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Original Article

# Immunoproteomic analysis of *Mycobacterium leprae* derived cell membrane antigens

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ABSTRACT

The aim of the study was serological characterization of antigens of cell membrane proteins (MLMA) of armadillo derived *Mycobacterium leprae,* a causative organism of leprosy. Cell membrane proteins of *Mycobacterium* leprae were separated using two dimensional gel electrophoresis (2-DGE) and then immunoblotted with sera from leprosy patients, tuberculosis patients, healthy individuals and anti-human IgG/IgA/IgM antibodies. The immunoblots thus obtained were compared and proteins corresponding to spots detected only in leprosy immunblots were finally analyzed for their characterization by MALDI-TOF/TOF. Employing this approach, 14 (6 with anti-IgA, 5 with anti-IgM and 3 with anti- IgG) immuno-reactive proteins with anti-human IgA/IgM/IgG were recorded. Out of these 14 proteins only 8 proteins (encoded as: MAL 1, MAL 4, MAL 5, MAL 6, MML 5, MML 6, MML 7 and MML 8) were identified to be *M. leprae* specific by MALDI-TOF/TOF and MASCOT search. Of these 8 proteins, 1 protein was (MAL1) identified as bacterioferritin (MMP-II) and remaining 7 proteins appeared to be as 5 isoforms of major membrane protein-1 (MMP-1), a 35kDa protein. To our knowledge this is the first report regarding existence of isoforms of MMP-1/35kDa protein. On preliminary examination for serological potential of these antigens, MAL5 was found to be the best (sensitivity= 82.6 specificity = 54.6%, efficiency= 68.9%) among IgA reactive proteins. On the other hand, antigen encoded as MML5 was best (sensitivity 36.4% with a specificity of 100.0%, efficiency of 70.8%) among IgM reactive proteins. Though seroreactive, as such both of these antigens do not seem to be very promising serodiagnostic reagents due to low specificity in case of MAL5 and low sensitivity in case of MML5 information obtained out of this study has opened paths towards dissection of these proteins at the peptide level with the view to look for their efficient serological potential.



## 1. Introduction

Leprosy is caused by *Mycobacterium leprae* which is a non-cultivable bacterium in artificial media rather due to its dividing nature in cooler regions of body it can be grown in armadillo [1]. Leprosy has been classified by Ridley and Jopling [2] on the basis of histological, microbiological and immunological parameters. There is heightened cell mediated immunity (CMI) induced by T cells (Th1 type of immunity) at tuberculoid pole while due to specific suppression of CMI in the lepromatous pole with heightened antibody response induced by B cells (Th2 type of immunity). These classified clinical conditions of leprosy viz. tuberculoid (TT), borderline tuberculoid (BT), borderline (BB), borderline lepromatous (BL) and lepromatous (LL) are diagnosed clinically by indicating the presence of anaesthetic skin lesions, thickened nerve and presence of acid fast bacilli in the smears of skin lesions [3]. However, a population of cases continues to be wrongly diagnosed due to lack of any discriminatory test, which in turn, could add to the load of occurrence of leprosy cases. Therefore, it is desirable that clinicians should have reliable laboratory tool to support the clinical diagnosis of leprosy without much problem. Over the years, the native antigens derived from armadillo cultured *Mycobacterium leprae* or recombinant protein antigens have been used as diagnostic reagents by several scientists in serological assays. Both, the cell wall lipids and cytosolic proteins have been studied for their utility in the sero-diagnosis by several scientists [4-8]. There are several serodiagnostic assays for leprosy such as radioimmunoassay [4] by using antigen 7 of *M leprae,* fluorescent antibody absorption (FLA-ABS) test [5, 9] by using whole cell *M leprae*, and many other

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ELISA based assays; especially based on phenolicglycolipid-I (PGL-I) [6, 10, 11], 35 kDa [7] antigen, 45kDa antigen [12], CFP-10 [13] and ESAT-6 [14]. However, none of these antigens singly was sufficient to detect all types, particularly smear negative paucibacillary (PB), of leprosy patients. Therefore, a need for better serologically potential antigen is still felt.

