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RESEARCH ARTICLE

# An Integrated approach for Proteomic and Immunological analysis of Cell wall and Cell membrane proteins of *Mycobacterium tuberculosis*

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## ABSTRACT

Mycobacterium cell wall and cell membrane proteins serve as key modulators in the host immune response. Some of these proteins, like MPT63, are known as immunodominant antigens, and others, such as LpqS and LpqH, are being explored as subunit vaccine candidates. However, due to their hydrophobic nature, conventional separation methods are not able to solubilize and isolate these proteins individually, leading to limited research on their systematic separation and immunological characterization.

In this study, we successfully employed a combination of Preparative Isoelectric focusing and Preparatory SDS-PAGE to separate mycobacterial cell wall and membrane proteins into 234 fractions. These fractions were pooled based on the proteins molecular weight, resulting in 24 pooled fractions labelled as Ag1 to Ag24. Immunological analyses on these pooled fractions were carried out in 3 different study groups (subjects with latent tuberculosis infection (LTBI, N=20), tuberculosis-diseased patients (PTB, N=20), and healthy subjects (HC, N=20). In these study groups, Interferon-γ and TNF-α responses against these antigens were analysed using the cytokine ELISA method.

Notably, one pooled fraction (Ag24 - Cell wall high molecular weight proteins pool) induced significantly higher IFN gamma response in LTBI compared to PTB, while also triggering significantly higher TNF-alpha response in PTB compared to LTBI. Additionally, three other pooled fractions (Ag6 - Cell Membrane Medium molecular weight proteins Pool, Ag11 - Cell Membrane high molecular weight proteins Pool, Ag23 - Cell wall high molecular weight proteins pool) induced significantly higher IFN gamma response in LTBI compared to PTB.

Proteomic analysis of the Ag24 fraction identified four proteins (ACN, KatG, Rv2623, and Rv0404). Among them, KatG and Rv0404 were previously reported as immunodominant T-cell antigens. On the other hand, ACN lacks immunological information, and limited information is available on protein Rv2623. In conclusion, extensive immunological studies on these four proteins could enhance their development as tuberculosis prognostic biomarkers, while our systematic 2-dimensional separation method shows promising potential for characterizing cell wall and membrane proteins in other pathogens

**Keywords:** *Mycobacterium tuberculosis*; cell wall; cell membrane proteins; Protein purification; Preparative electrophoresis; Rotofor; Latent TB.

## Introduction

It is estimated that 2 billion people are having latent tuberculosis[1]. Among, the LTBI population, 5 to 10% of them develop tuberculosis in their lifetime. It makes LTBI a large reservoir for active tuberculosis and therefore management of the LTBI plays an important role in tuberculosis control. LTBI is identified based on the tuberculosis-specific immune response to Tuberculin Skin Test {TST} and Interferon Gamma Release Assay {IGRA}. The major disadvantage of the above assays is that they are unable to differentiate latent and active tuberculosis. In this context, the identification of latent tuberculosis-specific markers helps in the development of newer and better tests for LTBI identification.

In our earlier studies, to identify latent tuberculosis-specific antigens, we analyzed whole culture filtrate proteome immune response in human tuberculosis *in vitro* [2]. Based on our data, we shortlisted 16 proteins that specifically induce immune response only in LTBI, and not in active TB[2,3]. Further studies are in progress to utilize them as a marker for latent tuberculosis.

However, recent immunological studies have shown that apart from culture filtrate proteins, the human immune response to TB is also directed against cell wall[4] and cell membrane[5] proteins of *M. tuberculosis*. Cell wall and cell membrane proteins play an important role in pathogen recognition in the human immune system. Some of these proteins are reported as immunodominant antigens and some are reported as pathogen-associated molecular patterns (PAMPs) that are recognized by immune cells, initiating an immune response. Mycobacterial lipoprotein LprG, LpqH, LprA, and PhoS1 were shown to be recognized by the Toll-like receptor 2. These proteins are shown to modulate the antigen-presenting cell function as well as CD 4 T cell function in tuberculosis pathogenesis[6,7,8,9,10,11,12]. Except for the above-reported proteins, the immunological role of the majority of the cell wall and membrane proteins of *M. tuberculosis* is unknown.

Genomics studies showed that 99 putative membrane-associated lipoproteins were coded by *M. tuberculosis*[13]. Due to the physicochemical characteristics of lipoproteins, most of these are found to be associated with the cell wall and cell membrane of mycobacteria[14,15]. Hence, isolation and immunological characterizations of these mycobacterium surface-associated proteins play an important role in the understanding of the pathogenesis of tuberculosis.

The hydrophobic nature of cell wall and membrane proteins make them technically challenging in terms of solubilization and separation. Studies have shown that membrane protein constitutes approximately 20 to 30% of the coding regions of all the organisms including mycobacteria[16,17]. Studies have shown that mycobacterial cell wall and cell membrane proteins plays important cell functions that includes energy production, signal transaction, cell communication, Ion and molecule transport, nutrient uptake and host immune response. Since these membrane proteins are involved in the central role in the mycobacterial cell physiological process, many of cell wall and cell membrane proteins are the target for the drug development[18]. All of the above facts, stress the importance of separation and characterisation of the cell wall and cell

membrane protein in understanding tuberculosis biology.

Due to the technical challenges and their intrinsic nature, cell wall and cell membrane proteins are difficult to solubilise and isolate like the soluble proteins. Due to these limitations in membrane purification only 2% of the membrane protein structure was reported[19]. Many factors make these cell wall and cell membrane protein isolation challenging. First main reason is that these proteins are low in the cell. This low level expression of the proteins makes it difficult to isolate them as individual protein for further characterisations[20]. Another important problem in cell wall and cell membrane protein separation is their solubility. These proteins have the intrinsic capacity to form aggregates. This problem can be overcome by the addition of detergents which helps to solubilise them. Some of these proteins tend to form aggregates even in the presence of detergents. In those cases, additional components such as chaiotropic agents and reducing agents are able to improve the solubility.

On the other hand using the detergents for the solubilisation of cell wall and cell membrane protein will interfere with the subsequent separation methods. Additionally, charged detergents cannot be used for the ion exchange chromatography, and may also interferes with the hydrophobic interaction chromatography separation[21]. Choice of the detergents used should be compactable with the separation approach applied. Another important step in the cell wall and cell membrane protein isolation is the removal of the detergents that is used for solubilisation. These detergents will interfere with the subsequent functional analysis of the proteins.

In this manuscript, we describe the methodology we have developed to overcome the shortcoming of membrane protein purification and analysis. We had developed this methodology using *M. tuberculosis* cell wall and cell membrane proteins as model. We have used a novel two-dimensional separation approach for the separation of these hydrophobic proteins. In the first step, we had fractionated the hydrophobic membrane proteins by their isoelectric point by using rotofor IEF system. Rotofor separated fractions were then separated again based on molecular weight of the proteins using SDS-PAGE. Then the separated proteins were eluted using whole gel eluter for the second-dimensional separation. Thus, we had combines these two successfully used experimental methods for separation of membrane proteins. The combination of these two approaches enabled us to separate the membrane proteins as less complex mixtures and then as individual antigens. Separated protein fractions were then subjected to immunological analysis in 20 LTBI subjects, 20 PTB patients and 20 healthy controls. This analysis identified LTBI specific fractions based on IFN-gamma and TNF-alpha response. These identified fractions can be considered as potential candidates for LTBI specific biomarkers.

## Methodology:

CULTURING OF THE *M. TUBERCULOSIS*:

We conducted a large-scale culture of the S7 strain, which previous studies[21;22] had identified as predominantly associated with infection. This culture was established using the protocol as described below. *M.*

*tuberculosis* S7 clinical strain colonies were revived and transferred from Lowenstein-Jensen slants to 2 ml of 7H9 broth medium (Himedia Laboratories), and the cells were dispersed using glass beads under sterile conditions. These bacterial cell suspension was then transferred to 10 ml of 7H9 broth medium in a McCartney bottle for incubation at 37°C for 2 weeks. The log phase culture was then up scaled to 200 ml of 7H9 broth medium and grown in shaker culture for 4 weeks. Further scaling up was done in 1-liter culture flask containing 500 ml of 7H9 broth medium as shaker culture for 4 weeks at 37°C. The mycobacteria in the culture were then pelleted by centrifugation at 3000 rpm for 30 min.

