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IDENTIFICATION OF CELL-WALL SPECIFIC PROTEIN OF *MYCOBACTERIUM TUBERCULOSIS* BY VISUAL COMPARISON OF 2-DIMENSIONAL CYTOSOLIC, MEMBRANE AND CELL-WALL FRACTIONS PROTEINS GELS OF *M. TUBERCULOSIS*.

DIVYA PANDEY[1], SUDHIR K. SINHA[2] AND VINEET K. MAURYA*[2,3]

[1]Modern Institute of Technology, Rishikesh, India.
*[*2]Central Drug Research Institute, Lucknow, India.*
[3]Deptatment of Microbiology, H.N.B. Garhwal University, Srinagar Garhwal. India.

ABSTRACT

Cell-wall is a very important component of bacteria, which besides making a protective outer covering, saves bacteria from harmful effects of antimicrobials. *Mycobacterium tuberculosis* (*Mtb*), causative agent of tuberculosis caused around 10.4 million new infections and 1.8 million deaths worldwide, during 2015. Despite all scientific advancement no new drug or vaccine has released in market for control of TB. Although global incidence rate of TB incidence has shown 1.5% declination (from 2014 to 2015), cases of TB-HIV alliance and single/multiple drug resistance are increasing. Lipid enriched thick cell-wall of *Mtb* is one of the reason for success of *Mtb* as human pathogen because it imparts an impervious covering against many drugs. Cell-wall of *Mtb* can be probed for possible drug targets against tuberculosis, because like other bacteria *Mtb* cell-wall is of utmost importance to it and any weakening or damage to it would directly affect the survival of bacteria. Concept of visual comparison of classical 1D gel and 2D gels of CW, CM and CST fraction proteins was followed in this study to find out true cell-wall proteins. Comparison of 1DGE gels showed four clear bands, while comparison of 2DGE gels showed six proteins which are supposed to be true cell-wall proteins. This study offers the identification of cell-wall specific proteins with higher probability and saving of time and resources both, compared to methods followed by other.

KEY WORDS: ***Mycobacterium tuberculosis, Cell wall, Drug targets, 2-Dimensional gel electrophoresis and comparative proteomics.***

VINEET K. MAURYA

Deptatment of Microbiology, H.N.B. Garhwal University, Srinagar Garhwal. India
Central Drug Research Institute, Lucknow, India

