antigenic peptides against latency-specific antigens. Designed peptides will be screened by In-vitro experiments after which development & evaluation of whole blood assay will be done using selected peptides (Schematic plan is given in Fig. 1).

2. METHODOLOGY

Study site: Samples for the study will be collected from OPD patient enrolled at CIIMS hospital Nagpur, Wards of CIIMS hospital Nagpur, and through camps conducted by CIIMS at the periurban areas of Nagpur as well as Melghat Amravati with help of the satellite center of CIIMS, i.e. Dr. G.M Taori Tribal Health research center Melghat, Amravati, (M.S) India. Thus the Place of the study will include Nagpur, & Melghat.

Study Participant: All participants will be recruited using predefined inclusion-exclusion criteria. There will be three major groups of participants for sample collection as follows:

- Active TB subjects (n=62)
- House hold contacts (latent cases) (n=89)
- Healthy controls (n=96)

Samples: Blood samples (6-8ml) in sterile EDTA tubes and QFT tubes (1 to 2 ml each tube)

3. INTERFERON-γ RELEASE ASSAY

Interferon-γ release assay (IGRAs) is blood tests that detect the level of Interferon gamma (IFN-γ) produced after treatment with sample after stimulation with 2 or 3 M. tuberculosis-specific proteins. [16] (Table 1).

3.1 Material Required

- Specimen: 1 to 2 ml of blood
- Kit component: QFT tubes (Nil tube, TB Antigen tube, Mitogen tube)
- Instrument required: ELISA reader, Incubator, Computer for result analysis, etc

3.2 Procedure

1. Take 1 ml blood in every tube
   - Nil tube (without any antigen)
   - Antigen tube (TB 7.7, CFP-10 and ESAT-6.)
   - Mitogen tube (control tube)

2. Incubate tubes for 16 to 18 hrs at 37°C.
3. Remove plasma & Store at -20°C for INF estimation
4. IFN-γ will be estimated as per kit protocol (Note: Above 0.35IU/ml is considered as a positive QFT result)

Keywords: M. tuberculosis; tuberculosis; latent TB infection; IFN-γ; QFT; peptide.
5. Positive and Negative outcomes will be defined by adding the IFN-γ level in the software provided with the kit. (As shown in Fig. 2)

3.3 Caution

1. Blood samples should be stored at 4°C.
2. Do not use the haemolysed sample for the QFT analysis.

3.4 Expected Outcomes

IGRA done in the subjects will give the idea about the subjects to be included in the latent category based on the positivity as described in inclusion-exclusion criteria and history.

4. TUBERCULIN SKIN TESTS (TST)

Tuberculin Skin Test (TST) is been developed and provided by MDH (Minnesota Department of Health). TST is a test used for the detection of M. tuberculosis. The test will be performed and used for the identification of sample groups i.e. Positive, Latent, and control groups [17] (Table 2).

4.1 Materials Required

- 10 TU of PPD
- Sterile 1ml TB syringe (27G x 1/2 needle) (Note: Do not use Insulin syringe)
- Disposable gloves
- Alcohol Swabs
- Discarder

4.2 Procedure

1. On the inner surface of the forearm inject 0.1 ml of tuberculin (PPD)
2. After 48 to 72 hrs Read skin test reaction (Fig. 3)

4.3 Expected Outcomes

1. ≤10mm induration in the injected site – Negative
2. Indurations is ≥ 10 mm within 72 hours - Positive result

4.4 Caution

1. No indurations – Re-inject after 7 days
2. Do not measure erythema (redness).

6. DESIGNING ANTIGENIC PEPTIDES AGAINST LATENCY SPECIFIC ANTIGENS OF M. tuberculosis

For designing of antigenic peptides against latency specific antigens of M. Tuberculosis.

5.1 Procedure

1. The sequence of Latency specific protein (Rv1978, Rv2623, Rv2029, Rv3133, Rv2624c, Rv2626c, and Rv2628, etc) will be extracted from proteomic data repository for pathogenic mycobacteria i.e. Mycobrowser (Mycobacterial browser) (https://mycobrowser.epfl.ch/)
2. The antigenic peptide in each targeted protein molecule will be predicted using the immune epitope database (IEDB) website

- Latent TB specific protein sequence will be taken from mycobrowser will be then entered to predict T cell epitopes for MHC class I and II binding.
- The IEDB recommended prediction method (Default method) will be used for the prediction of the antigen.
- The results will be obtained from the iedb.org via email.

