Send Orders for Reprints to reprints@benthamscience.net

Current Protein & Peptide Science, 2024, *25*, 613-625

RESEARCH ARTICLE

Dominant Circulating Cell-free Mycobacterial Proteins in In-use Machining Fluid and their Antigenicity Potential

Harish Chandra[1], Bethany Ahlers[2], Ying Wai Lam[2] and Jagjit S. Yadav[1,*]

[1]*Pulmonary/Microbial Pathogenesis and Immunotoxicology Laboratory, Department of Environmental and Public Health Sciences, University of Cincinnati College of Medicine, Cincinnati, OH45267-0056, USA;* [2]*Vermont Biomedical Research Network Proteomics Facility, University of Vermont, Burlington, VT05405, USA*

Abstract: ***Background*:** Occupational exposure to industrial Metalworking Fluid (MWF) colonized by *Mycobacterium immunogenum* (MI) has been associated with immune lung disease hypersensitivity pneumonitis (HP) in machinists. This warrants regular fluid monitoring for early detection of mycobacterial proteins, especially those with antigenic potential.

***Objective*:** To detect and identify dominant MI proteins and antigens directly from the field-drawn in-use MWF using an integrated immunoproteomic-immunoinformatic approach.

***Methods*:** An MI-positive MWF selected by DNA-based screening of several field-drawn MWF samples was cultured to isolate the colonizing strain and profiled for dominant circulating cell-free (ccf) MI proteins, including antigens using an integrated immunoproteomic (1D- and 2D-gel fractionation of seroreactive proteins combined with shotgun proteomic analysis using LC-MS/MS) and immunoinformatic strategy.

***Results*:** A new MI strain (MJY-27) was identified. The gel fractionated MI protein bands (1D-gel) or spots (2D-gel) seroreactive with anti-MI sera probes (Rabbit and Patient sera) yielded 86 MI proteins, 29 of which showed peptide abundance. T-cell epitope analysis revealed high (90-100%) binding frequency for HLA-I & II alleles for 13 of the 29 proteins. Their antigenicity analysis revealed the presence of 6 to 37 antigenic determinants. Interestingly, one of the identified candidates corresponded to an experimentally validated strong B- and T-cell antigen (AgD) from our laboratory culture-based studies.

***Conclusion*:** This first report on dominant proteins, including putative antigens of *M. immunogenum* prevalent in field in-use MWF, is a significant step towards the overall goal of developing fluid monitoring for exposure and disease risk assessment for HP development in machining environments.

ARTICLE HISTORY

Received: November 08, 2023
Revised: February 15, 2024
Accepted: February 21, 2024

DOI:
10.2174/0113892037291635240405042554

Keywords: Metalworking fluid, *Mycobacterium immunogenum*, protein, T-cell antigen, hypersensitivity pneumonitis, epitope, HLA allele.

1. INTRODUCTION

Machining fluids, a.k.a. metalworking fluids (MWFs) are used in metal operations for lubrication, cooling, and metal removal. Modern MWF formulations are complex aqueous fluids that can be either oil-in-water emulsions (semi-synthetic fluids) or water-based synthetic fluids. The complexity of these fluids can favor the growth of a variety of microorganisms, including pathogens, particularly non-tuberculous mycobacteria [1]. According to the National Institute for Occupational Safety and Health (NIOSH) estimates, about 1.2 million workers in different machining environments are potentially exposed to the aerosol mist of in-use MWF [2]. Machinists repeatedly exposed to microbial-contaminated MWF aerosols may develop respiratory conditions including hypersensitivity pneumonitis (HP), asthma, or other respiratory symptoms over a period of time [3, 4]. HP is a particularly difficult disease to diagnose because of the inhalation of antigens [5-7]. In recent decades, several cases and outbreaks of machinist's HP a.k.a machine operator's lung (MOL) have been reported in automotive plants and other machining operations [2]. According to recent reviews [8, 9], MWF exposure has become the most common and recognized cause of occupational HP, having been rare before 2000. It is now well recognized, based on human and animal studies, that machinist's HP pathology can be caused by repeated exposures to bioaerosols generated from the my-

* Address correspondence to this author at the Pulmonary/Microbial Pathogenesis and Immunotoxicology Laboratory, Department of Environmental and Public Health Sciences, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0056, USA; E-mail: jagjit.yadav@uc.edu

cobacteria-contaminated MWFs, particularly those containing *Mycobacterium immunogenum* that frequently colonizes these industrial fluids [3, 10-20]. In this regard, our laboratory has been instrumental in isolating multiple genotypes of MI from different MWF samples originating from diverse industrial MWF operations [21, 22]. Despite a recognized etiological role of MI in MWF-linked HP, it is not known which cell-free circulating proteins of this agent accumulate in the machining fluids that actually contribute to the immunogenicity leading to a MOL/HP lung in the exposed machinists. Our working hypothesis is that *M. immunogenum* proteins selectively induced under the prevailing field MWF conditions contribute to the circulating antigenic pool in the contaminated MWF. While recent efforts by us [20, 23-25] and other labs [26] have led to identification of immunoreactive proteins of MI from its laboratory-grown cultures in defined media, nothing is known on such antigenic proteins elaborated in industrial in-use MWF matrix. Our focus in the current study was to detect and identify dominant MI antigenic proteins directly from the field-drawn in-use MWF using an integrated immunoproteomic-immunoinformatic approach. Dominant MI proteins identified using LC-MS/MS were subjected to *in silico* prediction of T-cell and B-cell epitopes using Propred analysis and antigenicity analysis. Field MWF-associated mycobacterial proteins and their antigenic peptides/epitopes identified in this study could be exploited for the development of assays for routine fluid monitoring, including point-of-use tests/platforms to assess exposure and disease risk in machining environments.