After whole genome sequencing of Mycobacterium leprae [15] it was found that there are about 2720 protein coding genes. And probably these genes are capable of producing some sero-reactive proteins specific to *M leprae.* If such proteins are available in proteome of *M leprae* then these sero-reactive proteins could be useful for development of better diagnostic tool(s) for leprosy. Brennan and his colleagues [8, 16] have accomplished first ever proteomics studies with *M. leprae*. They have identified/isolated 177 proteins (86 novel proteins) from cytosolic fraction corresponding to 27 genes, and 66 proteins (16 novel proteins) from membrane fraction corresponding to 29 genes. However these appear to be a fraction of proteome of *M. leprae* which probably have 1604 proteins. Looking at the technological developments, we are now ideally positioned to adopt a more convincing route to antigen identification. In the present study an attempt has been made employing immunobloting approach to detect *M. leprae* specific antigen with sera from leprosy patients, tuberculosis patients and healthy individuals. After comparing the patterns of seroreactive proteins (as indicated by spots) in the immunoblots, the antigens specific to *M.leprae* were identified and characterized by MALDI-TOF/TOF.

## 2. Materials and methods

### *2.1. Antigens*

*M leprae* cell membrane protein fraction (MLMA) was received as a gift from Department of Microbiology, immunology and pathology, Colorado State University, USA. The *M. leprae* used for preparation of this antigen was Armadillo derived.

### *2.2. Sera*

Serum samples were taken from clinically defined leprosy patients who visited out patients department (OPD) in Clinical Unit II of National JALMA Institute for Leprosy and other Mycobacterial Diseases (NJIL&OMD), Tajganj, Agra, India. Patients were considered to be untreated as per their statements. Sera of tuberculosis patients who visited a reputed Paramhans Yoganand pathology laboratory, Agra and who were positive for anti-M.Tuberculosis antibody employing ANDA-TB IgG, IgA and IgM ELISA kits (Anda Biologicals, France) were employed for the present study. Sera from healthy individuals working in our institute (National JALMA Institute for Leprosy and Other Mycobacerial Diseases, Taj Ganj, Agra, INDIA) were collected. An approval from the institutes' ethical committee was obtained for the study. All serum samples were collected after consent of participants of the investigation.

### *2.3. Two dimensional gel electrophoresis and electro-transfer of MLMA*

The cell membrane fraction from *M.leprae* was mixed with rehydration buffer (BioRAD 2D starter kit). Then isoelecrofocussing (IEF) was performed on ReadyStripsTM IPGstrips 7cm, 4-7pH (BioRAD). The program was set for passive re-hydration for 16 hrs at 20°C, step 1 for 1 hr at 250V, step 2 for 1 hrs at 250V constant, step 3 for 4 hrs at 3000V then step 4 for 15000VH at 3000V. The final concentration of the protein was kept as 50-60 g per IPG strip. The isoelectric focused gel strip was then placed in equilibration buffer I and II (2D starter kit BioRAD) for 15-20 minutes each. The equilibrated strips were then loaded on to 12% polyacrylamide gel. The current applied for resolving the proteins was 0.04 Amp (BioRAD protean MP3). The separation of proteins in the gel was visualized with silver staining. The electro transfer of the separated proteins was carried out on polyvinylidene fluoride (PVDF, Immobilon-P, 0.45 m Millipore) membrane for 2 hrs at 56V by using tris glycine buffer with 10% methanol (BioRAD Transblot System).

### *2.4. Immunobloting*

After transfer of separated proteins the PVDF membranes were dried at room temperature. Before processing for immunobloting the empty sites on membranes were blocked by incubating the membrane using 1% BSA (Bangalore Genei) in Tris-Buffer saline pH-8.0 (50mM Tris-HCl, 138 mM NaCl, 2.7mM KCl, pH 8.0) for 1hr at 37°C, followed by treatment with sera from leprosy, tuberculosis and healthy individuals for 2 hrs at 37°C. The antigen antibody complexes thus formed were visualized by using metal enhanced 3,3'-diaminobenzediene (Pierce biotechnologies) after treating the complex with biotin SP-conjugated antihuman IgG, IgA and IgM (Jackson Immunoresearch Inc., USA) followed by peroxidase labeled streptavidin.