Table 1. Troubleshooting of Interferon-γ release assay

<table>
<thead>
<tr>
<th>Step</th>
<th>Possible problem</th>
<th>Possible reason</th>
<th>Possible solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>IGRA may give false positive result</td>
<td>Client might have received any live vaccines</td>
<td>Delay testing until 4 weeks after live vaccine was received</td>
</tr>
</tbody>
</table>

Table 2. Troubleshooting of Tuberculin Skin Tests (TST)

<table>
<thead>
<tr>
<th>Step</th>
<th>Possible problem</th>
<th>Possible reason</th>
<th>Possible solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Potency of the PPD may be diminished</td>
<td>Preload of syringes for later use</td>
<td>Drawing up the solution just before injecting</td>
</tr>
</tbody>
</table>
3. The results of several peptides with information like rank, score, start, and endpoint along with the length of peptides will be obtained.
4. As per IEDB recommendation, the peptides with less than 1 rank will be filtered for the further analysis.
5. Repeated/Duplicate peptides will be removed from the obtained results sheet.
6. These peptides will be then selected for checking the MHC Class I and II immunogenicity scores by the use of the IEDB website in the epitope analysis resource section.
7. The top 25% peptides with the highest immunogenicity score will be then selected from each protein set.
8. By applying all the above parameters, peptides will be selected for the blast analysis.
9. The peptide, which will be found non-specific in Blast analysis, will be excluded, and the remaining peptide will be sent for synthesis.

6. SYNTHESIS OF PEPTIDE

Based on Blast result, the most potent and specific peptides will be selected and synthesize, through custom services with suitable company.

6.1 Reconstitution Synthetic Peptides Storage

1. Peptides prepared will be first dissolved in DMSO or other suitable media for cell culture Experimental purpose at a stock concentration (10mg/ml) (Main stock).
2. Stock will be stored at -20°C.
3. For experiment purposes, the sock peptide will be further diluted with PBS to get the final concentration of 1mg/ml (Working Stock).
4. The diluted peptide will be used to induced PBMC in a cell culture experiments at various concentrations.

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**Fig. 1. Systematic workflow for the LTBI study**
Fig. 2. Schematic representation of interferon gamma release assay

Fig. 3. Representation of administration pf PPD and reading TST result by measurement of induration after 72hrs

Fig. 4. Anticoagulated and diluted blood layered over histopaque and the buffy coat of lymphocytes obtained after centrifugation

(A): Anticoagulated and diluted blood layered over histopaque

(B): Lymphocytes and other mononuclear cells separate out as Buffy Coat at Plasma histopaque
6.2 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

The PBMC is consists of leukocytes which are isolated from whole blood and contain lymphocytes, monocytes, and a small percentage of some other immune cells, such as dendritic cells. PBMCs can be stimulated or treated with or drugs in-vitro [18-19]. Peripheral blood lymphocytes from heparinized blood will be isolated by gradient centrifugation using HISTOPAQUE – 1077 (Fig. 4) (Table 3).

6.3 Materials Required

6.3.1 Reagents
- Ficoll (Histopaque 1077)
- Phosphate Buffered Saline
- RPMI - 1640 Medium

6.3.2 Glass ware / plasticware
- 15 ml Falcon tubes
- Eppendorf Tubes
- Micropipette and Tips
- Tube Stand

6.3.3 Equipment
- Refrigerated centrifuge
- Light Microscope
- Laminar Air Flow

6.3.4 Procedure
1. Withdrawal 2ml of heparinized blood from a patient and dilute in 4 ml of RPMI - 1640.
2. In a 5 ml falcon tube layer blood gently over 2-3ml ficoll-histopaque
3. Spin the tube in 1878g for around 20-25 min.
4. Remove the upper layer of plasma
5. In another falcon, tube take the lymphocytes from the buffy coat (Fig. 4. A and B)
6. Wash the lymphocyte cell suspension two times with RPMI medium
7. Gently mix the lymphocytes pellet in 1 ml RPMI medium
8. Do the cell count by heamocytometer.