2. MATERIALS AND METHODS

2.1. Molecular Screening for MI-contaminated Field in-use MWF Sample and Strain Identification

The rabbit anti-MI serum was commercially obtained. Microbial-contaminated in-use water-based metalworking fluid (MWF) samples used in this study were obtained from an industrial collaborator. The samples were screened for the presence of mycobacteria using a genus-specific PCR method developed in our earlier studies [27] based on the 65 kilodalton heat shock protein (hsp65) gene, as elaborated in the later part of this section. When combined with restriction analysis, this PCR-based assay can further differentiate *M. immunogenum* from closely related species of the *M. chelonae - M. abscessus* complex [28]. A mycobacteria-positive ($Myco^+$) MWF sample was thus selected, and a new *M. immunogenum* genotype/strain (designated MJY-27) was isolated and identified from this fluid as a part of this study. This involved culturing the Myco+ MWF sample using conventional microbiological approach and identifying the resulting mycobacterial isolates using morphological and DNA-based techniques [27]. Briefly, the selected field MWF sample was centrifuged (8000 rpm for 20 min. at 4°C), and the resulting cell pellet was spread-plated on Middlebrook 7H10 agar (30^oC) to isolate the colonizing Mycobacterium species. The mycobacterial nature of the colonies was tentatively identified based on colony morphology and acid-fast staining. The putative colonies were individually grown in Middlebrook 7H9 broth (Difco Laboratories, Sparks, MD, USA) using a shaker incubator (30^oC and 225 rpm) for further identification and speciation using our Amplified hsp65 Restriction Analysis (AHSPRA) method developed for the purpose [27]. This PCR-based detection and speciation method involved the amplification of a 667 bp fragment of the mycobacterial heat shock protein (*hsp*) gene using the following primer pairs: Hsp667 Forward: (5'-GGCCAAGACAATTGCGTACG-3') and Hsp667 Reverse: (5'-GGAGCTGACCAGCAGGATG-3'). The PCR amplicons were identified by restriction pattern analysis using *Bbv*I. All the isolates from Myco+ sample were confirmed as *M. immunogenum*. Further analysis of this MI isolate using amplicon DNA sequencing coupled with BLAST search and multiple sequence alignment against the hsp gene's 667 bp sequences for the previously known MI genotypes confirmed it to be a new genotype (designated as MJY-27).

2.2. Preparation of Total Microbial Protein Samples Directly from field MWF Samples

$Myco^+$ MWF sample was centrifuged as above to separate the cell pellet and the supernatant fractions. The cell pellet was stored at -80^oC for future use after washing thrice with 10 mM Tris-HCl (pH 7.5) containing protease inhibitor cocktail (Sigma, St. Louis, MO). The supernatant was further made cell-free by filtration using a 0.22-micron sterile filter. Filtered MWF supernatant was precipitated using 10% TCA to isolate the total microbial proteins present in the fluid [25]. The precipitated proteins were washed thrice with acetone and the washed protein pellet was air-dried and suspended in 10 mM Tris-HCl (pH 7.5) containing protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentration in this total protein extract was estimated by DC assay (Bio rad).

2.3. Immunoproteomic Identification of MI-specific Proteins in MWF

The total protein extract from the $Myco^+$ MWF sample was subjected to 1D- and 2D- SDS-polyacrylamide gel electrophoresis (SDS-PAGE). With an aim to capture antigenic proteins, the gels were subjected to immunoblot analysis using anti-MI rabbit sera (RS) probe or human HP patient sera (HS) probe as primary antibody (1:200) followed by reaction with HRP-conjugated goat anti-rabbit antibody (1:3000 dilution) or goat anti-human IgG antibody (Sigma USA). Finally, the blot was developed by chemiluminescence detection using ECL kit (Pierce Chemical, Rockford, IL, USA). RS probe represented anti-MI polyclonal serum raised in rabbits against a mixture of extracellular and intracellular protein extracts pooled from previously isolated six different MI genotypes, namely MJY-3, -4, -10, -13, -14, and -22 [22]. HS probe was derived by pooling sera from five physician-diagnosed HP patients (machinists), as described previously [24]. Total protein extract was directly separated on 12% SDS-PAGE using the conventional 1D-gel electrophoresis. The 2D-gel electrophoresis was performed as described previously [25] with slight modifications. Briefly, the isoelectric focusing (IEF) step was performed on a 7 cm

immobiline dry strip pH 4-7 (GE Healthcare, Piscataway, NJ). The strip after rehydration was focused using sequential steps with the following parameters: step 1 (50 V, 12 h), step 2 (100 V, 1 h), step 3 (500 V, 2 h), step 4 (1000 V, 1 h), step 5 (2000 V, 2 h), step 6 (8000 V, 2 h). The proteins resolved on the IEF strip were then separated in the second dimension using 12% SDS-PAGE at 70 V. The 1D- and 2D-gels were stained with SYPRO Ruby (Bio rad) for band/spot visualization and imaging. For Western blot analysis, a repeat gel (each for 1D and 2D) run in parallel was blotted onto nitrocellulose membrane using wet transfer protocol and analysis was performed using the anti-MI RS probe or HS probe (generated as described above). Protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The immunoreactive gel bands (1D) and gel spots (2D), picked up using direct excision and spot picker, respectively, were destained with 50% acetonitrile (ACN)/25 mM NH_4HCO_3 for 2–3 h. After reduction by 10 mM DTT and alkylation with 55 mM iodoacetamide, the gel pieces were washed with 100 mM NH_4HCO_3. They were dehydrated with ACN, dried in a SpeedVac, and subjected to digestion with trypsin (Promega, Madison, WI) for 18 h at 37°C. Peptides were extracted successively with 5% formic acid (FA)/50% ACN and 100% ACN. The dried digests were re-suspended in 20 µl of a solution of 2.5% ACN and 2.5% FA in water and 4 µl of the digest was analyzed by LC-MS/MS on a linear ion trap (LTQ) mass spectrometer (ThermoFisher Scientific). Peptides were loaded onto a 100 µm x 120 mm capillary fused silica column packed with MAGIC C18 (5 µm particle size, 20 nm pore size, Michrom Bioresources, CA) at a flow rate of 500 nL/min. Peptides were separated by a gradient of 5-40% ACN / 0.1% FA over 33.9 min, 40-100% ACN /0.1% FA in 1 min, and 100% ACN /0.1% FA for 5 min, followed by an immediate return to 2.5% ACN /0.1% FA and an isocratic hold at 2.5% ACN /0.1% FA until the next injection. Peptides were introduced into the linear ion trap *via* a nanospray ionization source and a laser-pulled ~3 µm orifice with a spray voltage of 1.8 kV. Mass spectrometry data were acquired in a data-dependent "Top 10" acquisition mode, in which a survey scan from *m/z* 360-2000 is followed by 10 collision-induced dissociation (CID) tandem mass spectrometry MS/MS scans of the most abundant ions. MS/MS scans were acquired with the following parameters: isolation width: 2 *m/z*, normalized collision energy: 35%, Activation Q: 0.250 and activation time = 30 ms. Dynamic exclusion was enabled (repeat count: 2; repeat duration: 30 sec; exclusion list size: 180; exclusion duration: 60 sec). The minimum threshold was 500. Product ion spectra were searched using the SEQUEST HT search engine implemented on the Proteome Discoverer 1.4 (ThermoFisher Scientific, Waltham, MA, USA) against a curated Uniprot *Mycobacterium immunogenum* database (UP000037962) in forward and reverse orientations. Search parameters were as follows: (1) full trypsin enzymatic activity; (2); maximum missed cleavages = 2; (3) min. peptides length = 6; (4) mass tolerance at 2 Da for precursor ions and 0.8 Da for fragment ions; (5) Dynamic modifications on methionine (+15.9949 Da: oxidation); (6) 4 maximum dynamic modifications allowed per peptide; and (7) static modification on cysteine (+57.0215 Da: carbamidomethylation). For the 2D-gel spot identification, peptides were filtered according to XCorr criteria of 1.5, 2.0, 2.25 for singly, doubly, and triply charged peptides, and a minimal number of peptides = 2. All protein identification information, including the proteins that were identified with only one peptide, are summarized in the Tables **S1-S4**. For the secretory proteome profiling by 1D- proteomics (analyzed in duplicate using rabbit and human sera as probe), the false positive rate of the search results was limited to less than 1% by the Target/Decoy PSM Validator node in the Proteome Discoverer workflow. Proteins that were identified in both replicates with more than 2 peptides were included for further analysis.