PREPARATION OF WHOLE CELL LYSATE PROTEINS:

Sonication of the isolated bacterial pellet was carried out by using the standard protocol. Briefly, for every 2 g of the bacterial cell pellet, 1 ml of breaking buffer was added and sonication was carried out at 9 seconds on and 9 seconds off cycle for 30 minutes with 40% amplitude using vibra cell instrument (Sonics). Prepared lysate was than centrifuged at 5000 rpm for 15 minutes to remove the unbroken cells. The supernatant was concentrated using amicon centrifugal concentrators. Protein concentration in the collected supernatant was estimated by BCA kit (Thermofisher scientific).

ISOLATION OF CELL WALL AND CELL MEMBRANE PROTEINS FROM THE WHOLE CELL LYSATE BY ULTRA-CENTRIFUGATION:

From the whole cell lysate, the cell wall, cell membrane and cytosol proteins were isolated by using protocol as described previously[23]. Briefly, whole cell lysate was centrifuged at 10,000 rpm for 30 minutes. This procedure separated the cell wall proteins as a pellet. The obtained supernatant was subjected to 100,000 x g for 4 hr in ultra-centrifuge (Beckmen). This procedure separated cell membrane as pellet and cytosol as a supernatant. Protein concentration in the cell wall and cell membrane fractions were estimated by BCA protein assay (Thermofisher scientific).

PREPARATIVE LIQUID-PHASE ISOELECTRIC FOCUSING; (ROTOFOR)

Cell wall and cell membrane proteins isolated from ultra-centrifuge procedure were solubilized in a IEF buffer {8 M urea, 1 mM dithiothreitol (DTT), 5% glycerol, 2% digitonin, and 2% ampholytes (pH 3.0 to 10.0 and pH 4.0 to 6.0 at a ratio of 1:4) (Bio-Rad Laboratories, Hercules, CA, USA)}. The solubilised cell wall and cell membrane protein was fractionated using a liquid isoelectric focusing (IEF) system (Rotofor, Bio-Rad Laboratories, Hercules, CA, USA) and maintained at 4°C using cooling water bath. Preparative IEF separation of the proteins was carried out by following manufacturer's instructions. Separation of the proteins in the IEF system was carried out by applying constant power (12 W) until the voltage stabilized. The voltage is stabilised between 1300 V.-1600 V range. After this IEF run was continued for an additional 30 min and then terminated (total IEF run had taken approximately 5- 6 hours depending on the sample). The individual IEF fractions were collected by using vacuum pump. pH value of the separated fractions were determined. Separated fractions were subjected to SDS–PAGE and the proteins were visualized by CBB/ silver staining.

PREPARATIVE SDS-PAGE AND WHOLE GEL ELUTION:

IEF separated cell wall and cell membrane fractions were mixed with 6X SDS-PAGE sample buffer and heated at 95°C for 5 min before SDS-PAGE analysis. IEF separated protein fraction were further subjected to second dimensional separation using 16 X20-cm polyacrylamide gels comprising a 12.5% resolving gel and 4% stack over of it. Single 13-cm-long sample well was used for the loading of the sample. Electrophoresis was carried out by setting constant current of 50 mA/gel until the dye front reached the bottom of the gel. After the electrophoresis run is over, the gel was initially equilibrated in elution buffer (60 mM Tris (pH 9.4), 40 mM CAPS) for 10 min. after equilibration, gel was transferred to a Whole Gel Eluter apparatus (Bio-Rad,) by following the manufacturer's instructions.

The proteins were eluted from the gel by running the eluter at constant current of 250 mA for 1 hour. After the elution, eluted protein fractions were harvested using the Vacuum pump. 30 protein fraction (approximately 3ml volume) were collected from every gel. BCA protein assay Kit (Thermofisher scientific) was used for quantification of the protein concentration in the eluted fractions. 10 µg of eluted fraction was subjected to SDS-PAGE analysis and silver stained.

IMMUNOLOGICAL INVESTIGATIONS:

Immunological characterization of the separated fractions was carried out in 1/10 diluted whole blood assay as described previously[24]. Briefly, whole blood was diluted 1:10 with RPMI medium. To the diluted blood, cell wall and cell membrane protein fractions and the standard protein (*M. tuberculosis* whole cell lysate) were added at the concentration of 5µg/ml and incubated at $37^0$C in 5% $CO_2$ for 6 days. After the culture period, supernatant will be isolated from the culture and stored in the -$80^0$C until use.

IFN-GAMMA & TNF-ALPHA MEASUREMENT:

Cytokine production was determined by a standard ELISA technique using commercially available BD OptEIA kits (BD Biosciences) according to the manufacturer's instructions. The OD values were read at 450 nm using an ELISA reader (MolecularDevices, Sunnyvale, CA).

PROTEOMIC ANALYSIS OF THE IMMUNOLOGICALLY IMPORTANT FRACTIONS:

**Protein sample preparation and Mass Spectrometry:**

100 µg of protein in 100 µl was used for the mass spectrometry based proteomic analysis. Proteomic analysis of the trypsin digested protein was carried out by using 1D nano-LC. (LC/MS/MS) (Synapt G2-HDMS from Waters).

**Liquid chromatography analysis conditions:**

A nano ACQUITY UPLC® chromatographic system (Waters, Manchester, UK) was used for the trypsin peptides separation. MassLynx4.1 SCN781 software, (Waters) was used as the analysis software.

Binary solvent system with solvents detailed below was used for the separation of the peptides.

Solvent A (0.1% formic acid in Water)

Solvent B (0.1% formic acid in Acetonitrile)

We used Symmetry® 180µm x 20mm C18 5µm (Waters) used as the trap column. 75 µm X 200 mm HSS T3 C18 1.8 µm (Waters) was used as the Analytical column.

The solvent flow rate was kept at 300 nL/min and the column temperature was maintained at 35$^0$C. Reverse phase Chromatography mode was used and the auto sampler temperature was fixed at 4°C.

### Details of Liquid chromatography gradient:

Total run time: 60 minutes

The gradient profile was set as follows:

Time (min) Flow (µl/min) % A (Water) %B (Acetonitrile)

Initial: 99.0% A (Water) and 1.0% B (Acetonitrile)

3.00 minutes: 99.0% A and 1.0% B

43.00 minutes: 60.0% A and 40.0% B

46.00 minutes: 20.0% A and 80.0% B

50.00 minutes: 20.0% A and 80.0% B

51.00 minutes: 99.0% A and 1.0% B

60.00 minutes: 99.0% A and 1.0% B

### Mass spectrometry conditions:

Synapt G2 High Definition MS™ System (HDMSE System) from Waters was used for mass spectrometry analysis of trypsin digested peptides. MS runs were performed using ion mobility enabled separation with calibration carried out with Sodium iodide: ESI positive Acquisition mode was used. Online mass correction was carried out using Leucine encephalin in positive ion mode (m/z =556.2766). Nano ESI capillary voltage was fixed at 3.4 KV. Sample cone value fixed at 40 V and extraction cone was maintained at 4 V. IMS gas (N2) flow rate was 90 (mL/min). IMS T-Wave™ pulse height was kept at 40 V.IMS T-Wave™ velocity was set as 800 m/s. IMS voltage was 8 V and 20 V. Resolution mode was used as mode of operation and Continuum Data acquisition format was employed. Collision energy was maintained from 20 eV to 45 eV.

## Data analysis:

Progenesis QI for Proteomics V4.2 (Non Linear Dynamics, Waters) was used as the analysis software. We utilized the UniProt Mycobacterium tuberculosis (https://www.uniprot.org/) database for protein identification. False positive rate of 4 was maintained, allowing one missed cleavage. The number of fragments per peptide was limited to 1 and number of fragments per protein was limited to 3. At least 1 unique peptide was required for protein identification. Cysteine carbamidomethylation was used as a variable modification and oxidation of methionine was used as a fixed modification.

## Results

PREPARATION OF WHOLE CELL LYSATE PROTEIN:

We earlier carried out a systematic and immunological characterisation of mycobacterial culture filtrate protein in which we had initially separated the culture filtrate proteins based on isoelectric point using preparative IEF system (Rotofor, Biorad) and Preparative IEF.The separated fractions were then immunologically and proteomically characterised[2&25]. We had used the above explained approach in separating the hydrophobic cell wall and cell membrane proteins of mycobacteria in this study. From our mycobacterial cultures we were able to obtain 10 mg of the whole cell lysate protein per 100 ml of the culture. We had produced 2 gm whole cell lysate protein preparation from 20 litre of *M. tuberculosis* culture. **Figure 1** represents the SDS-PAGE analysis of the prepared whole cell lysate proteins.