7. CELL VIABILITY AND CELL COUNT ASSAY

Trypan blue staining is used to check cell membrane integrity and cell viability. The isolated and washed lymphocytes are checked for viability and cell count using a heamocytometer.

7.1 Principle
The viable and living cells do not dye in Trypan blue staining as they expel the dye intruding the cell as metabolically active cells while non-viable and dead cells fail to do so and stain blue in Trypan Blue staining.

7.2 Materials Required
Reagents
- Trypan Blue dye solution (0.4%)
- Phosphate Buffered Saline
- RPMI - 1640 Medium

Apparatus & Equipment
- Eppendorf Tubes
- Micropipette and Tips
- Light Microscope
- Heamocytometer with coverslip

7.3 Procedure
1. In a 1.5ml Eppendorf tube take 100 µl of cell suspension
2. Add 800µl PBS and 100 µl of trypan blue solution in the tube and vortex it
3. In a heamocytometer with the clean coverslip on it add 10µl of Stained cell suspension
4. Count all the colorless viable cells visible in heamocytometer with a light microscope (Fig. 5) (Table 4)

The formula for cell count:

\[
\text{Cell count /ml} = \frac{\text{Average cell count in 4 chamber}}{10^{2}} \times 10^{4} \times 10^{2} \times 10^{3} \times 10^{3}
\]

If the average cell count is 50 then

\[
\text{Cell count /ml} = 50 \times 10 \times 10^{4} = 5 \times 10^{5} \text{ cell /ml}
\]

8. In vitro SCREENING OF DESIGNED PEPTIDES

PBMC will be incubated in the presence and absence of different concentrations of peptide and its cocktails.
Table 3. Troubleshooting of Isolation of Peripheral Blood Mononuclear Cells (PBMC)

<table>
<thead>
<tr>
<th>Step</th>
<th>Possible problem</th>
<th>Possible reason</th>
<th>Possible solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Buffy coat may not be observed</td>
<td>Blood sample could not have been added slowly through</td>
<td>Blood sample should be layered carefully and slowly</td>
</tr>
<tr>
<td></td>
<td></td>
<td>the wall of centrifuge tube</td>
<td>by the walls</td>
</tr>
</tbody>
</table>

Table 4. Trouble shooting of cell viability and cell count assay

<table>
<thead>
<tr>
<th>Step</th>
<th>Possible problem</th>
<th>Possible reason</th>
<th>Possible solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Improper cell count</td>
<td>Debris and other RBC in pellet are also counted</td>
<td>Only the unstained viable cells should be counted. One should be able to compare between the non-viable and viable cells properly.</td>
</tr>
</tbody>
</table>

Fig. 5. Overview of cell counting

8.1 Materials Required

- **Reagents**
  - RPMI - 1640 Medium
  - PBMC cell suspension

- **Glassware / plasticware**
  - 15 ml Falcon tubes
  - Eppendorf Tubes
  - Micropipette

8.2 Procedure

1. Cells will be cultured in 2 x 10^5 cells per well in a 96 well tissue culture plate.
2. The cells will be divided into four groups and treated as follows:
Group I: Only PBMC cell line
Group II: Cells + LPS
Group III: Cells+ LTBI specific peptide (Conc. 1)
Cells+ LTBI specific peptide (Conc. 2)
Cells+ LTBI specific peptide (Conc. 3)
Group VI: Cells+ LPS+ LTBI specific peptide (Conc. 1)
Cells+ LPS+ LTBI specific peptide (Conc. 2)
Cells+ LPS+ LTBI specific peptide (Conc. 3)

3. Incubate for 24 to 48 hours
4. Collect the supernatant & pallet and store at -20°C until further use (or proceed with the XTT assay) (Fig. 6) (Table 5)

Note
- Cell concentration for the experiment will be optimized
- LPS concentration will be used as reported in the literature
- The concentration of each peptide will be optimized

9. CELL VIABILITY ASSAY (XTT ASSAY)

9.1 Principle
In XTT assay, Mitochondrial enzymes of the live cells reduce the tetrazolium salt into orange-colored compounds of formazan dye, which directly proportional to the live cells. The dye formed is water-soluble and can be easily read at 450nm. The assay is mainly used for assessment of the cell viability and cytotoxicity, in comparison to the untreated control set (Table 6).