2.4. *In-silico* Identification of T-cell Epitopes in the Dominant MI Proteins

The dominant MI proteins, including putative immunoreactive candidates identified above using LC-MS/MS were further analyzed by subjecting their derived amino acid sequence to *in silico* prediction of T-cell epitopes using Propred I program on the Propred analysis server (www.imtech.res.in) as described elsewhere [29]; this program predicts promiscuous binding regions for 47 and 51 different HLA class I and II alleles, respectively. This allowed us to predict the candidate T-cell antigens based on detecting T-cell epitopes in their sequence.

2.5. Prediction of Antigenic Determinants (B-cell Epitopes) in the Identified MI Proteins

Antigenic regions (antigenic determinants) within a protein that elicit an antibody response (B-cell epitopes) were predicted using antigenic peptide prediction tool (http://imed.med.ucm.es/Tools/antigenic.pl) [30]. This tool's prediction is based on the amino acid residues present in the experimentally known segmental epitopes.

3. EXPERIMENTAL DESIGN

An overall study design and workflow is depicted in Fig. (**1**).

4. RESULTS

4.1. *M. immunogenum* Genotype Isolated from the Field in-use MWF

Screening of field-drawn MWF samples using a combination of molecular and microbiological approaches enabled identification of the required $Myco^+$ MWF sample (Fig. **1**). Culturing the Myco+ sample followed by molecular speciation yielded a new *M. immunogenum* isolate arbitrarily designated as MJY-27 based on our ongoing numbering scheme for field MWF isolates. Individual colony isolates were identified using acid-fast staining (genus level) and our previously developed [27] PCR-restriction analysis method (species level); the latter involved amplification of a 667 bp product from the mycobacteria-specific *hsp* gene (Figs. **2A, B**) followed by *Bbv*I restriction pattern analysis. Two restriction

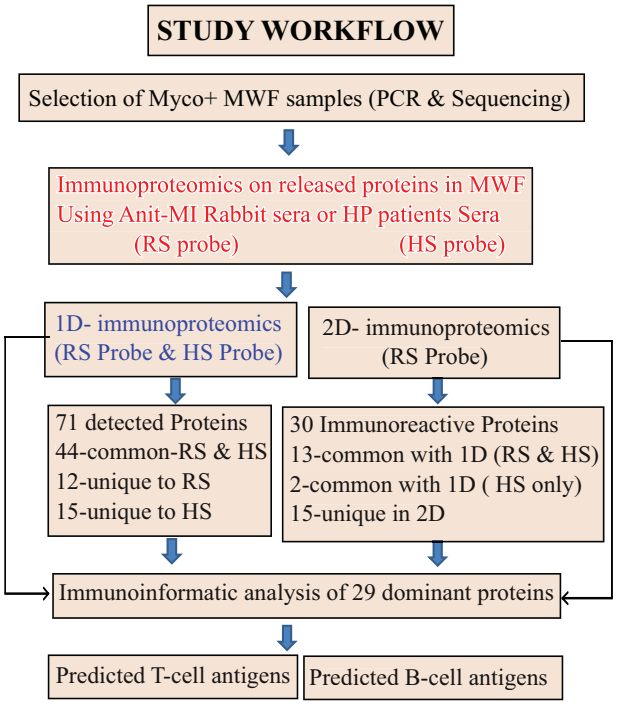

Fig. (1). Overall study design and workflow.

ction bands on the gel confirmed the isolated species as *M. immunogenum* (Fig. **2D**). This was further confirmed by determining the PCR amplicon sequence (Fig. **2C**), which showed the closest match (> 97% identity) with known *M. immunogenum* sequences; however, there is a lack of absolute similarity with the *hsp*667 amplicon sequences from any of the known MI genotypes [27] indicating that this isolate is a new genotype of *M. immunogenum*.

4.2. Identification of Cell-free MI Proteins Predominant in the Field in-use MWF

In 1D-immunoproteomic analysis of the total protein extract from a field-drawn MWF sample ($Myco^{+}$), the immunoblots showed five large immunoreactive bands (light to strong intensity) with both anti-MI rabbit serum (RS) probe and human sera (HS) probe (Figs. **3A** & **B**).

LC-MS/MS analysis of these immunoreactive bands led to the identification of a total of 71 proteins (Table sheets **SI** and **S2**). Forty-four proteins were common in both, while 15 and 12 proteins were unique to the human HP patient-sera probe and rabbit- anti-MI serum probe, respectively. To confirm these and to identify any additional seroreactive proteins, 2D immunoproteomics was performed using RS probe on both Myco+ (Fig. **4A**) and Myco- (Fig. **4B**) MWF samples. A total of 30 seroreactivity proteins were identified in the 2D immunoproteomic analyses for the Myco+ MWF sample (Table **S3**) while the MWF^{-} (negative) sample did not react to the RS probe used.

Of these, 15 proteins were the same as detected by 1D-proteomic analysis above (13 were detected by both RS and HS probes while 2 were detected by only the HS probe). The remaining 15 seroreactive proteins were uniquely detected in 2D-proteomic analysis (Fig. **5**).