***Corresponding Author**

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INTRODUCTION

Cell-wall is a very important component of bacteria. Besides providing an outer firm, protective covering it also allows transport of nutrient, waste material, secretory products[1,2,3] and play important role in establishing host-pathogen interaction,[4,5] in case of pathogenic bacteria. Cell-wall remains a primary site of action of many known antibiotics such as penicillin and due to its paramount importance to bacteria, [6-10] it worth to be probed for search of new drug targets. Tuberculosis (TB), remains a serious threat to human health worldwide, causing an estimated 10.4 million new active TB cases and 1.8 million deaths, during 2015.[11] Among global active TB cases, 95% cases were reported in low and middle income countries, including 2.72 million active-TB cases in Africa and 4.74 million cases in South- East- Asia. Six countries, accounting for 60% of the global TB burden are; India, Indonesia, China, Nigeria, Pakistan and South Africa. In this group India is leading with 2.84 million cases followed by 1.02 million cases in Indonesia, 0.918 million cases in China and 0.454 million cases in South Africa, in 2015.[12] One million cases of child morbidity and death of 0.17 million children were also reported in 2015 due to TB. Although global incidence rate of TB incidence has shown 1.5% declination (from 2014 to 2015), cases of TB-HIV alliance and single/multiple drug resistance are increasing. Out of 1.8 million global deaths due to TB, 0.4 million cases were due to HIV-TB both and TB proves to be more dangerous than HIV, claiming 1.4 million deaths compared to 0.8 million deaths due to HIV alone. Among HIV infected patients, HIV-TB alliance caused death of 35% patients in 2015.[11,12] There were 0.48 million case of MDR-TB with additional 0.1 million cases of rifampicin resistance.[12] Despite of all scientific advancement now new drug or vaccine has released in market for control of TB[13] and emerging cases of MDR/XDR TB,[14-16] coalition of TB-HIV[17,18] are deteriorating the situation.[13] Success of Mycobacterium tuberculosis (*Mtb*) as intracellular pathogen can be accounted for many reasons and mycolic acid rich thick cell-wall is one of them, which provides impervious barrier for many of drugs.[19-22] When a pathogenic bacterium like *Mtb* infects the human body, the pattern recognition receptors on innate immune cells recognize the pathogen associated molecular patterns (PAMPs) on cell-wall of *Mtb.*[23,24] This interaction is responsible for final outcome of infection, whether it will be cleared without causing TB or active/latent tuberculosis would be established.[25,26] Considering the importance of PAMPs, it can be hypothesized that cell-wall proteins which are important players of *Mtb* PAMPs, can be probed as possible drug targets. Cell-wall associated proteins of *Mtb* can be targeted to affect cell-wall structure/function or to modify the PAMPs so that *Mtb* infection could be controlled by innate immune system at initial stages of infection. There are many proteins which has been identified as potential drug target which includes; *Icl*, *PcaA* , *KasA* and *KasB*, *Erp, fadD28, mmpL7, RpoV* and *SigB,* out of which *KasA* and *KasB* have important role in cell-wall biosynthesis *of Mtb.*[27] For all these purposes, detailed information about cell-wall associated proteins of *Mtb* are urgently required, which is obstructed till date, owing to complex nature of *Mtb* cell wall. Genome of *Mtb* predicts for 4012 proteins[28], out of which only about 72% has been identified till date, and only few of them are cell-wall associated ones.[29] Studies focusing solely on cell-wall proteins of *Mtb* are sparse. First global proteomics study of *Mtb* cell-wall was performed by Ida Rosekrands,[30] who identified 23 proteins in their work. Studies by He Z. and Buck De-J. on *M. avium* cell-wall,[31] Gengenbacher M. *et al* with *Mtb*,[32] Lisa M et al., on *Mtb*,[33] Mattow J. 2001 and 2003 with *M. bovis* BCG[34,35] can be taken as example of *Mtb* cell-wall associated proteins studies. All these studies showed a considerable overlapping of membrane and cytoplasmic proteins in cell-wall fraction. Since the cell-wall is in close contact with membrane and all secreted proteins have to passed through it, isolation of pure cell-wall associated proteins, free from cytosolic, membrane and secreted proteins is a cumbersome task. There is no any extraction protocol available till date which could isolate the cell-wall associated proteins in pure form. So here we focused on finding true cell-wall associated proteins by gel comparison method, in the present study. We followed a unique but simple concept, based on comparing protein profiling of cytosolic (CST), membrane (CM) and cell-wall (CW) fraction proteins of *Mtb,* using conventional 1-Dimensional SDS-PAGE gels (1DGE) and 2-Dimensional gel electrophoresis (2DGE), which had not been followed by any other research group. The concept says "membrane and cytosolic protein have close association with cell-wall of *Mtb*, hence these CM/CS proteins contaminate the CW fraction.[36,37] Due to this phenomenon, protein analysis of all CS, CW and CM fraction will show presence of CS proteins in all three (CS,CM and CW) fraction, CM protein in 2 (CM and CW)fractions but presence of CW proteins exclusively in CW fractions only. So those proteins which will be present exclusively in CW fraction are supposed to be true cell-wall associated protein."

MATERIAL AND METHODS

Growth and harvesting of Mtb H37Rv bacilli.

Lowenstein-Jensen Medium slants were prepared in inspissator, followed by inoculation with *Mtb* H37Rv strain and incubation for 3-4 weeks at 37ºC. After incubation period, bacilli was harvested in sonication buffer (50 mM Tris,10mM $MgCl_2$,1mM EGTA, 1mM PMSF and 1mM Na_2Z, pH 7.4) by loop scraping.[38]

Preparation of Cell-wall (CW), TCA precipitated Cytosolic (CST), Cytosolic (CS) and Membrane (CM) fractions.