9.2 Reagents

**XTT Reagent:** 10 mg XTT is dissolved in 10ml warm PBS. Aliquots are prepared in vials and are stored at -20°C.[XTT-2,3bis (2-methoxy-4 nitro 5-sulphonyl) 2h-tetrazolium-5 carboxanilide sodium salt].

**Activation Reagent:** A sterile solution containing PMS (0.383 mg/ml) is prepared. Aliquots are prepared in vials and are stored at -20°C in dark. [PMS – N-methyl dibenzopyrazine methyl sulfate]

9.3 Procedure
1. Culture 2 X 10^5 Cells/200 µl in 96 microwell plate.
2. Defrost XTT reagent solution and activation solution just before use.
3. Prepare reaction solution by adding 50µl of PMS to 950µl of XTT.
4. Add 10 µl (50 µg/ml) of the reaction solution to each well and incubate the plate for about 4 hrs.
5. Measure absorbance at 450 nm

![Fig. 6. In vitro screening of designed peptides with PBMC cell model by cytokine assay](image-url)

**Table 5. Troubleshooting of in vitro screening of designed peptides**

<table>
<thead>
<tr>
<th>Step</th>
<th>Possible problem</th>
<th>Possible reason</th>
<th>Possible solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Contamination could be seen in the experimental set</td>
<td>Contamination could occur due to use of unsterilized equipment and handling issues</td>
<td>To avoid contamination UV treatment should be given to the equipment in the Biosafety cabinet for at least 20min</td>
</tr>
</tbody>
</table>
Fig. 7. Schematic presentation of improved whole blood assay development procedure

Table 6. Trouble Shooting of cell viability assay (XTT assay)

<table>
<thead>
<tr>
<th>Step</th>
<th>Possible problem</th>
<th>Possible reason</th>
<th>Possible solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>No proper results obtained</td>
<td>XTT reagent used alone without activation reagent</td>
<td>Prepare XTT reagent along with activation reaction solution and repeat assay</td>
</tr>
<tr>
<td>6</td>
<td>Optical density too high</td>
<td>Cell numbers per well are too high</td>
<td>Reduce number of cells plated per well</td>
</tr>
</tbody>
</table>

Table 7. Trouble shooting of cytokine assay

<table>
<thead>
<tr>
<th>Step</th>
<th>Possible problem</th>
<th>Possible reason</th>
<th>Possible solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Improper results</td>
<td>Reagents used without bringing them at room temperature</td>
<td>Keep the reagents at room temperature for about 15 min before use</td>
</tr>
<tr>
<td>3</td>
<td>No proper results</td>
<td>Reagents might be contaminated</td>
<td>Use fresh reagents and repeat the test</td>
</tr>
</tbody>
</table>

10. CYTOKINE ASSAY

The levels of cytokines (INF-α/INF-γ/IL-6/IL-10/IP-10/GM-CSF) will be estimated in the supernatants using a commercially available kit (Table 7):

10.1 Materials Required

**Specimen**
- Blood sample

**Equipment**
- Elisa reader
- Incubator
- Shaker
- Vortex

**Kit reagents**
- Standards
Cytokine Ab solution  
Streptavidin-HRP  
Wash buffer  
Substrate solution  
Stop solution

**Procedure**

1. Prepare the reagents and standards at room temperature.
2. Add 50ul of standards in standard well and 40ul of samples in sample wells.
3. Add 10ul of respective cytokines Ab to sample well and 50ul streptavidin-HRP to sample well and standard well.
4. Incubate the plate for 1hour at 37ºC.
5. Wash the wells 5 times with wash buffer.
6. Add 50ul of substrate solution to both the wells and incubated for 10mins at 37ºC in the dark.
7. Stop the reaction by adding 50ul of stop solution
8. Measure the optical density at 450 nm using ELISA reader

**11. DEVELOPMENT OF IMPROVED WHOLE BLOOD ASSAY**

**11.1 Materials Required**

- Potential peptides sorted by In-vitro analysis
- Active TB cases, Latent TB cases, and Household control category blood samples
- Vacutainers
- LPS and PHA

**11.2 Instrument Required**

- ELISA reader
- Incubator etc.