We further narrowed the candidate count based on abundance of the peptides (cut off ≥4) in LC-MS/MS analysis. A total of 29 proteins were considered abundant. Ten of these top abundant candidates are listed here as putative antigens with accession numbers and protein names in decreasing order of abundance: A0A0N1CHW4 (Dioxygenase) > A0A0N1CG85(homocysteine methyltransferase)> A0A0N1CD96 (Catalase) > A0A0N1LYK1 (Dehydrogenase) ≥ A0A0N0KP56 (Enolase) ≥ A0A0N0KQE7 (Copper oxidase) ≥ A0A0N1LVD8 (Uncharacterized protein) ≥ A0A0N1CEW0 (Adenosylhomocysteinase) > A0A0N0KNL1 (Succinate-CoA ligase) > A0A0N0KM55 (Aldehyde dehydrogenase).

4.3. Functional Distribution of the Abundant Proteins

The dominant proteins identified in this study were analyzed for functional clustering using the information retrieved from the UniProt Knowledgebase (UniProtKB) (http://www.uniprot.org/). Functional distribution of these proteins for biological processes (Fig. **6**) showed that 32% belonged to oxidation-reduction processes, 14% to metabolic processes, and 46% to other processes, including glycolytic process (7%), TCA cycle (4%), aminopeptidases (4%), methyl transferases (4%), response to stress conditions (3%), fatty acid synthesis (4%), protein synthesis (7%) and refolding (3%), amino acid biosynthesis (4%), and nucleotide metabolism (3%). The remaining 11% belonged to the 'unknown function' category.

4.4. *In silico* Prediction of T-cell Epitopes in the Abundant Immunoreactive Proteins

To predict putative T-cell antigens among the identified 29 abundant proteins from the seroreactive fractions (bands/spots), T-cell epitope analysis was performed using their full amino acid sequences retrieved from the Uniprot protein database for *M. immunogenum*. All 29 proteins showed strong HLA I and HLA II binding potential, albeit with varying frequency, based on Propred1 and Proped analysis for HLA class I and II alleles, respectively. For HLA I binding (47 alleles), all candidate proteins were found to bind with more than 50% frequency, of which 13 candidates showed binding to 90-100% of the alleles (Fig. **7**). For HLA II binding (51 alleles), 13 of the candidate antigens could bind with 90-100% frequency, whereas only 2 candidate proteins showed binding with less than 50% frequency.

The predicted HLA I and II binding epitopes and corresponding HLA I and II alleles are presented in Table **1**. The top 10 protein IDs with binding to the highest number of HLA I alleles (in parenthesis) in decreasing order are: **A0A0N0KP56** (47) > A0A0N1LWE8 (46) ≥A0A0N1CBE4 (46) > A0A0N1LVN8 (45) ≥ A0A0N1LV45(45) ≥A0A0N1CJV0 (45) ≥ **A0A0N1CG85**(45) > A0A0N1CI37(44) ≥A0A0N1CFU4 (44) ≥**A0A0N1CEW0** (44). The top 10 protein IDs with binding to the highest number of

HLAII alleles (in parenthesis) in decreasing order are: **A0A0N1CG85** (50) ≥ A0A0N1CI37 (50) ≥ A0A0N1CFU4 (50) ≥ A0A0N0KPF9 (50) ≥ A0A0N0KLT1 (50) ≥ **A0A0N1CD96 (50)** > **A0A0N1CEW0 (49)** ≥ **A0A0N1-LYK1** ≥ A0A0N1CH54 (49) > A0A0N1LWE8 (48). The protein IDs shown in bold are also part of the 'top 10 list of most abundant proteins'.