Harvested bacilli were suspended in sonication buffer and probe sonicated for lysis of bacterial cells. Sonication was performed for 20 min with pulse on time for 10 seconds and pulse off time for 30 seconds. After sonication, lysate was centrifuged at 14000 rpm for 20 min, to separate insoluble cell-wall fraction (CW) and soluble lysate (supernatant). Supernatant was ultra centrifuged at 150000*xg* for 90 minutes, to separate insoluble membrane (CM) fraction and soluble cytosol (CS and CST) fraction.[39] a. Recovery of proteins from CW: CW fraction was first treated with a cocktail of Chloroform:Methanol:Water (10:10:3 v/v) for 2hrs at room temperature, under constant agitation. Then it was

centrifuged at 27000×g for 15 minute, followed by washing the pellets twice with Chloroform:Methanol:Water (10:10:3 v/v) and once with chilled acetone.[33] b. Recovery of proteins from CST: Proteins from soluble cytosol fraction were precipitated using 15% TCA (v/v) under cold conditions. Precipitate was washed with chilled acetone to remove TCA.[38,39] c. Recovery of proteins from CS: A small part of soluble cytosol fraction was kept untreated from TCA and used as such for next steps. This fraction was called CS. d. Recovery of proteins form CM: CM fraction was resuspended in TDW and used as such for further experimental procedures.[38,39] Protein estimation was performed using 'Modifed Lowry's method',[40] after estimation proteins were aliquoted, lyophilized and stored at -20°C.

1D Electrophoresis or SDS-PAGE of CW, CST, CS and CM fractions

100µg of lyophilized proteins of CW fraction was solubilised in 100µl of sample loading buffer (having 5% v/v β-mercaptoethanol) and incubated in boliling water for 10 minutes. 30µl and 20µl of this mixture was incubated into two separate wells of SDS-PAGE. Same procedure was repeated for CS and CW fraction. After sample loading, gel electrophoresis was performed at 15mA current for 90 minutes and gel was stained with Coomassie brilliant blue R250.[38,39]

2-Dimensional gel electrophoresis (2D-GE) of CW, CM and CST fractions

2D GE was performed using IPGs either of 7 cm (Bio-rad, USA). 200 µg lyophilized proteins of CW fraction was mixed with 200 µl sample solubilization buffer. After centrifugation (12,000 rpm x 10 min, to remove insoluble matter), 150 µl of this mixture was applied to IPG strips of 4-7 pH range and incubated overnight at 25°C. Isoelectric focusing (IEF) of the rehydrated strips was performed at 20°C temperature, using Protean IEF (Bio-rad, USA) with following parameters: (i) 0–250 V in 1:30 h, (ii) 250 V- 2500 V in 3 hrs, (iii) 2500 V constant for 3hrs, and maximum current of 50µA was allowed for each strip. After IEF, IPG strips were equilibrated for 10 min each in solutions 'A' (0.05 M Tris–HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, 1% DTT) and 'B'(0.05 M Tris–HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, 4% iodoacetamide, 0.005% bromophenol blue). Later, the strip was loaded on top of a SDS-polyacrylamide gel slab (12% gel, 1 mm thick) and electrophoresis was performed at a constant current of 15 mA.[41] Gels were stained with Coomassie brilliant blue R-250. The same processes were repeated for CM and CS fraction using separate IPG strips.

Identification of differentially expressed proteins on 2D gels.

Gel imaging and analysis was performed using ProExpress 2D Proteomic Imaging system (Perkin Elmer, USA) with exposure time of 900miliseconds.

RESULTS

1DGE gels and 2DGE gels of different fraction proteins.

1DGE gel images and their comparisons of CW, CS, CST and CM fraction proteins are shown in figure-1. Individual images of 2DGE gels of CST, CW and CM fraction proteins are shown in figure- 2, 3 and 4 respectively. 2DGE gels of CST, CW and CM fraction proteins showed 292, 128 and 222 spots, respectively.