Figure.1 SDS PAGE Analysis of whole cell lysate proteins

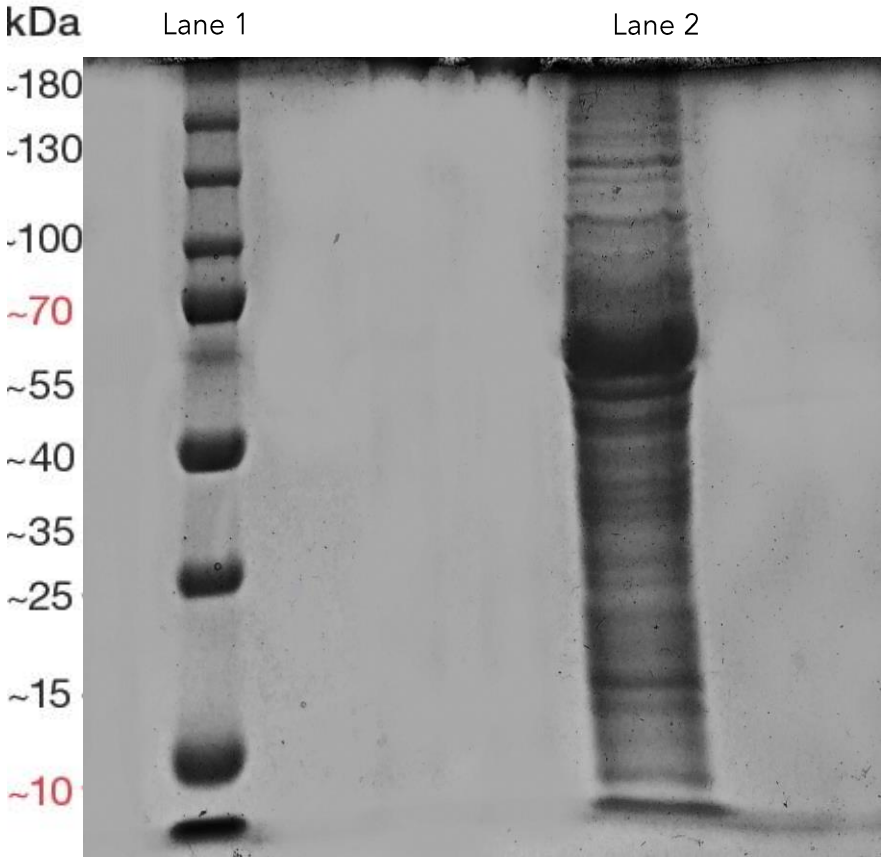



Figure 1 represented the SDS-PAGE analysis of the prepared whole-cell lysate proteins. Lane 1 represents the Molecular weight marker and lane 2 represents whole cell lysate protein fractions.

ISOLATION OF CELL WALL AND CELL MEMBRANE PROTEIN ISOLATION BY HIGH-SPEED ULTRA-CENTRIFUGATION:

The cell wall and cell membrane proteins were isolated from whole cell lysate protein by ultracentrifugation method. From 2 gram of whole cell lysate proteins we had obtained 300 mg of the cell wall protein and 100 mg of cell membrane proteins.

Figure 2 represented the SDS-PAGE analysis of the separated cell wall, cell membrane and cytosol proteins. Initially we carried out the RP-HPLC separation of cell wall and cell membrane proteins. We loaded 10 mg of cell wall and cell membrane proteins separately for the isolation. HPLC separated fractions had a protein concentration that was too low for further purification (data not shown). Since two dimensional separation is mandatory to reduce the complexity of mycobacterial proteins as observed in the earlier studies[2], we decided to implement an alternative method for the first dimensional separation.

Earlier we had successfully used Preparative liquid-phase isoelectric focusing separation of proteins using Rotofor IEF system for the fractionation of mycobacterial Culture filtrate proteins[25]. So we used similar approach for the fractionation of the cell wall and cell membrane proteins **as explained in flow chart 1**

**Figure. 2. SDS-PAGE analysis of cell wall and cell membrane proteins**:

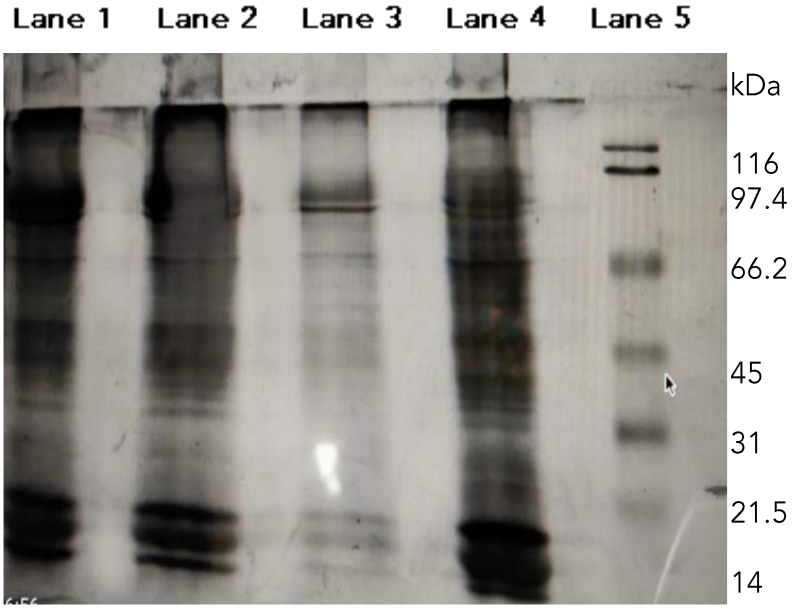
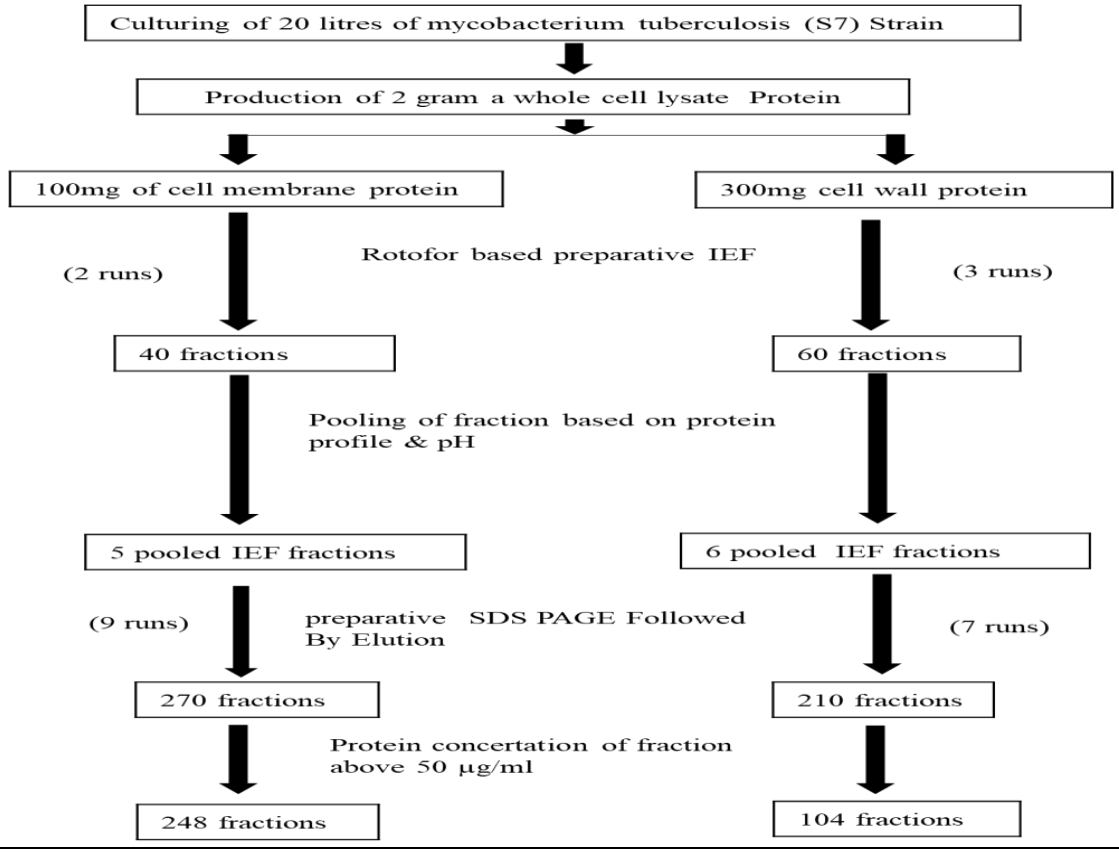



Figure 2 represented the SDS-PAGE analysis of the separated cell wall, cell membrane, and cytosol proteins. In that lane 1 represent whole cell lysate, lane 2- cell wall fraction, lane 3 represents cell membrane fraction, lane 4 represents cytosol fraction, and lane 5 represents protein molecular weight marker.