### *2.5. Documentation and Image Analysis*

Digital image of each immunoblot and gel was made using high resolution CCD Camera (Chemidoc XRS System BioRAD). The *.tif and *.1sc images were then analysed by 'PDQuest Discovery series' software (BioRAD) and 'Decodon Delta 2D ver2.0'.

### *2.6. In-gel digestion of specifically reactive proteins and analysis by MALDI TOF/TOF*

The in-gel digestion of protein was carried out according to manufacturer's protocol provided with 'IGD profile Kit' (Sigma) Briefly, the spots corresponding to the specifically reactive proteins to leprosy sera (selected after matching the staining patterns obtained with sera from leprosy or tuberculosis or healthy individuals) were cut from two dimensionally electrophoresed gels and placed into a 0.5ml low binding PCR grade tubes (Eppendorf). For this purpose the silver stained gel pieces were de-stained by using freshly prepared 1:1(v/v) solution of 30mM $K_3Fe(CN)_6$ (Qualigens) and 100mM $Na_2S_2O_6.5H_2O$ (CDH) in 40mM $NH_4HCO_3$ (Qualigens). After this the gels were washed two times with 1:1 v/v solution of 40mM $NH_4HCO_3$ and acetonitrile (LobaChemi) for 10 minutes each and two times with 100% acetonitrile (ACN). These gel pieces were dried for an hour at 37°C. For alkylation of protein 40mM dithiothreitol $NH_4HCO_3$ was applied for 45minutes at 56°C and for reduction 50mM iodoacetamide (Sigma) in 40mM $NH_4HCO_3$ was applied for 30 minutes at 37°C to dried pieces. After this the gel pieces were washed again with 50% $NH_4HCO_3$/ACN and 100% ACN as before. After this 0.1ng/ml solution of trypsin (IGD Profile Kit Sigma) was used to digest the protein for 16 hrs at 37°C and 0.1% triflouroacetic acid (TFA) prepared in 50% acetonitrile was used for peptide extraction. The peptide extract thus prepared was then mixed with 20 mg/ml HCCA/CHCA (α-cyano-4-hydroxycinnamic acid) matrix in 0.1% TFA prepared in 50% acetonitrile (Bruker Daltonics) and was applied to Autoflex III MALDI-TOF/TOF (Bruker Daltonics) for analysis.

### *2.7. Protein identification*

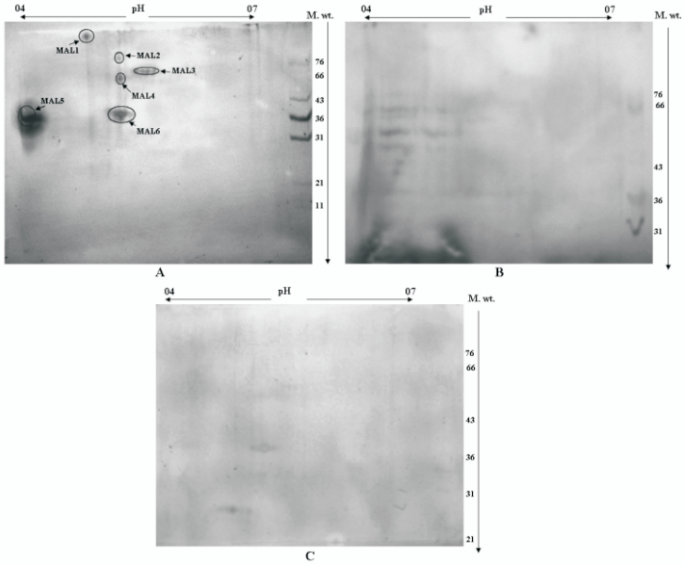
Employing the peptide mass fragment data obtained from MALDI-TOF/TOF analysis, proteins having the corresponding

mass were identified using MASCOT software (http://www.matrixscience.com). The missed cleavage sites were allowed and the tolerance mass error was up to 100 ppm. The criteria used to accept protein identifications were based on the extent of MASCOT score (minimum of 50 at p value >0.5). Lower scoring proteins were rejected. The NCBI database (http://www.ncbi.nlm.nih.gov) was searched for the identification and information about the potential function of the identified proteins.