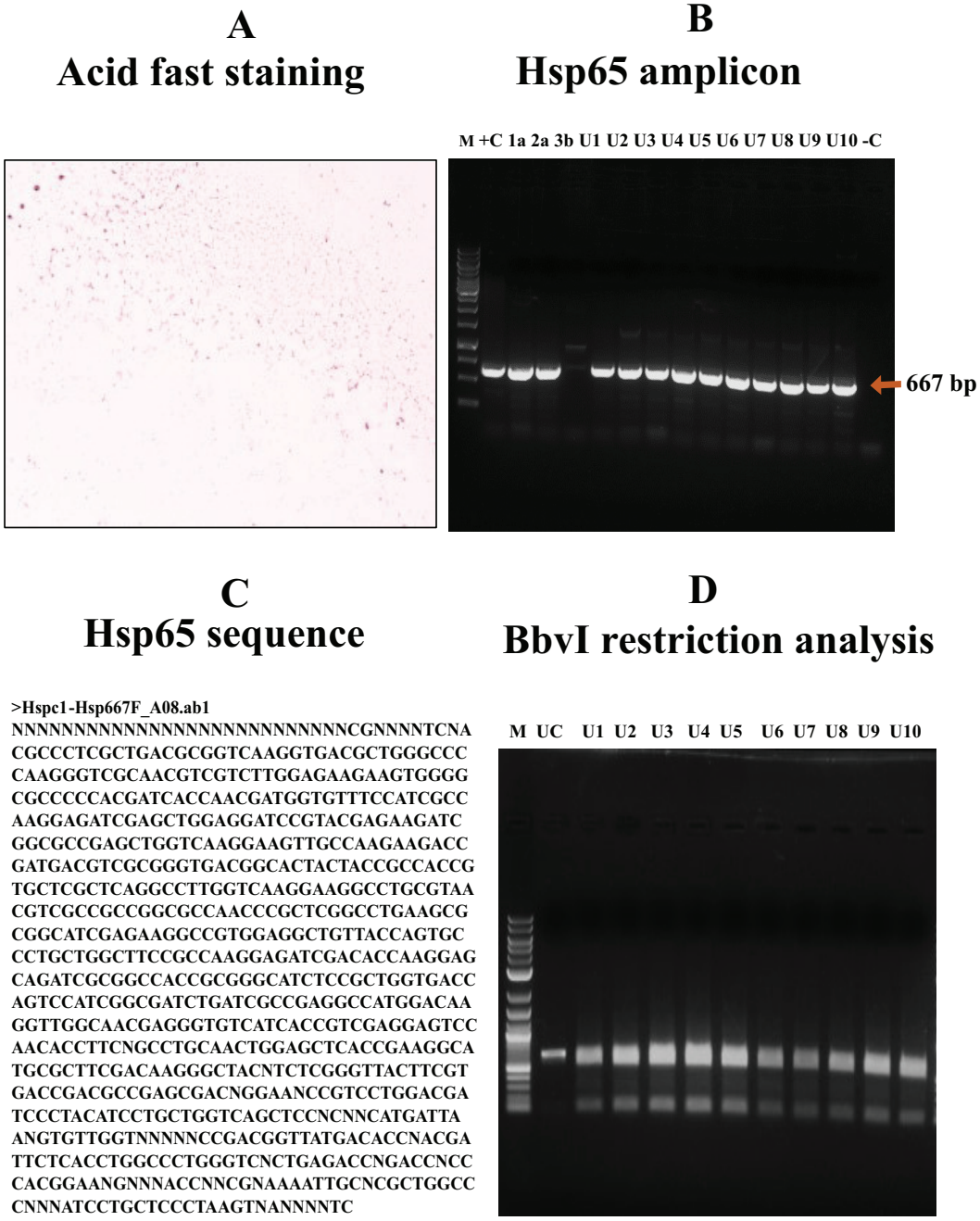

Fig. (2). Isolation and identification of *M. immunogenum* strain MJY-27 from MWF. Panel **A**: Acid-fast staining confirming mycobacterial identity of the isolate from MWF. Panel **B**: PCR amplification of 667 bp mycobacterial heat shock protein (hsp65) gene fragment, resolved on agarose gel (Lane 1, 100 bp DNA ladder; Lane 2, positive control; Lanes 3, 4, & 5, different MWF samples; Lanes 6-15, unknown colonies isolated from MWF; Lane 16, negative PCR control). Panel **C**: DNA sequencing result for the 667 bp PCR product. Panel **D**: *Bbv*1 restriction pattern analysis of the 667 bp PCR product generated from different colony isolates (U1-U10) as compared to the negative control (UC, abbreviated for Uncut DNA); Results in Panels C and D confirmed the *M. immunogenum* identity of the mycobacterial isolate. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

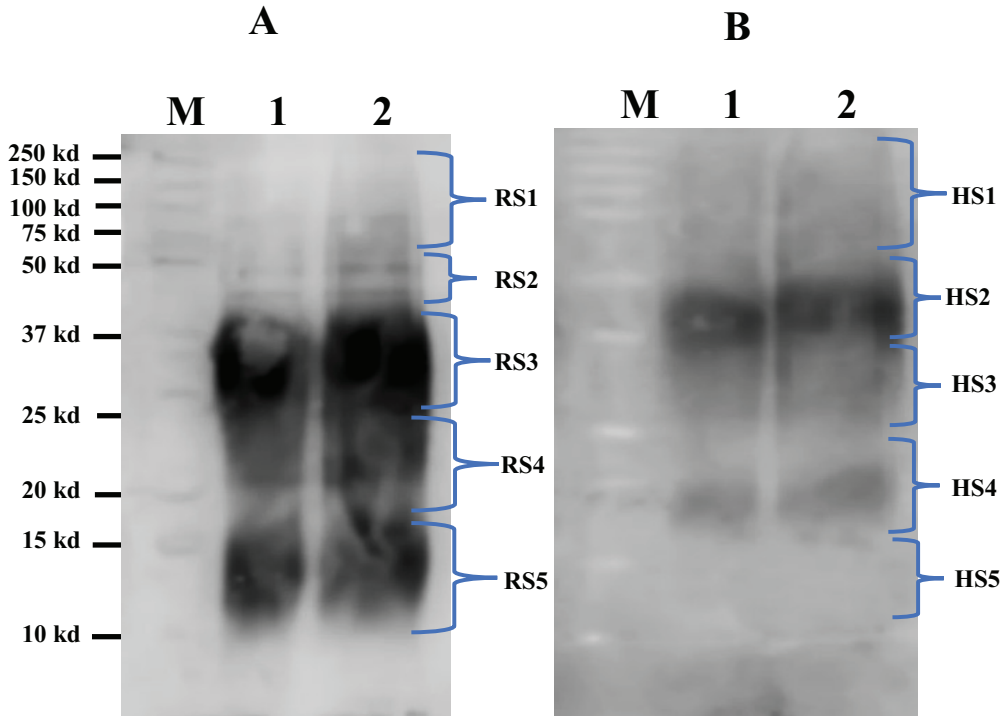

Fig. (3). Identification of dominant MI proteins from MWF by 1D-immunoproteomics (shot-gun approach). Panels **A** and **B**: Immunoblot profiles for 1D-gels of total protein extract when probed using anti-MI rabbit serum (Panel **A**) and pooled sera from machinist HP patients (Panel B). Lanes 1& 2, MI-positive total protein sample (50 and 100 µg per lane). The protein mixture was separated in the 10kDa-250 kDa range on the gels and was harvested as five gel fractions (RS1-RS5 or HS1-HS5) for downstream proteomic analysis. Representative images from multiple runs are presented. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

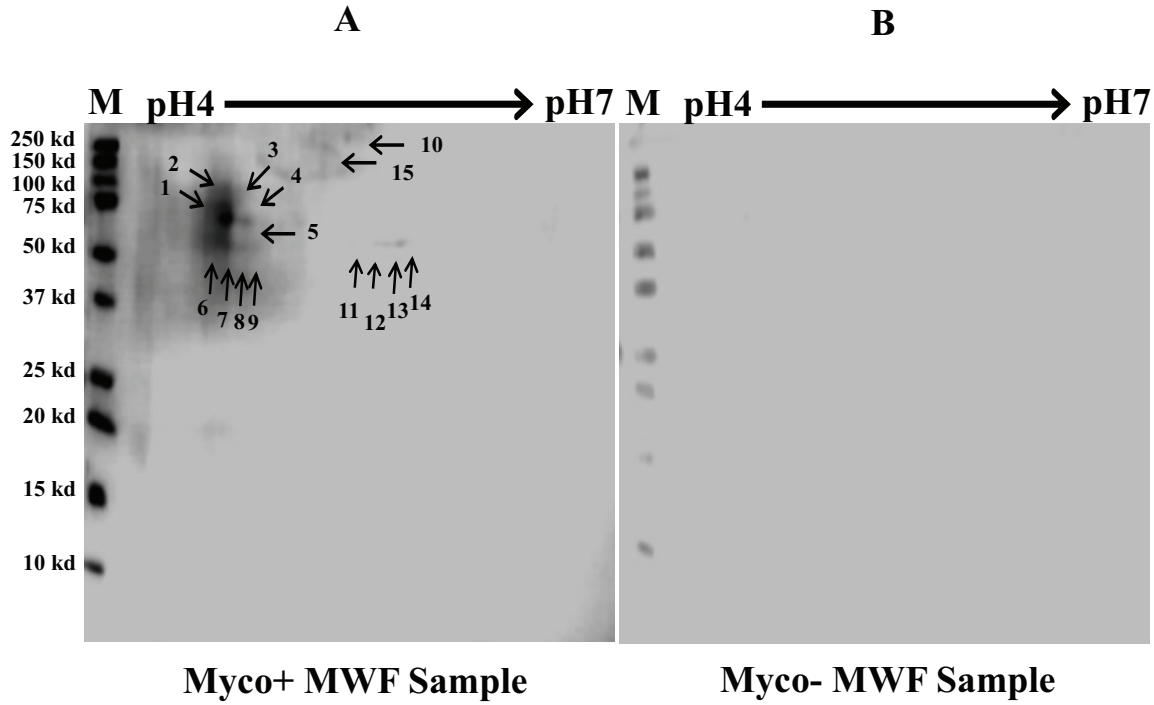

Fig. (4). Identification of dominant MI seroreactive proteins from in-use MWF by 2D-immunoproteomics using anti-MI rabbit serum probe. Immunoblot images of the 2D-gel profile of proteins isolated from Myco+ MWF (Panel **A**) and Myco- MWF (Panel **B**) were generated using an anti-MI rabbit serum (RS) probe. Representative images from multiple runs are presented. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