Flow Chart 1. Separation of cell wall and cell membrane proteins



SEPARATION OF CELL WALL PROTEINS BY PREPARATIVE LIQUID-PHASE ISOELECTRIC FOCUSING: (ROTOFOR)

By using the IEF buffer, we were able to dissolve 100 mg of the cell wall proteins. Higher protein concentration above 100 mg resulted in the precipitation of the cell wall proteins. So, 300 mg of cell wall proteins were subjected to 3 separate IEF runs with 100 mg each. Each preparative IEF run separates the cell wall proteins into 20 fractions. 300 mg cell wall proteins were separated into 60 fractions (3 runs). The pH of these separated fractions ranged from 1 to 11.45. The protein concentration of these fractions ranged from 16 µg to 795 µg **(Figure. 3).** It is interesting to note that cell wall proteins are focused and isolated majorly on the acidic pH range. Separated rotofor fractions were then subjected to SDS-PAGE analysis to identify their protein profile (**Figure.4**). Fractions which had similar pI value and proteins profiles were pooled together. By this approach, we were able to pool the 60 IEF fractions into 6 pooled IEF fractions. IEF pooled rotofor (pH based) separated cell wall fractions were further subjected to preparative SDS-PAGE separation (based on Molecular weight) followed by whole gel elution.

**Figure.3: pH Vs protein concentration of the rotofor separated cell wall fractions (**Run 1 of cell wall protein separation**)**

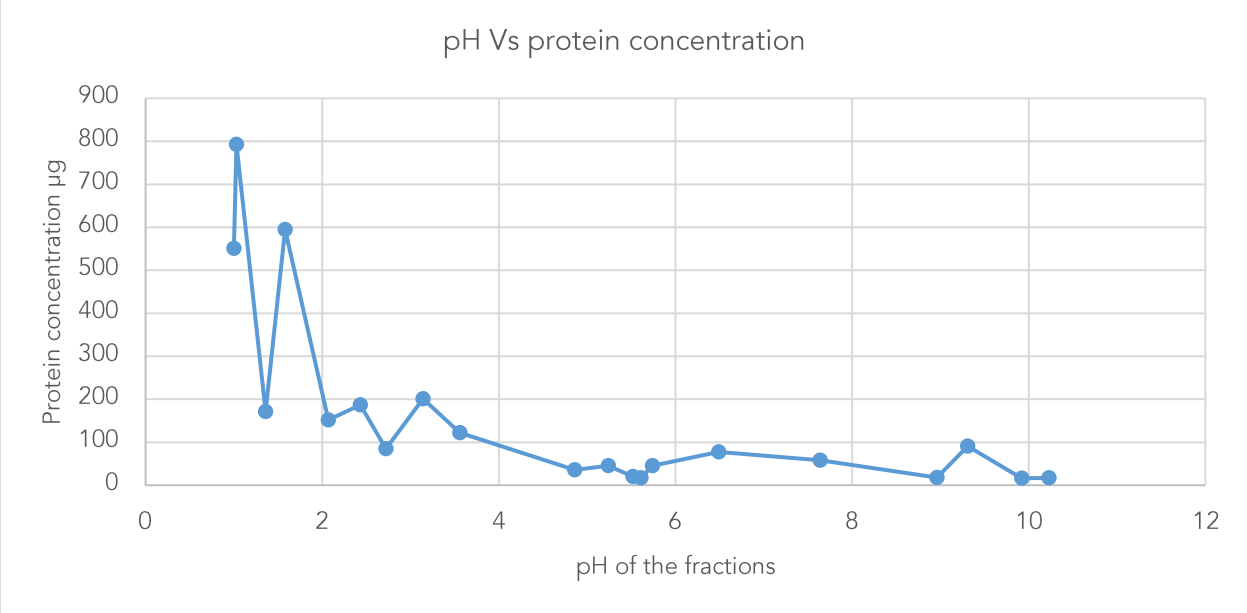



Figure 3 represents the pH versus protein concentration of the rotofor separated cell wall proteins. The numbers 0 to 12 represent the pH values. values 0 to 900 represent the protein concentration in micrograms per ml.

**Figure 4. SDS PAGE analysis of rotofor separated cell wall fractions**

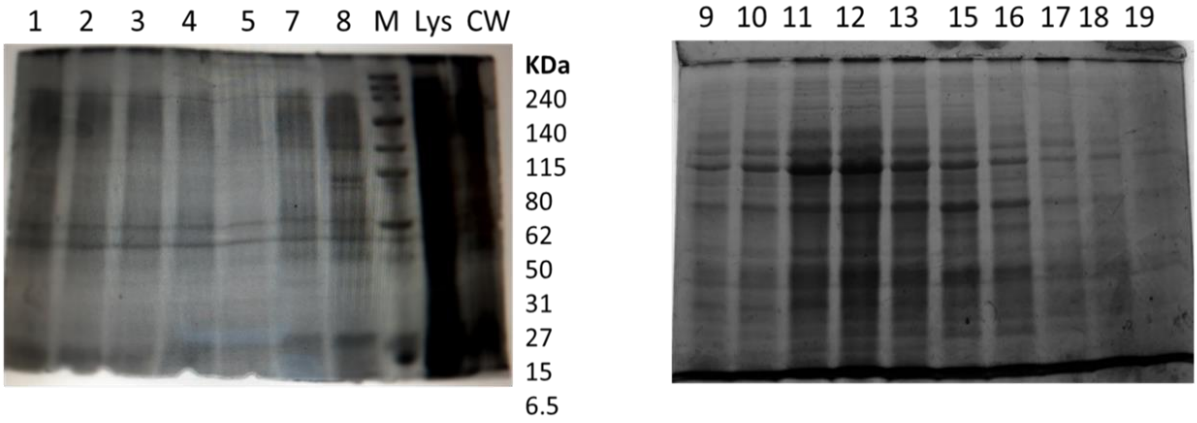



Figure 4 represents the SDS-PAGE analysis of rotofor separated cell wall fractions. The numbers 1 to 19 represents rotofor separated fractions. M. represents molecular weight marker. Lys represents whole cell lysate. CW represent cell wall proteins. Numbers 240 to 6.5 is protein's molecular weight in kilo Daltons.

SEPARATION OF CELL MEMBRANE PROTEINS BY PREPARATIVE LIQUID-PHASE ISOELECTRIC FOCUSING:

By using the IEF buffer, we were able to dissolve 50 mg of the cell membrane proteins. Protein concentration more than 50 mg resulted in the precipitation of cell membrane proteins during IEF separation. So, the 100 mg of the cell membrane protein was subjected to 2 separate IEF runs. Each preparative IEF run separated the cell membrane proteins into 20 fractions. 100 mg of cell membrane proteins were separated into 40 fractions. The pH of these separated fractions ranged from 3 to 11. The protein concentration of these fractions ranged from 50 µg to 1200 µg (**Figure. 5**). It is interesting to note that unlike the cell wall proteins which are focused and isolated majorly on the acidic pH range, cell membrane proteins are focused and separated in in all pH range (acidic, basic as well as neutral pH range). Separated rotofor fractions were subjected to SDS-PAGE analysis to identify the protein profile (**Figure. 6**). The SDS-PAGE protein profile and pH value of the 2 different IEF runs of cell membrane were compared. Fractions which have the similar pI value and similar protein profile were pool together. We were able to pool the 40 IEF fractions into 5 pooled IEF fractions. This 5 IEF pooled rotofor separated cell membrane fractions were subjected to preparative SDS-PAGE separation followed by whole gel elution.