### 3. Results and Discussion

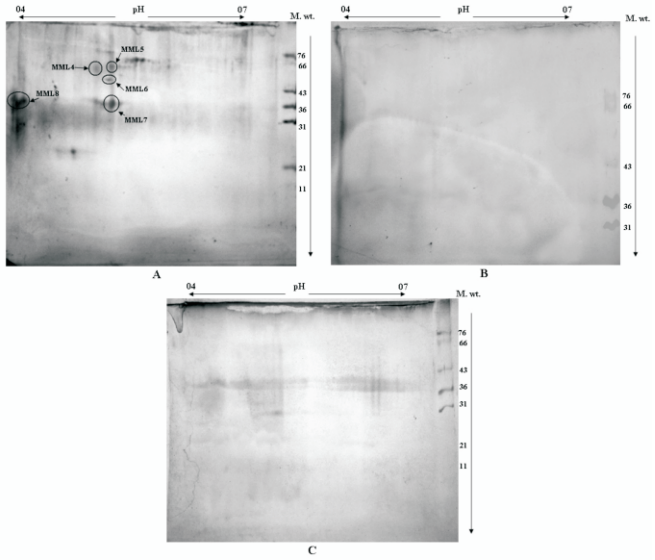
Tests based on humoral responses against *M. leprae* (serological tests) are very affordable and easy to perform. Therefore improvement of such tests is highly needed and in our opinion it can be achieved by identification of better antigens which must be serologically specific to *M. leprae.* To identify specific antigen we have used two-dimensional gel electrophoresis followed by immunoblotting and MALDI-TOF/TOF analysis. The *M.leprae* specific sero-reactive proteins were identified by comparing the immunoblots obtained with sera from leprosy, tuberculosis and healthy persons. The proteins reactive with leprosy sera but not with sera from tuberculosis patients and healthy individuals were considered to be *M.leprae* specific. To our knowledge it is first attempt of its kind towards identification of seroreactive proteins existing in *M.leprae* cell membrane.

Figure 1. IgA reactive antigens observed by immunobloting of *M. leprae* membrane antigen (MLMA) with sera from (A) Leprosy (B) Tuberculosis (C) Healthy subjects




To achieve the aimed target towards investigating specific antigen of *M. leprae,* we have analyzed 2-DGE separated proteins by developing respective immunoblots. The staining pattern (represented by spots) depicting the reactive proteins have been shown in Figure 1 and Figure 2. In the immunoblots various proteins of MLMA were found to be located between pH 4.0-5.5; and most of seroreactive protein antigens were of molecular weights from 25kDa to 45kDa. On comparing with reactivity towards tuberculosis and healthy sera, 11 immuno-reactve proteins (6 with anti-IgA only, 5 with anti-IgM only) were observed to be reactive specifically with sera from leprosy patients. None of the IgG reactive spots was found to be specific to *M.leprae* (Figure not shown). Earlier studies at genetic levels have reported that though, the gene encoding the 35kDa protein is not found in the *Mycobacterium tuberculosis* complex or *Mycobacterium bovis* BCG alone , its homologue with 82.0% DNA and 90.0% amino acid identity, respectively, has been found in *Mycobacterium avium* as well (17). However, knowledge regarding the serological cross-reactivity of *M.leprae* derived MMP-I/35kDa protein with the proteins from other microbes is scanty.

Figure 2. IgM reactive antigens observed by immunobloting of *M. leprae* membrane antigen (MLMA) with sera (A) Leprosy (B) Tuberculosis (C) Healthy subjects