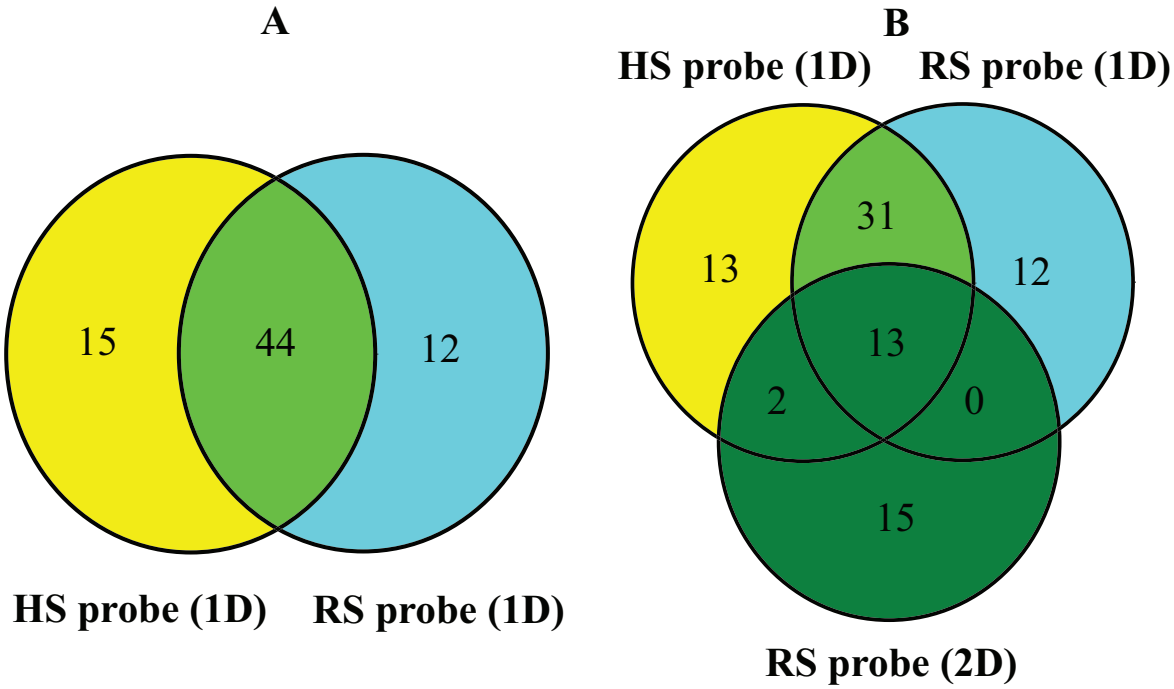

Fig. (5). Venn diagrams of MI proteins identified from MWF in 1D- and 2D-immunoproteomics based on anti-MI rabbit serum (RS) probe and human patient sera-based (HS) probe. Panel **A**: Proteins identified by 1D-immunoproteomics using RS and HS probes. Panel **B**: Comparison of all proteins identified by 1D- and 2D- immunoproteomics using RS and HS probes. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

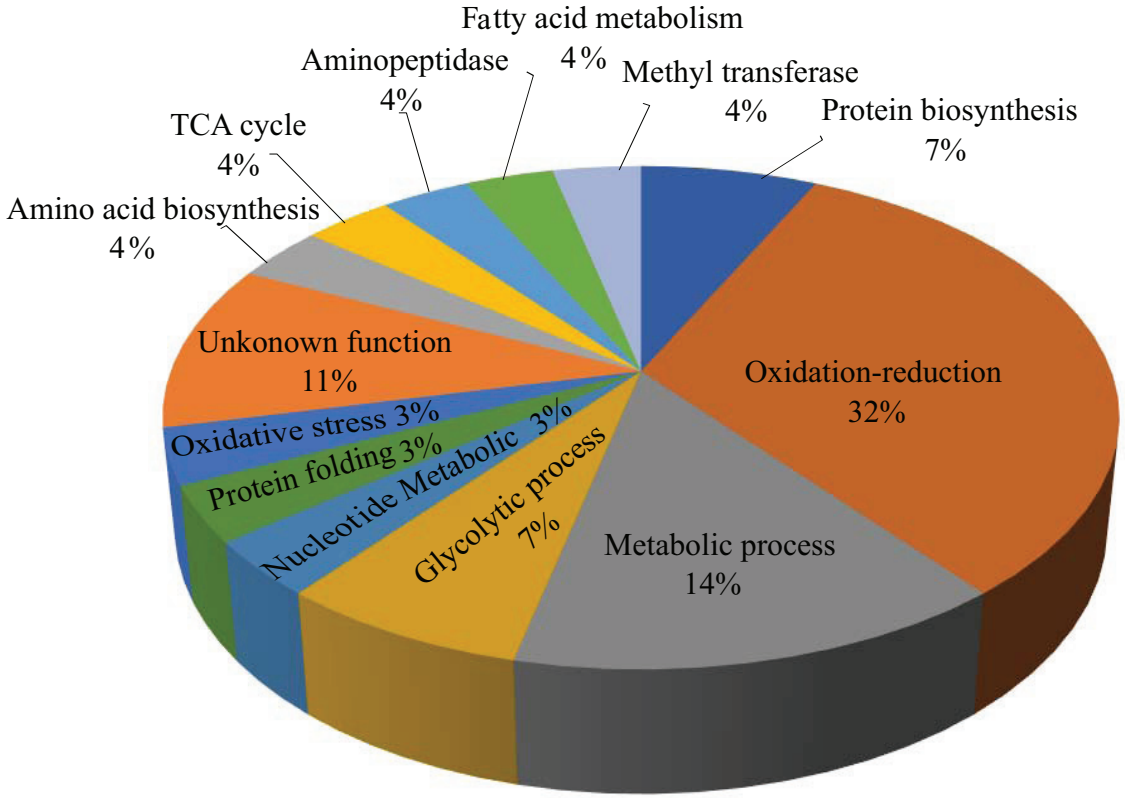

Fig. (6). Functional distribution of the abundant MI proteins identified from MWF. Pie-chart showing functional clustering of the abundant proteins of *M. immunogenum* identified from in-use MWF. Clustering for various biological processes was done using the information retrieved from UniProt Knowledgebase (UniProtKB) (http://www.uniprot.org/). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