**Figure.5: pH Vs protein concentration of the rotofor separated cell membrane fractions** (Run 1 of cell membrane protein separation)

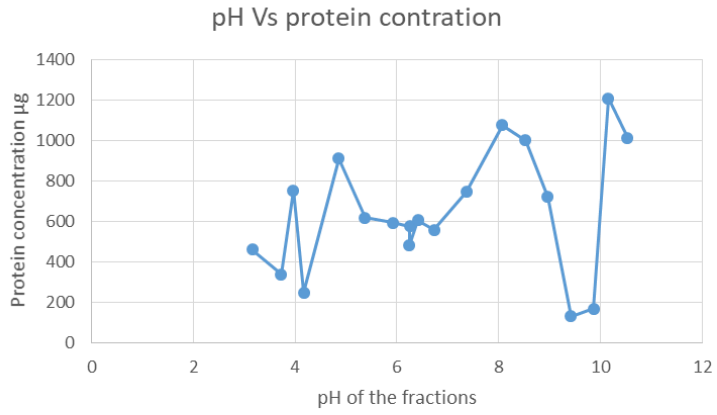



Figure 5 represents the pH versus protein concentration of rotofor separated cell membrane fractions. The numbers 0 to 12 represent the pH values. Values 0 to 1400 represent the protein concentration in micrograms per ml

Figure 6. SDS PAGE analysis of rotofor separated cell membrane fractions

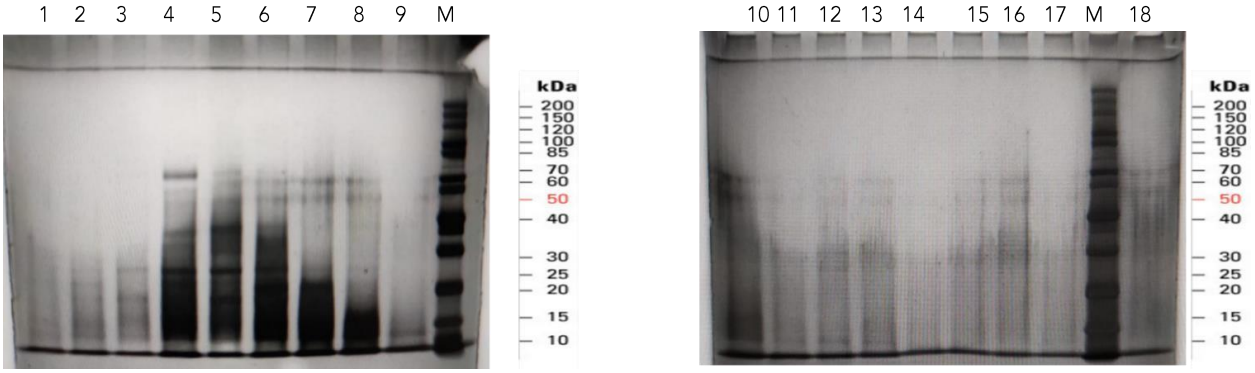



Figure 6 represents the SDS-PAGE analysis of rotofor separated cell membrane fractions. Numbers 1 to 18 represent rotofor separated cell membrane fractions. M. represents molecular weight marker. Numbers 200 to 10 represent protein molecular weight in kilo Daltons.

PREPARATIVE SDS-PAGE SEPARATION OF CELL WALL PROTEINS:

Six pooled rotofor separated cell wall fractions were subjected to the preparative SDS-PAGE followed by whole gel elution. Totally 7 preparative SDS-PAGE were run for the separation of cell wall proteins. This SDS-PAGE run separated cell wall proteins were eluted using the whole gel elutor. This procedure separated cell wall proteins into 210 fractions. Proteins concentration in the separated fractions ranged from 25µg to 650 µg (**Figure. 7**). The whole gel eluted fraction were subjected to SDS-PAGE analysis (**Figure.8**).

Figure 7.Protein concentration of whole gel eluted cell wall fractions

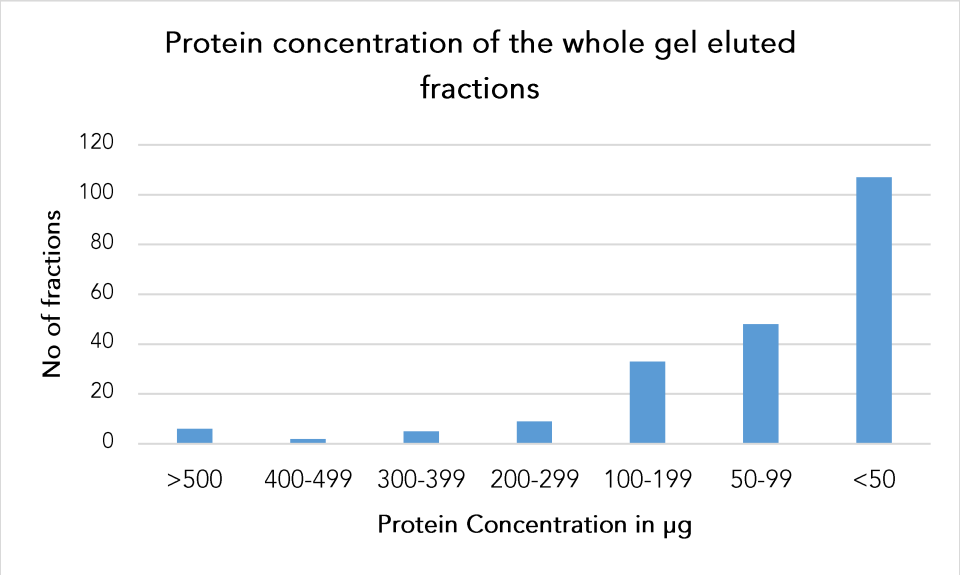



Figure 7 represented the protein concentration range of the whole gel eluted cell wall fractions. Numbers 0-120 represent the number of fractions in the particular protein concentration range. Numbers 500 to 50 represents protein concentration in microgram

Figure 8: SDS-PAGE analysis of whole gel eluted cell wall fractions

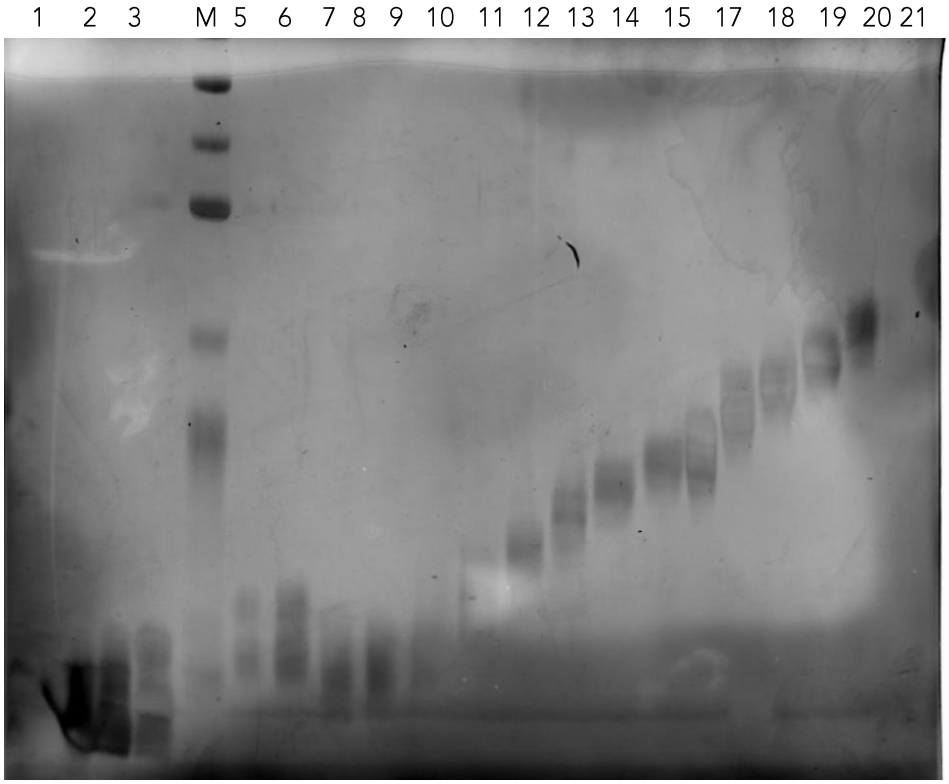



Figure 8 represents the SDS-PAGE analysis of the whole gel eluted cell wall fractions. Numbers 1 to 21 represent the whole gel eluted fractions. M represents the marker

PREPARATIVE SDS-PAGE SEPARATION OF CELL MEMBRANE PROTEINS:

Five pooled rotofor separated cell wall membrane were subjected to the preparative SDS-PAGE followed by the whole gel elution. Totally 9 preparative SDS-PAGE were run for the separation of cell membrane proteins. SDS-PAGE separated cell membrane proteins were eluted by using the whole gel elutor. This procedure separated cell membrane proteins into 270 fractions. Protein concentration in the separated fractions ranged from 20 µg to 650 µg (**figure .9**). The whole gel eluted cell membrane fractions were subjected to SDS-PAGE analysis (**Figure. 10**). As observed, majority of the fractions were found to have the single band in the SDS-PAGE. The above explained results showed that, we had separated cell wall into 104 fractions and cell membrane into 248 fractions.