**11.3 Procedure**

1. Take Blood in three EDTA vacutainers as follows:
   - Tube 1: Nil Tube containing no peptides and Mitogen
   - Tube 2: containing the synthesized potential peptides (Concentration to be optimized)
   - Tube 3: containing Mitogens LPS and PHA (Concentration to be optimized)
2. Incubate tubes for 16 to 24hrs
3. Centrifuge the tubes at 5634g
4. Collect the plasma from tubes
5. Measure the level selected cytokine panel (Identified by cytokine assay protocol) as per the instructions of manufacturers of kit (Fig. 7)

**11.4 Calculation**

ROC curve analysis will be performed over the cytokine value observed in a known set of Latent, Active, and Control blood samples, using statistical software for calculation of cut off value.

**12. EVALUATION OF IMPROVED WHOLE BLOOD ASSAY**

For evaluation, the developed assay will be compared with the existing, QFT assay & TST assay using the statistical tools.

**13. STATISTICAL ANALYSIS**

All the analysis will be performed using standard statistical software. The receiver operating curve (ROC) will be used for determining the cut-off values for individual cytokines. Subjects who will have cytokine and chemokine levels above the cut-off point will be considered as positive.

**14. RESULTS**

IGRA done in the subjects will give the idea about the subjects to be included in the latent category based on the positivity as described in inclusion-exclusion criteria and history. Latent specific peptides will be identified and synthesized for further evaluation through In-vitro protocols and will be further used for the development of whole blood assay. From the collected samples the PBMC will be isolated which further will be useful for in vitro screening of designed peptides. Identification of cytokine panel for diagnosis of the LTBI will take place and these identified cytokines will be used in the development of improved whole blood assay. By doing cell count the accurate number of cells (viable and non-viable) present in 1ml of pellet will be obtained which will be useful in In-vitro screening of the designed peptides. Evaluation of multi-cytokine in response to the disease-specific antigen will help us in understanding the role of other cytokine involved in latency and active infection. Also, our peptide-based approach may reduce the cost of developed Whole Blood Assay.
15. DISCUSSION

One of the study mentioned that there are significant differences in IFN-γ responses for DosR antigens (Rv1735c, Rv2006, Rv2625c, Rv1996, Rv2032, Rv2629, Rv3126c, Rv0081, Rv2631, Rv3130c, Rv2624c, Rv2007c, Rv2028c, and Rv3134) in healthy household contacts compared with patients with active TB, as reported by a study in whole blood that included a wide range of stage-specific antigens to assess IFN-γ response in a long-incubation assay [20].

In the similar way we will identify major DosR antigens based on literature and will extract their peptides which will be further filtered by the use of online software’s such as www.idbi.org and other online software’s.

Another study showed that the stimulation of peripheral blood mononuclear cells (PBMC) with Rv1737c and Rv2029c seem to increase the IFN-γ or TNF-α-producing CD4 and CD8 T cells in individuals with LTBI compared to active TB [21].

Related relevant literature was reviewed [22-24]. Sharma et al. reported on molecular infection biology, pathogenesis, diagnostics and new interventions [25]. Other related studies were reviewed [26]. Different aspects of tubercular infections in this region were addressed by Singhal et al. [27,28], Bawankule et al. [29] and Dholakia et al. [30-34].

16. CONCLUSION

Thus in our study further evaluation of the filtered potential peptides will be done and these peptides will be analyzed by In-Vitro studies using the peripheral blood mononuclear cells (PBMC) in the animal cell culture laboratory to see the cytokine response of these peptides. The identified potential immunogenic peptide target will be useful for studying the patho-physiology, of latent tuberculosis & development of alternative diagnostic protocol, for Latent TB infection.

17. LIMITATIONS

IGRA’s are developed and evaluated in the western population hence are less sensitive and specific for the Indian population and there is a need for its modification and also IGRA is a costly affair.

CONSENT

As per international standard or university standard, patient’s written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

The study was approved by the Institutional Ethical Committees of both, Central India Institute of Medical Science (CIIMS) Nagpur and Datta Meghe Institute of Medical Sciences (DMIMS), Sawangi, Wardha.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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