1DGE Gel image of isolated protein fractions of *Mtb*

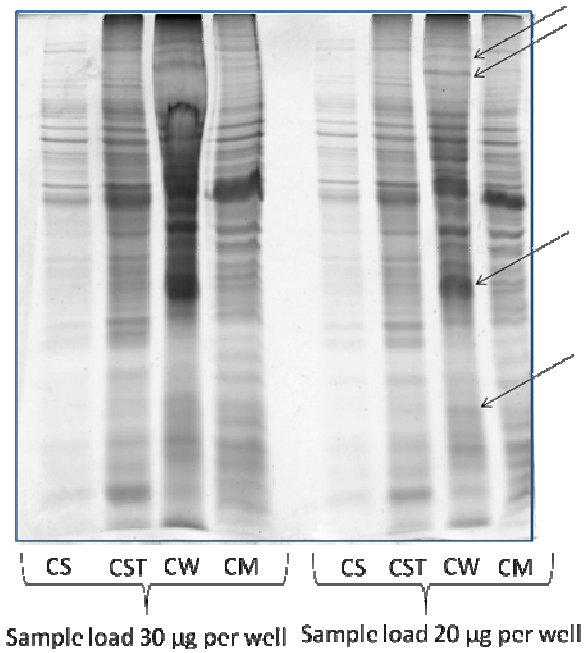

Figure 1

1DGE gel image and their comparison of CS, CST, CW and CM fractions proteins of Mycobacterium tuberculosis H37Rv. Four bands, appearing exclusively in CW fraction are marked with arrow.

2DGE image of Mtb cytosolic fraction proteins

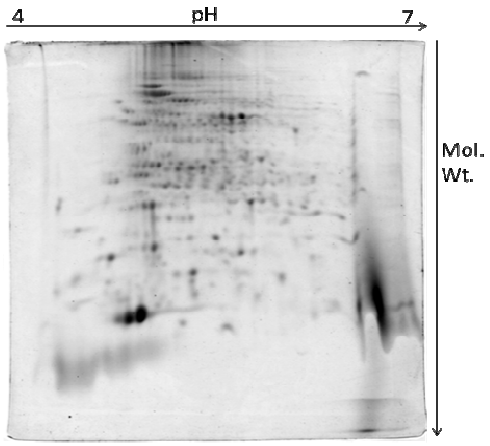

Figure 2
2DGE gel image of CST fraction proteins of *M. tuberculosis* H37Rv, using 7cm IPG strip of pH range (4-7) from Bio-rad.

2DGE image of cell wall fraction proteins of *Mtb*

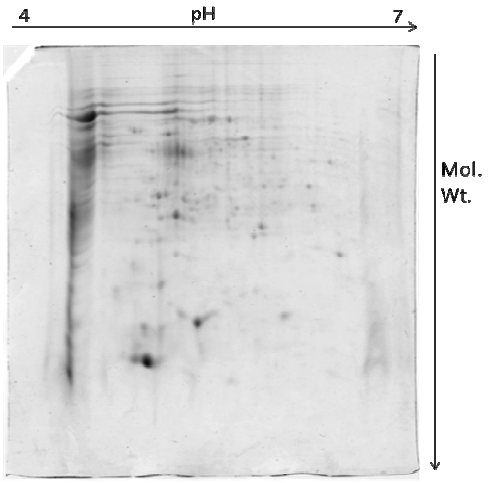

Figure 3
2DGE gel image of CW fraction proteins of M. tuberculosis H37Rv, using 7cm IPG strip of pH range (4-7) from Bio-rad.