In the separated fractions, 234 fractions having 50 µg/ml or more protein concentration (110 cell membrane fractions & 124 cell wall fractions, 234 fractions). These fractions were subjected to immunological and proteomic analysis as described in **Flow chart 2**.

**Figure 9. Protein concentration of whole gel eluted cell membrane fractions**

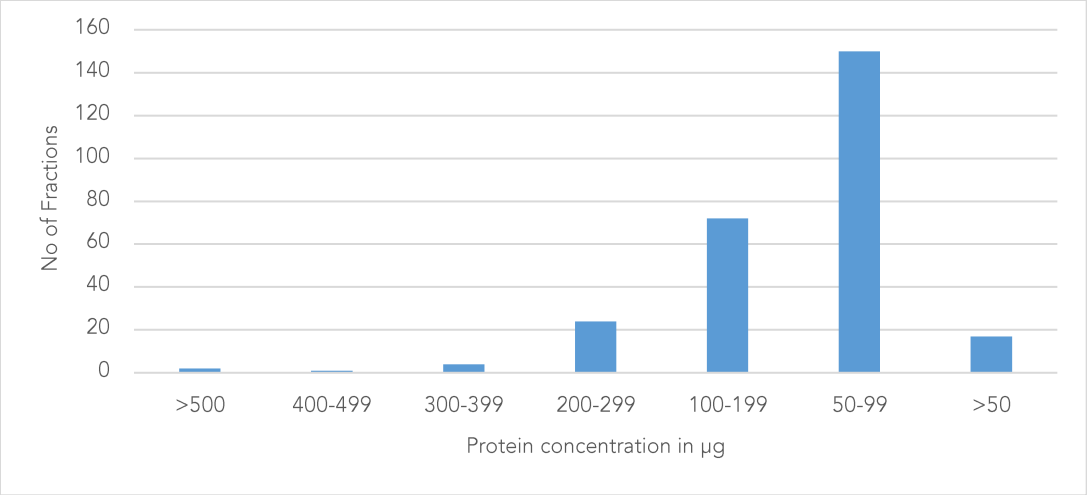



Figure 9 represented the protein concentration range of the whole gel eluted cell membrane fractions. Numbers 0-160 represent the number of fractions in the particular protein concentration range. Numbers 500 to 50 represent protein concentration micrograms.

Figure 10: SDS-PAGE analysis of whole gel eluted cell membrane fractions

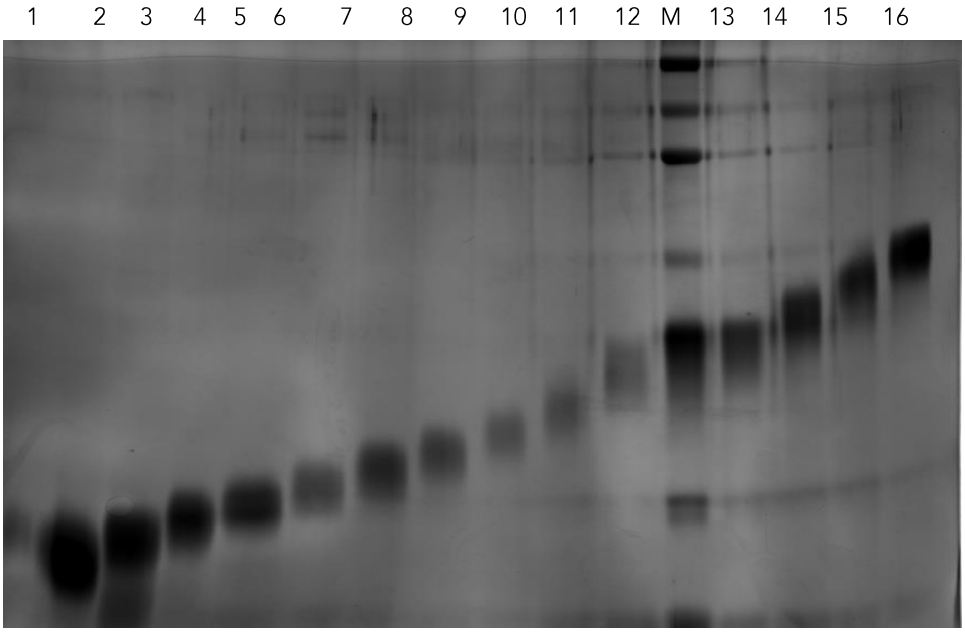



Figure 10. represents the SDS-PAGE analysis of whole gel eluted cell membrane fractions. Numbers 1 to 16 represents the whole gel eluted cell membrane fractions. M. Represents the molecular weight marker

**Flow Chart 2. Immunological and proteomic of cell wall and cell membrane proteins**

352 fractions (104 cell wall fractions & 248 cell membrane fractions

↓

Based on their molecular weight above mentioned fractions pooled as 24 pooled fraction

↓

Pooled fractions subjected Immunological analysis by whole blood assay in three study groups

↓

IFN γ Response | TNF α response

↓

Identification of four Immunologically important pooled fractions

↓

Proteomic characterisation by mass spectrometry

↓

Identification of four proteins (ACN, KatG, Rv2623, Rv0404) as LTBI specific proteins

Since the number of fractions screened was larger (234 fractions), we had grouped the isolated fractions as 3 major groups as described below

1. High molecular weight protein fractions (Protein with molecular weight more than 50 Kda),
2. Medium molecular protein fractions (protein with molecular weight in the range of 49-20 Kda),
3. Low molecular weight fractions (protein having molecular weight less than 20 kda),

In each group, 10 fractions were pooled **(Table 1)**. By this approach, we obtained 11 pooled cell wall protein fractions. It included 2 pools of low molecular weight cell wall fractions (labelled as Ag 14 & Ag 15), 5 pools of medium molecular weight cell wall fractions (labelled as Ag 16 to Ag 20), and 4 pools of high molecular weight cell wall fractions (labelled as Ag 21 to Ag 24).

**Table 1.** Details of the pooled fractions

| S. No | Pooled fraction name | Pooled fraction details |
|---|---|---|
| 1 | Ag 1 | Cell Membrane low molecular weight proteins Pool A |
| 2 | Ag 2 | Cell Membrane low molecular weight proteins Pool B |
| 3 | Ag 3 | Cell Membrane low molecular weight proteins Pool C |
| 4 | Ag 4 | Cell Membrane low molecular weight proteins Pool D |
| 5 | Ag 5 | Cell Membrane low molecular weight proteins Pool E |
| 6 | Ag 6 | Cell Membrane Medium molecular weight proteins Pool A |
| 7 | Ag 7 | Cell Membrane Medium molecular weight proteins Pool B |
| 8 | Ag 8 | Cell Membrane Medium molecular weight proteins Pool C |
| 9 | Ag 9 | Cell Membrane Medium molecular weight proteins Pool D |
| 10 | Ag 10 | Cell Membrane Medium molecular weight proteins Pool E |
| 11 | Ag 11 | Cell Membrane high molecular weight proteins Pool A |
| 12 | Ag 12 | Cell Membrane high molecular weight proteins Pool B |
| 13 | Ag 13 | Cell Membrane high molecular weight proteins Pool C |
| 14 | Ag 14 | Cell wall low molecular weight proteins pool A |
| 15 | Ag 15 | Cell wall low molecular weight proteins pool B |
| 16 | Ag 16 | Cell wall medium molecular weight proteins pool A |
| 17 | Ag 17 | Cell wall medium molecular weight proteins pool B |
| 18 | Ag 18 | Cell wall medium molecular weight proteins pool C |
| 19 | Ag 19 | Cell wall medium molecular weight proteins pool D |
| 20 | Ag 20 | Cell wall medium molecular weight proteins pool E |
| 21 | Ag 21 | Cell wall high molecular weight proteins pool A |
| 22 | Ag 22 | Cell wall high molecular weight proteins pool B |
| 23 | Ag 23 | Cell wall high molecular weight proteins pool C |
| 24 | Ag 24 | Cell wall high molecular weight proteins pool D |

In each pooled fractions, 10 -20 fractions were pooled

Similarly, we obtained 13 pooled cell membrane fractions including 5 pools of low molecular weight cell membrane fractions (labeled as Ag1 to Ag 5), 5 pools of medium molecular weight cell membrane fractions (labeled as Ag 6 to Ag 10), and 3 pools of high molecular weight cell membrane fractions (labeled as Ag 11 to Ag 13).