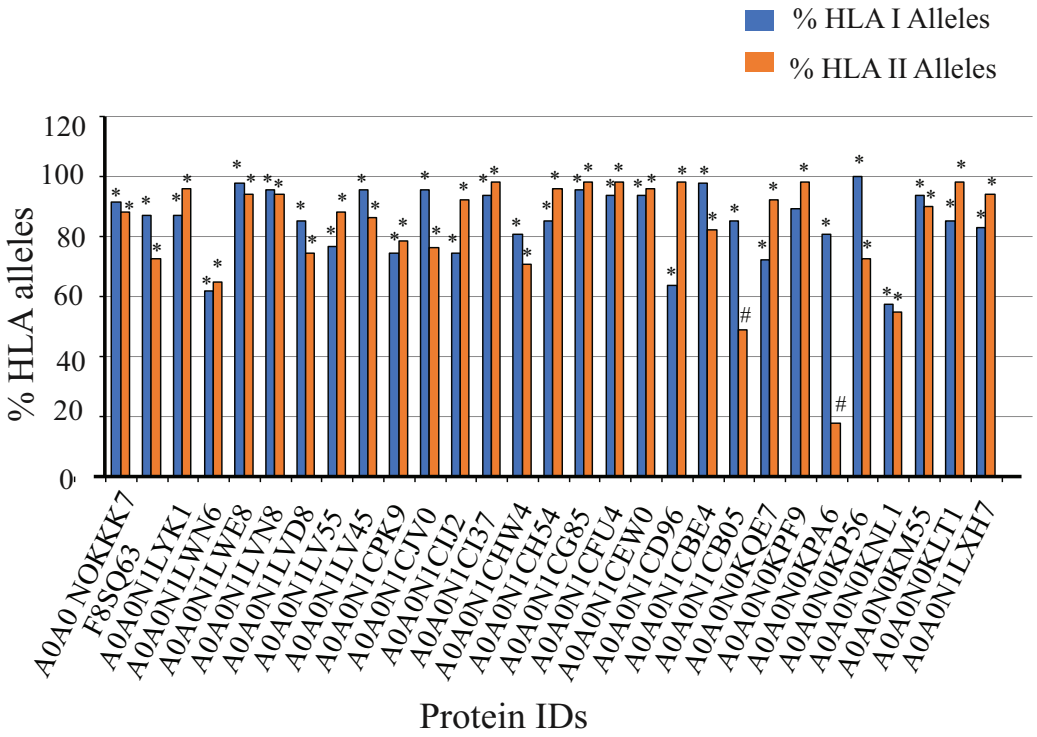

Fig. (7). *In-silico* prediction of T-cell epitopes in the identified abundant proteins. T cell antigenic epitopes were predicated using Propred1 for HLA I and Propred for HLAII alleles. Asterisk (*) Sign on the bars shows antigenic epitopes binding to more than 50-100% HLA alleles, whereas the Number (#) sign on the bars shows epitopes binding to less than 50% HLA alleles. The Y-axis represents % binding to HLA alleles; the X-axis represents the MI protein IDs. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

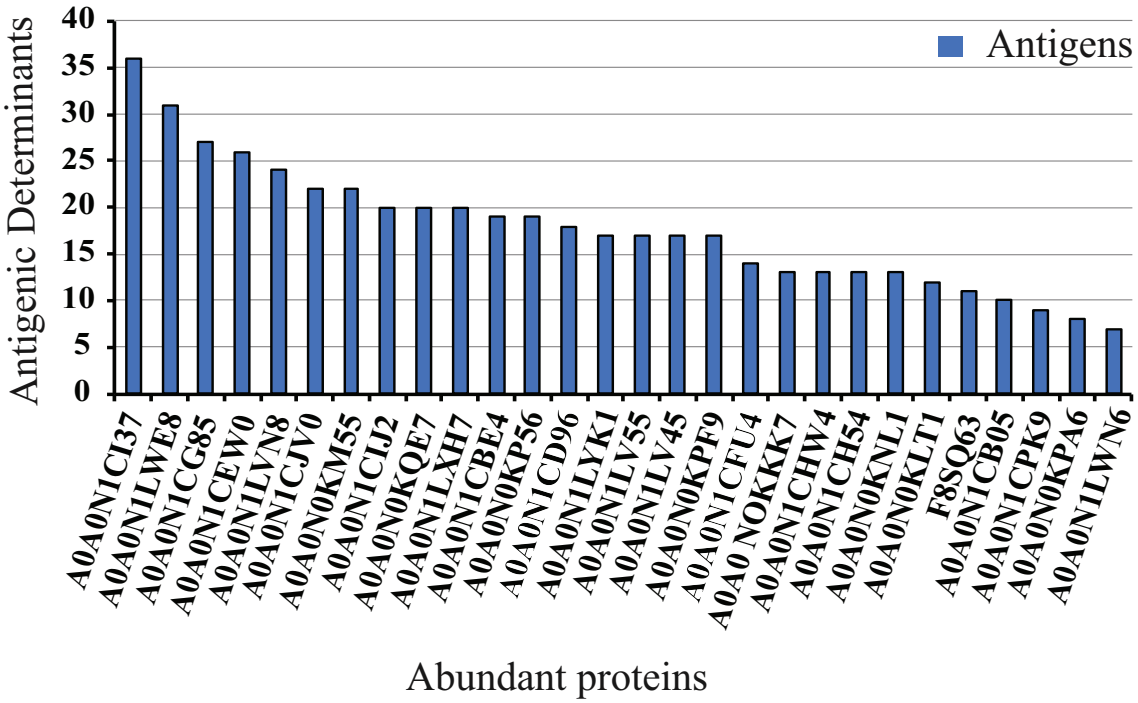

Fig. (8). *In-silico* prediction of antigenic determinants (B cell epitopes) in the abundant proteins identified from the seroreactive fractions of MI proteins. Antigenic determinants/epitopes within a protein that elicit an antibody response were predicted using antigenic peptide prediction tool (http://imed.med.ucm.es/Tools/antigenic.pl). Y-axis represents number of antigenic regions found within a protein sequence ; X-axis represents the MI protein IDs. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

4.5. Identification of Metal-binding T-cell Antigens

We analyzed the predicted T-cell antigens for metal-binding domains. Surprisingly, eight of the candidate T-cell antigens (those showing HLA-binding T-cell epitopes) were found to be metal binders. These proteins were predicted to possess metal ion binding domains for metals such as Cu, Mg, Fe, Mn, and Zn. The identified metal ion-binding proteins and their corresponding domains are presented in Table **2**.