2DGE gel image of membrane fraction proteins of *Mtb*

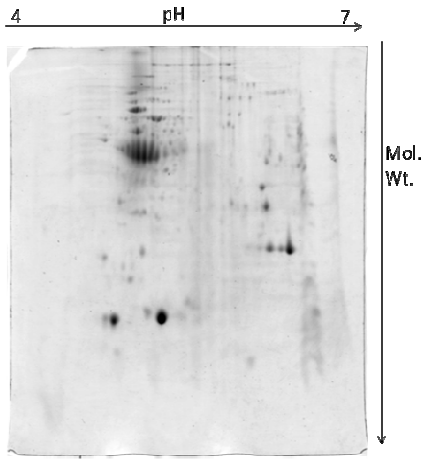

Figure 4
2DGE gel image of CM fraction proteins of *M.tuberculosis* H37Rv, using 7cm IPG strip of pH range (4-7) from Bio-rad.

Comparison of 1DGE and 2DGE gel images of cell-wall fraction proteins with that of CM and CST fraction protein gel images.

Comparison of 2DGE images of CST, CW and CM fraction proteins

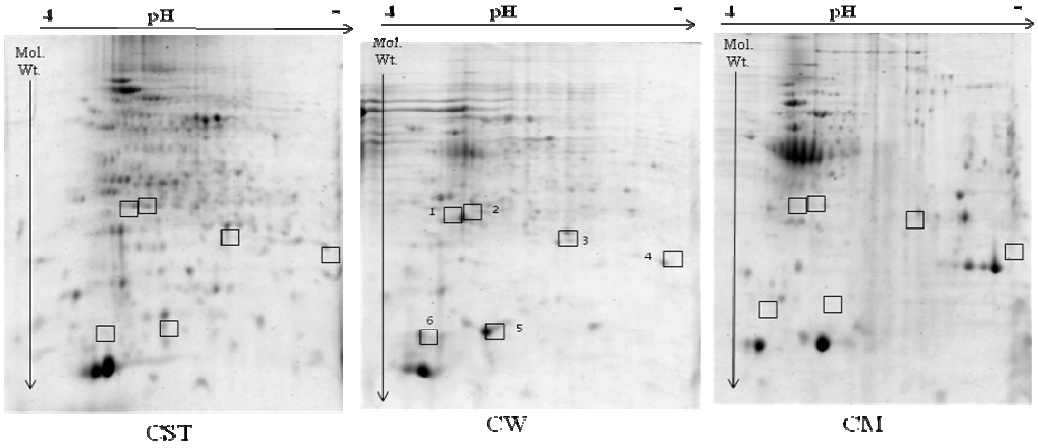

Figure 5

Comparison of 2DGE gel images of CW fraction proteins of M. tuberculosis H37Rv with that of CST and CM fraction proteins. 6 protein spots appearing exclusively in CW fraction gels are shown in rectangles and numbered accordingly.

There is a clear difference in 1DGE band pattern of all the three samples, and cell-wall fraction contains four extra bands which represent cell-wall proteins. Bands present in CST are intense compared to CS, which shows the efficacy of TCA precipitation.[46] Comparison of 2DGE gel images of all three CW, CS, CST and CM fraction proteins is shown in figure.5. Comparison of 2DGE gel images of all three fractions shows presence of six cell-wall proteins specific spots, which are present in cell-wall fraction only. These six spots can be considered as true cell-wall proteins. There are many spots which are common in all three fractions, which confirm the concept that CW fraction is contaminated by other fraction, hence it contains the proteins from both CST and CM fractions.