Immunological characterisation of these pooled fractions had been carried out in 20 QFT positive (LTBI) subjects, 20 healthy controls (QFT negative) subjects, and 20 smear positive tuberculosis patients (PTB). IFN-γ and TNF-α cytokine response were studied by cytokine ELISA method.

IFN-GAMMA RESPONSE:

We observed that QFT positive subjects showing increased antigen specific response against tested antigens compared with TB patients and QFT negative subjects. We found 4 pooled fractions (Fractions that induced significant IFN-γ response (Ag6 {Cell membrane medium molecular weight pool, **Figure 11**}, Ag11 {Cell membrane high molecular weight pool, **Figure 12**}, Ag23 {Cell wall high molecular weight pool, **Figure 13**}, Ag24 {Cell wall high molecular weight pool, **Figure 14**}) induced significantly higher IFN gamma response in QFT +ve (LTBI) compared with the tuberculosis patients (PTB) (**Figures 11 to 14**).. There is no significant difference in antigen-specific IFN-gamma secreted between QFT positive (LTBI) and QFT negative (Healthy controls) subjects. Similarly, there is no significant difference in the IFN-gamma secreted between tuberculosis patients and QFT-negative (Healthy controls) subjects in all the antigens analyzed (Data not shown).

**Figure 11. IFN γ RESPONSE aganist pooled Antigen- 6 fraction in LTBI & PTB**

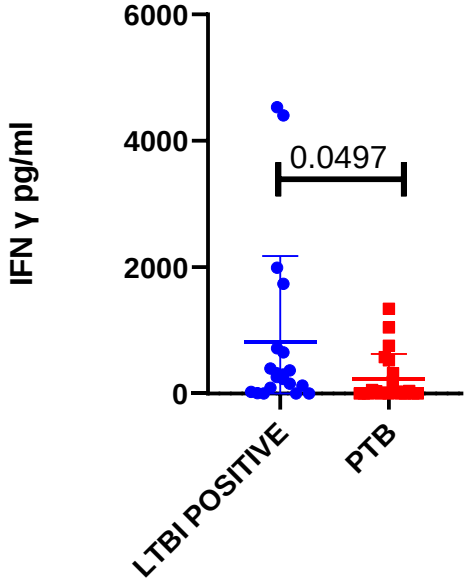



Figure 11. IFN- γ level after antigen 6 stimulation. 0.0497 Refers to the P value which is a significant value ($p < 0.05$); statistical analysis was performed using Mann–Whitney U test Filled Blue circles represent the LTBI Individual response for antigen 6. Red Square boxes represent the Individual patient response to antigen 6

## Figure 12. IFN γ response aganist pooled Antigen- 11 fraction in LTBI & PTB

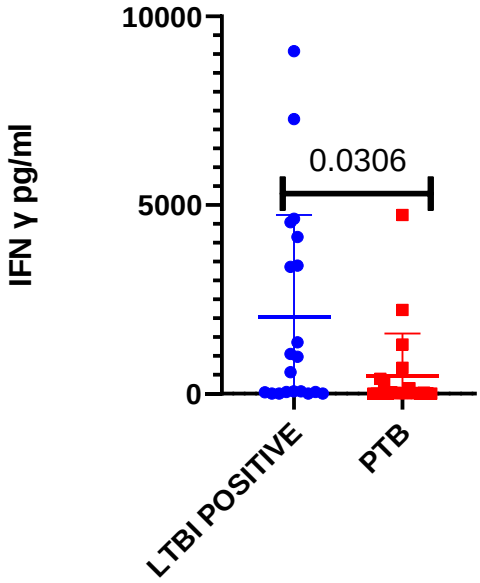



Figure 12. IFN- γ level after antigen 11 stimulation. 0.0306 Refers to the P value which is a significant value ($p < 0.05$); statistical analysis was performed using Mann–Whitney U test Blue circles represent the LTBI Individual response for antigen 11. Red square boxes represent the Individual patient response to antigen 11.

## Figure 13. IFN γ response aganist pooled Antigen - 23 fraction in LTBI & PTB

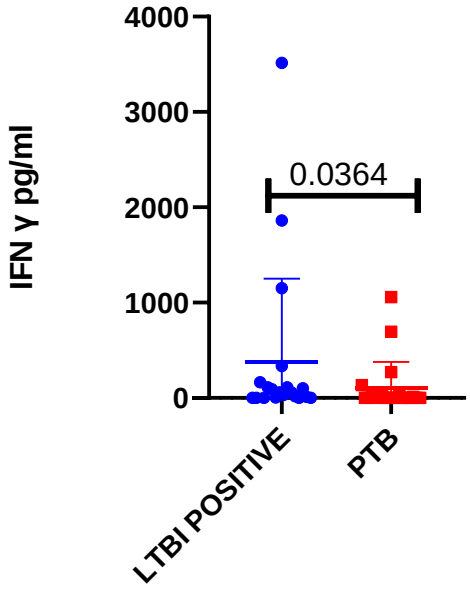



Figure 13. IFN- γ level after antigen 23 stimulation. 0.0364 Refers to the P value which is the significant value ($p < 0.05$); statistical analysis was performed using Mann–Whitney U test Blue circles represent the LTBI Individual response for antigen 23. Red square boxes represent the Individual patient response to antigen 23

**Figure 14. IFN γ response aganist pooled Antigen- 24 fraction in LTBI & PTB**

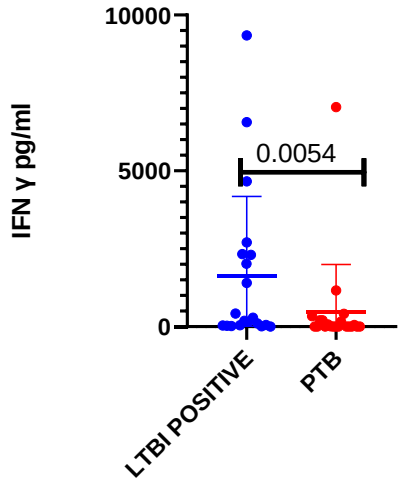



Figure 14. IFN- $\gamma$ level after antigen 6 stimulation. 0.0054 Refers to the P value which is the significant value ($p < 0.05$); statistical analysis was performed using Mann–Whitney U test. Blue circles represent the LTBI Individual response for antigen 24. Red circle represent the Individual patient response to antigen 24

TNF-ALPHA RESPONSE:

Our results showed that TB patients had increased antigen specific TNF-alpha response against the tested antigens compared with QFT positive subjects and QFT negative subjects. We found that only one pooled fraction (Ag24 {Cell wall high molecular weight pool}) induced significantly higher TNF-alpha response in tuberculosis patients (PTB) compared with QFT +ves (LTBI) (Figure. 15). There is no significant difference in antigen-specific TNF-alpha secreted between QFT positive (LTBI) and QFT negative (Healthy controls) subjects. Similarly, there is no significant difference in the TNF-alpha secreted between tuberculosis patients and QFT-negative (Healthy controls) subjects in all the antigens analyzed (Data not shown).

**Figure 15. TNF-α response aganist pooled Antigen- 24 fraction in LTBI & PTB**

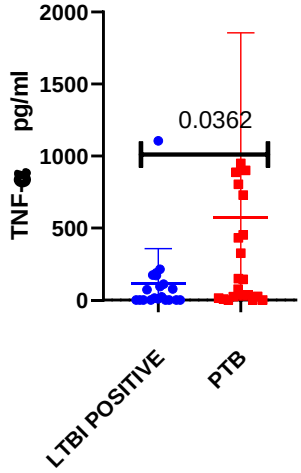



Figure 15. TNF- $\alpha$ level after antigen 24 stimulation. 0.0362 Refers to the P value which is the significant value ($p < 0.05$); statistical analysis was performed using Mann–Whitney U test. Blue circles represent the LTBI Individual response for antigen 24. Red Square boxes represent the Individual patient response to antigen 24

PROTEOMIC CHARACTERISATION OF IMMUNOLOGICALLY IMPORTANT FRACTIONS: Proteomic characterisation of the immunologically important fractions were carried out as described. Due to the issues in trypsin digestion, we were not able to identify proteins in the fractions Ag 6, Ag 11 and Ag 23 **(Table 2).** In the fraction Ag 24, {Cell wall high molecular weight pool}. , we had identified 4 proteins in (ACN, KatG, Rv2623, Rv0404).