The 11 *M. leprae* specific proteins as observed with immunoblots were further processed for their characterization by MALDI-TOF/TOF. The mass fragments obtained were searched in database (SwissProt 51.6) of 'other Actinobacteria class' by using "MASCOT search engine". The MASCOT provides probability based Mowse score of protein. The proteins found with homology (significant score with other bacteria) to other bacteria could not be taken as specific antigens as such antigens could give false results for leprosy diagnosis if used for the purpose. As shown in Table 1, of the 11 proteins only 10 were found to be suitable for their identification/prediction as a proteins. Of the 10 significantly scoring proteins only 8 were found to be *M.leprae* specific. Among these 8 spots, 4 were IgA reactive and other 4 were IgM reactive. Of the 8 proteins 1 spot (MAL1) was predicted to be bacterioferritin (MMP-II) where as remaining 7 proteins were predicted to be MMP-I /35kDa protein. In the recent past, MMP-II has been reported to be a serological reagent for detection of IgG type of antibodies for diagnosis of leprosy [18]. However, its performance with reference to detection of IgA and IgM type of anti-*M.leprae* antibodies for diagnosis of leprosy still remains unexplored. The presence of 35kDa protein in leprosy bacillus has been established by a series of studies following immuno-biochemical purification techniques [19-21]. It is one of the membrane components of *M. leprae* and comprises 0.05% of the total M. leprae protein. Another interesting observation noticed in the study was that of the 7 sero-reactive MMP-I proteins, 3 IgA reactive proteins encoded as MAL4, MAL5 and MAL6 were similar (considering their PI values as well as molecular weights as features) to the 3 IgM reactive proteins encoded as MML7, MML8 and MML6 were respectively (Table 2.). Thus present findings indicated the existence of 5 isoforms (in all) of MMP-I /35 kDa protein. To our knowledge present report is the only report in the literature indicating the existence of various iso-forms of MMP-I/35kDa protein of *M.leprae*. Previous studies regarding conventional

35-kDa protein of *M. leprae* have demonstrated 35kDa to be a major target for the IgG antibody response that too primarily in lepromatous leprosy [22-24]. Since the searched isoforms were reactive against IgA or IgM antibody, serologically also it appears regarding *M.leprae* infection. that various isoforms of protein described by us could be variants of the conventional 35kDa protein which might have been generated by post translational modifications occurring inside the bacteria and host. It would be interesting to understand their immuno-biological significance, if any, of these isoforms.regarding *M.leprae* infection.

The specificity and sensitivity of the identified eight specific antigen of *M. leprae* were analysed from results of immunoblots obtained with against sera from leprosy patients, tuberculosis patients and healthy subjects. The respective results with various proteins have been displayed in Table 3 and 4. From the findings we could observe that of these 8 antigens, one IgA reactive antigen (encoded as MAL5) was found to be the best (sensitivity= 82.6 (19/23)

Table 1. Identification/prediction of IgA and IgM reactive proteins from MLMA

| Spot ID | DetectorAntibody | Protein identified /predicted | Remarks |
|---|---|---|---|
| MAL 1 | IgA | Bacterioferritin (BFR) (Major membrane protein II) (MMP-II) - *M. leprae,* Score: 51 | Specific to *M. leprae* |
| MAL 2 | IgA | 50S ribosomal protein L27 - *Nocardia farcinica,* Score: 51 | Non specific to *M. leprae* |
| MAL 3 | IgA | No significant score results ( Score less than 50) | No conclusion |
| MAL 4 | IgA | Major membrane protein I (MMP-I) (35 kDa antigen) - *M. leprae,* Score: 51 | Specific to *M. leprae* |
| MAL 5 | IgA | Major membrane protein I (MMP-I) (35 kDa antigen) - *M. leprae,* Score: 164 | Specific to *M. leprae* |
| MAL 6 | IgA | Major membrane protein I (MMP-I) (35 kDa antigen) - *M. leprae,* Score: 135 | Specific to *M. leprae* |
| MML 4 | IgM | Ribosome recycling factor (Ribosome-releasing factor) (RRF) - *Streptomyces avermitilis,* Score: 57 | Non specific to *M. leprae* |
| | | Fumarate hydratase class II (EC 4.2.1.2) (Fumarase C) - *Streptomyces coelicolor,* Score: 51 | |
| MML 5 | IgM | Major membrane protein I (MMP-I) (35 kDa antigen) - *M. leprae,* Score: 72 | Specific to *M. leprae* |
| MML 6 | IgM | Same spot as MAL4 Major membrane protein I (MMP-I) (35 kDa antigen) - *M. leprae,* score: 51 | Specific to *M. leprae* |
| MML 7 | IgM | Same spot as MAL6 Major membrane protein I (MMP-I) (35 kDa antigen) - *M. leprae,* Score: 171 | Specific to *M. leprae* |
| MML 8 | IgM | Same spot as MAL5 Major membrane protein I (MMP-I) (35 kDa antigen) - *M. leprae,* score: 51 | Specific to *M. leprae* |