DISCUSSION

Comparative proteomics is powerful approach to decipher phenotypic, physiological, temporal difference of an organism and merits further application in tuberculosis too.[34,42] Proper solubilization and obtaining pure protein fraction are bottleneck for success of comparative proteomics.[43] Due to this reason membrane and cell-wall proteomics of *Mtb* has not gained much success, and lipid rich cell-wall increase the dimension to this difficulty. Due to various obstacles like; improper solubiliztion, contamination with other fractions, extreme pH, very low abundance etc, till date only ~72% of proteome of *Mtb* predicted by genome[28] sequence have been identified.[29] Critical observation of all these studies reveals that during all the studies different fractionation methods were unable to provide pure fractions and most of the time cytoslic, membrane and cell-wall associated proteins were present in all the fractions, due to which information about true cell-wall associated proteins is scarce. Comparative study of these entire three fraction was necessary because cell-wall fraction of bacilli contains proteins from both cytosol and membrane fractions. Contamination of cytosolic proteins in CW fraction is due to unbroken bacilli, which remain unlysed during sonication. Close physical association of membrane with cell-wall[36,37] and projection of many membrane proteins in cell-wall cause contamination of membrane proteins in CW fraction. To segregate these contaminating cytosolic and membrane proteins from cell-wall proteins, and to find out true cell-wall associated proteins, comparative study of these three fractions was performed which provided hopeful results. At first part of experiment, comparison of 1D gel pattern of CW, CST and CM fraction exhibited the presence of four clearly visible cell-wall specific protein bands that were missing in other two fractions. On the basis of 1D gel comparison results it was sought that comparison of 2D gels would definitely provide some more information about cell-wall specific proteins, and concept came true. 2D-GE offers a pattern of spots over entire gel area in which each spots represent a single protein. Comparative study of 2D gels of CW, CST and CM fraction proteins of *Mtb* showed that there were 6 protein spots, those were present in cell-wall fractions only. These 6 spots represent proteins from cell-wall of *Mtb* only. Identification of these 6 spots via MALDI-TOF would provide identification of proteins present in those spots [44, 45] and those proteins will be true cell-wall proteins of cell-wall associated proteins. These proteins can be validated further for their suitability as drug targets against tuberculosis. Not only this, these proteins can be used as a standard, and any new drug which is supposed to act via cell-wall synthesis mechanism can be tested against these proteins only, which will save time and cost of drug development. Comparison of CS and CST in present work shows efficacy of TCA precipitation using which intensity of protein band in clearly increased.[46] Present study has potential to provide information about cell-wall associated proteins of *Mtb*, which could be validated as potential drug targets. For their validation as drug-targets against tuberculosis, three dimensional structures of these proteins can be constructed *in-silico*

and their interacting partner can be identified using bio-informatics approaches. Once the protein is validated as potential drug-target, its *in-vitro* and *in-vivo* studies could be performed to check its efficacy in tuberculosis control. This study is extremely simple, time saving and can provide information about cell-wall proteins of pathogenic proteins other than *Mtb* too, thus can of great help in controlling other bacterial diseases.

CONCLUSION

This study came out with 6 spots that were present only in cell-wall. Identification of 6 spots instead of all 128 protein spots present on gels of CW fraction is comparatively easier, and offers higher probability of getting cell-wall proteins. As the cell-wall fraction is always contaminated with cytosolic and membrane proteins, identification of all the spots on CW fraction gel would be much time and resource consuming. Moreover identification of all proteins present on CW gel would lead to finding of most of the cytosolic or membrane proteins, and if CW specific spots could be selectively pick by gel comparison, as suggested in this work, the probability of getting true cell-wall protein will be much higher and would save time and resources both. The study could be extended by other research groups to the inclusion of MALDI-TOF-TOF and Nano-LC-MS-MS analysis of selected proteins spots, bands from gels, respectively. Due to limitation of fund and time this study was limited to visual identification of cell-wall specific proteins spots/ band of *Mtb* only and Identification of proteins present in selected spots/bands was not performed. Applying the same strategy of comparing cell-wall, cytosol and membrane fractions along with inclusion of MALDI-TOF-TOF/ Nano-LC-MS-MS analysis, cell-wall specific proteins of *Mtb* could be identified. Moreover by varying the length and pH of IPGs, and using gradient gels for instead of homogenous SDS-PAGE, more number of spots/bands could be identified from gels, which would add the knowledge of *Mtb* cell-wall protein in database of *M. tuberculosis complex*.

CONFLICT OF INTEREST

Conflict of interest declared none.

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Reviewers of this article

Dr. Rudra Dev Tripathi

Chief Scientist & Professor, Department of Environmental science, SCIR-National Botanical Research Institute, Lucknow 226001, India

G. Bakhya Shree M.S. (Research)

Coordinator and Trainer, Department of Biotechnology and Life Sciences, Dexter Academy, Madurai, Tamilnadu

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