Table 2. Proteins identified in the immunologically important fractions:

| Pooled fraction Name | No fractions pooled | Protein Identified | Rv number | Subcellular location | Immunological studies available | In our study Able to differentiate |
|---|---|---|---|---|---|---|
| **Ag 6** | 20 fractions- cell membrane medium molecular weight fractions | No protein identified | NA | NA | NA | QFT pos & PTB |
| **Ag 11** | 20 fractions- Cell Membrane high molecular weight proteins | Proteins yet to be identified | NA | NA | NA | QFT pos & PTB |
| **Ag23** | 10 fractions- cell wall high molecular weight fractions | Proteins yet to be identified | NA | NA | NA | QFT pos & PTB |
| **Ag24** | 10 fractions- cell wall high-weight fractions | Catalase-peroxidase (katG ) | Rv1908c | cell wall, cytosol, plasma membrane | No report | QFT pos & PTB |
| | | Aconitate hydratase A (ACN) | Rv1475c | cell wall, cytosol, plasma membrane | No report | QFT pos & PTB |
| | | Universal stress protein Rv2623 (TB31.7) | Rv2623 | cell wall, cytosol, plasma membrane | Yes | QFT pos & PTB |
| | | Chaperonin GroEL 2 | Rv0440 | Secreted, capsule, Cell surface, Secreted, cell wall | Yes | QFT pos & PTB |

The proteins in the bold letter represents novel T cell antigens identified in this study. NA – Not available.

## Discussion

In our study, we addressed the technical challenges associated with the hydrophobic nature of the cell wall and cell membrane proteins of *M. tuberculosis* and providing a novel two dimensional separation approach. This involved the fractionation of the hydrophobic membrane and cell wall proteins by isoelectric point by using the rotofor IEF system. We have improved the solibilization of these proteins with the help of a high concentration of the chaotropic agent (urea) and detergents (Digitonin). This important approach allowed for loading the higher concentration proteins without solubilization issues, and these solubilizing agents were integral part of the IEF buffer thus preventing further protein dilution. The use of Rotofor system separated the fractions based on the molecular weight and subsequently eluted using whole gel eluter for the immunological and proteomic analysis without any further purification steps involved.

Earlier studies[26] by others and our earlier study[2] have shown that these eluted fractions are non-toxic in nature and can be directly used for immunological analysis. Similarly, our earlier analysis has shown that these fractions can be directly subject to mass spectrometry-based proteomic analysis for the identification of the proteins by using in solution digestion approach[2]. Thus, we applied two successfully used experimental methods for the soluble proteins analysis for the separation of insoluble cell membrane and cell wall proteins. We separated the cell wall into 104 fractions and the cell membrane into 248 fractions. These separated fractions were subjected to immunological analysis using the whole blood culture method. Results of the immunological analysis show that, as observed in the earlier studies[27,28], the whole blood culture method is a convenient and efficient method of systematic screening of the large number of fractions of immunological analysis.

Earlier studies that aimed to use the identification and characterization of membrane and cell wall protein used 1D, 2D SDS-PAGE as a separation method[29]. In those studies identified proteins were not analyzed for their immunological role.

To our knowledge, our study marks the first successfully attempt in separating *M. tuberculosis* cell wall and cell membrane proteins into fractions that can be directly used for immunological and proteomic analysis. This is very significant given the importance of understanding tuberculosis immunology for development of new diagnostic tools for latent and active tuberculosis.

Earlier studies have shown that many mycobacterial proteins and cytokines were a potential marker for tuberculosis diagnosis[30]. These studies mainly used secreted or soluble proteins as their target of interest[31] and only limited information was available for the cell wall and cell membrane proteins as markers for tuberculosis diagnosis.

Many isolation and purification methods for mycobacterial cell wall and cell membrane were described[32, 33]. This approach is mainly used for isolation and mass spectrometry identification of these proteins, and research on Mycobacterial cell Membrane proteins is mainly focused as target for anti-mycobacterial drugs[34]. Our study has shown

a novel systematic approach for membrane protein useful in proteomic and immunological analysis. This analysis helped in the identification of fractions that induce high interferon-gamma in the LTBI subjects. In the LTBI-specific fractions,

In our analysis, also we also identified 4 cell wall high molecular weight proteins (ACN, KatG, Rv2623, Rv0404) in fraction 24two of which (ACN & Rv2623) had not been studied previously for their immunological role. The other two cell wall proteins (KatG, Rv0404) were previously reported to be involved in modulating anti tuberculosis immune response[35, 36].

Moreover, our findings highlights the significance of Rv2623 protein initially being identified as a highly expressed protein during hypoxic stress[37] and for establishment of infection in mouse models[38,39]. This protein also plays an important role in the basal level of drug resistance in *M. tuberculosis*[40]. Ofloxacin and Moxifloxacin drug treatment induced increased expression of this protein in *M. tuberculosis*[41]. The above-explained studies have shown that this protein plays an important role in drug resistance and the establishment of chronic tuberculosis infection. In CSF, antibody levels against this protein significantly increased latent and active tuberculosis meningitis subjects compared with healthy controls[42]. In the Chinese population, this antigen increased significantly higher IFN –gamma response in both tuberculosis patients and LTBI subjects compared with QFT-negative healthy controls[43]. Importantly our analysis, demonstrated significantly higher IFN gamma response in latent tuberculosis compared with PTB on stimulation containing this protein pool. In Wang et al. study[43] they used recombinant protein expressed in the E.coli as the testing antigen, whereas in our study, we used *M. tuberculosis* native antigen as the test antigen. The source of the antigen used in our study being native antigen may have contributed to this differential immune response. We identified Aconitase (Acn) protein (Rv1475c) in the LTBI-specific fractions. Aconitase is an iron-sulfur protein that catalyzes the inter-conversion of citrate and isocitrate via cis-aconitate in the Krebs and glyoxylate cycles[44].

*M. tuberculosis* aconitase was shown to have bifunctional activity[45]. Apart from functioning as a metabolic enzyme this protein also functions as an iron-responsive protein (IRP). This protein is involved in the iron homeostasis in *M. tuberculosis*. The immunological role of this protein so far has not been reported. Our study reported this protein as a potential LTBI-specific protein.

Our study findings suggest that the KatG protein is another potential LTBI-specific protein. Earlier studies on these proteins helped us to get a better understanding of this protein's molecular function as well as its role in *M. tuberculosis* drug resistance. But limited information is available on the immunological role of KatG protein in human tuberculosis infection. Further immunological analysis of this protein will help to better understand tuberculosis biology.

Another protein identified in this study GroEL2 is a well-reported immunodominant antigen.

Our study has thus introduced a systematic approach for cell wall and cell membrane protein analysis, aiding in the identification of

LTBI-specific proteins. Existing QFT IT test antigens are not able to differentiate LTBI and active tuberculosis infection in endemic settings. These findings hold the potential for differentiation of LTBI from tuberculosis disease along with QFT antigens in endemic settings, which is a critical need in the fight against tuberculosis.

Studies have shown that one-third of the world's population is infected with *M. tuberculosis*. A small fraction of this LTBI population (5 to 15%) develop active tuberculosis. A key part of the End TB Strategy is targeted toward the treatment of those infected and at risk for progression to active TB disease. These antigen-specific IFN-gamma responses could also serve as a potential biomarker for monitoring these LTBI subjects that could develop TB disease.

## Conclusion:

Based on the finding from this study, we were able to identify 4 proteins (ACN, KatG, Rv2623, Rv0404) from the cell wall of virulent clinical isolate S7, that was able to differentially induce IFN-Gamma response in TB-infected (LTBI) and TB disease subjects. It's important to note that the study had limitations, including a small sample size for immunological assays and a cross-sectional subject group. To validate the significance of these four antigens, further studies are warranted in larger follow-up cohorts. These studies could provide valuable biomarkers for identifying the 5% of TB-infected individuals at risk of developing TB disease, allowing for targeted interventions that can significantly reduce TB transmission. This work holds great promise for addressing this crucial issue in tuberculosis control.

## Conflicts of Interest Statement:

All authors would like to declare that, they do not have any conflict of interest

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## Author contributions:

Designed research studies: AD, RB and UR. Conducted experiments: AD, MD, SS and MT. Data analysis and interpretation: AD, MD, SS, MT, RA, VS, RB and UR. Contributed towards clinical and instrumental resources: RA, VS and JNS. Wrote the manuscript: AD, RB and UR. Manuscript review and editing: AD, JNS, RB and UR. All authors contributed to the article and approved the submitted version

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