Table 2. Molecular weights and PI values of various IgA and IgM reactive overlapping proteins.

| Protein Code | Molecular weight by MALDI | Molecular weight as calculated by Geldoc after 2 DGE separation | PI value by MALDI | PI value as calculated by Geldoc |
|---|---|---|---|---|
| MAL4 | 33.688 | 49 | 5.03 | 5.3 |
| MAL5 | 33.688 | 36 | 5.03 | 5.3 |
| MAL6 | 33.688 | 36 | 5.03 | 4.3 |
| MAL6 | 33.688 | 49 | 5.03 | 5.3 |
| MAL7 | 33.688 | 36 | 5.03 | 5.3 |
| MAL8 | 33.688 | 36 | 5.03 | 4.3 |

specificity = 54.6% (12/22); efficiency 68.9% (31/45). On the other hand, antigen encoded as MML5 was best (sensitivity 36.4% (4/11) with a specificity of 100.0% (13/13) and efficiency of 70.8% (17/24) among IgM reactive proteins. Though seroreactive, as such both of these antigens do not seem to be very promising serodiagnostic reagents due to low specificity in case of MAL5 and low sensitivity in case of MML5, information obtained out of this study has opened paths towards dissection of these proteins at the peptide level with the view to look for their efficient serological potential. Probably, this approach, eventually, may provide us some serological reagent for its application in detection of leprosy patients.

Table 3. Sensitivity and specificity of sero-reactive antigens from cell membrane of *M. leprae* employing anti IgA as detector antibody

| Groups | MAL1 | MAL4 | MAL5 | MAL6 |
|---|---|---|---|---|
| Leprosy | 7/23 (30.43%) | 8/23 (34.78%) | 19/23 (82.60%) | 21/23 (91.3%) |
| Tuberculosis | 4/9 (44.44%) | 5/9 (55.56%) | 6/9 (66.67%) | 8/9 (88.89%) |
| Healthy | 4/13 (30.77%) | 5/13 (38.46%) | 4/13 (30.77%) | 8/13 (61.54%) |
| Specificity | 14/22 (63.4%) | 12/22 (54.5%) | 12/22 (54.5%) | 6/22 (27.3%) |
| Efficiency | 21/45 (46.7%) | 20/45 (44.4%) | 31/45 (68.9%) | 27/45 (60.0%) |

Table 4. Sensitivity and specificity of sero-reactive antigens from cell membrane of *M. leprae* employing anti IgM as detector antibody

| Groups | MAL5 | MAL6 | MAL7 | MAL8 |
|---|---|---|---|---|
| Leprosy | 36.4% (4/11) | 36.4% (4/11) | 90.9% (10/11) | 72.7% (8/11) |
| Tuberculosis | 0% (0/7) | 0% (0/7) | 42.8% (3/7) | 42.8% (3/7) |
| Healthy | 0% (0/6) | 16.67% (1/6) | 66.6% (4/6) | 66.6% (4/6) |
| Specificity | 13/13 (100.0%) | 12/13 (92.3%) | 6/13 (46.2%) | 6/13 (46.2%) |
| Efficiency | 17/24 (70.8%) | 16/24 (66.7%) | 16/24 (66.7%) | 14/24(58.3%) |

## 4. Conclusions

Employing immunoblot followed by analysis by MALDI-TOF/TOF several isoforms of MMP-I/35kDa protein of *M.leprae* were identified. Of these proteins, two serologically better variants were detected. Considering their limited serological non-specific behavior these proteins need to be dissected to search serologically efficient peptides. The peptide(s) thus found could be useful additional reagents for detection of leprosy either alone or in combination with other antigens. This eventually could help early detection and treatment of leprosy patients and thereby in breaking of the transmission cycle. Additionally, investigations towards T stimulating property of MMP-I/35kDa proteins and their peptides could prove to be useful for developing diagnostic reagent involving cell mediated immunity; and also may prove to be worthwhile molecule for vaccine development against leprosy.

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