4.6. A Functionally Validated T-cell Antigen among the Identified Dominant Antigen Candidates

One of the predicted T-cell antigen candidates (Accession # A0A0N1CIJ2) identified from MWF in this study coincided with AgD, an experimentally validated T-cell antigen reported from Laboratory-grown MI cultures in our previous studies [20, 23, 25]. Briefly, this antigen showed a strong T-cell reactivity in the MI-exposed splenocytes from mice, in terms of IFN-γ release in re-challenge experiments. The antigen also activated the naive $CD4^+$ T cells as demonstrated by ELISPOT assay for IFN-γ.

4.7. *In silico* Predicted Antigenic Determinants (B-cell Epitopes) in the Identified Dominant MI Proteins

The abundant proteins were analyzed for the presence of antigenic determinants in their sequence by the method of Kolaskar and Tongaonkar (1990) [30], which is based on the occurrence of amino acid residues in experimentally determined epitopes. The algorithm calculates the average antigenic propensity of the whole proteins, and the peptides having an average propensity higher than one propensity of whole protein are considered antigenic. Our results for these abundant proteins are shown in Fig (**8**). The abundant proteins showed antigenic determinants ranging from 6 to 37. The top 10 antigenic proteins showing the highest antigenic determinants (in parenthesis) in decreasing order are A0A0N1CI37 (37) > 0A0N1LWE8 (31) > **A0A0N1CG85** (27) > **A0A0N1CEW0** (26)> **A0A0N1LVN8** (24)> A0A0N1CJV0 (22)≥ **A0A0N0KM55** (22)> A0A0N1CIJ2 (20) ≥A0A0N0KQE7 (20) >**A0A0N1LXH7** (20). Among these, five proteins (shown in bold) were also the most abundant proteins list. Furthermore, the antigen A0A0N1CIJ2 with 20 antigenic determinants coincided with a pre-validated T cell antigen (AgD) in our previous study. The antigenic epitopes of these top 10 proteins are shown in Table **S5**.

5. DISCUSSION

Machinists exposed to mycobacteria-colonized MWF are at an increased risk of developing MOL/HP or other respiratory conditions as evidenced by several cases and outbreaks [3, 11, 14]. *M. immunogenum* (MI) has been widely recognized as the etiological agent of Machinist's HP based on both epidemiological [1, 2, 14] and animal model [18] studies and many different strains of MI have thus far been

Table 1. Immuno-informatic analysis data for the identified T-cell antigens of *M. immunogenum* from in-use Myco-positive MWF.

Protein ID (Accession Number)	HLAI Alleles	HLAI Binders[a]	Promiscuous Epitope[b] / Frequency[c]	AA Start Position	HLAII Alleles	HLAII Binders	Promiscuous Epitope[b] / Frequency[c]	AA Start Position
A0A0 NOKKK7	43	26	NPTLLAKAV (11)	89	45	19	LRPNIPIMI (13)	171
F8SQ63	41	32	GMLDCKNAL (11)	19	37	14	LRIKGAKDV (17)	40
A0A0N1LYK1*	41	24	DPIARTVTL (18)	83	49	23	IVILGAGIG (15)	49
A0A0N1LWN6	29	14	EPQPELAAL(15)	30	33	9	YVLVFAVGA (13)	145
A0A0N1LWE8	46	48	IESRYFTSL (12)	276	48	29	IKKIGVLGA (16)	318
A0A0N1LVN8*	45	58	LPVASVLAV (10)	80	48	27	VLLVGVRPS (21)	23
A0A0N1LVD8	40	21	RAVTASLLL (16)	49	38	10	FRIKRAVTA (19) LKVLAKRGI (17) LINVLKLRS (26)	44 111 135
A0A0N1LV55	36	13	VAVVGLGGL (16)	178	45	25	IKFAGICHS (18)	34
A0A0N1LV45*	45	17	MTLTDGTEL (117)	117	44	22	VGRDDTVAV (10)	176
A0A0N1CPK9	35	12	VLFDAVTGL(14)	182	40	12	WRLMNLAVL (21)	45
A0A0N1CJV0	45	33	KPLAGKVAI (10)	209	39	17	VRFILSAKS (32)	177
A0A0N1CIJ2	35	13	APVVRVSAL (20)	170	47	25	VVRVSALKA (18)	171
A0A0N1CI37*	44	43	LPETNIIKL(12)	77	50	37	IRGLDTISA (21) MRPSATFNS (21)	535 729
A0A0N1CHW4*	38	14	DPAKVAELL (16)	160	36	17	YRQLPAALQ(17)	131
A0A0N1CH54	40	21	LPIALAHRL(16)	149	49	21	IVRERVLEI (20) VRLDTVVLS (22)	69 190

(Table 1) Contd……

Protein ID (Accession Number)	HLAI Alleles	HLAI Binders[a]	Promiscuous Epitope[b] / Frequency[c]	AA Start Position	HLAII Alleles	HLAII Binders	Promiscuous Epitope[b] / Frequency[c]	AA Start Position
A0A0N1CG85*	45	51	VPELAGKTL (8) WRTNLQSAL (8)	277, 296	50	33	VVVLARALS(30)	355
A0A0N1CFU4	44	16	TLYSSLAVL (16)	279	50	23	LRTISTLLD (20) WRGIPIIPS (34) VRFTGINRS (21)	91 212 262
A0A0N1CEW0*	44	30	DPINALQAL (18)	298	49	26	VRLNIKPQV(24)	369
A0A0N1CD96*	30	11	AGTLVREVL (10)	425	50	22	VQVMPVAEA (25)	257
A0A0N1CBE4*	46	50	KLQERLAKL(12)	362	42	21	LVVNKIRGT(11)	259
A0A0N1CB05	40	18	ITLNRPKAL (13)	17	25	10	MMCDLIIAA(8)	113
A0A0N0KQE7	34	20	GPGAAVRQF (10)	59	47	26	VRLRFINDS(23) VRLRIVNAG (18) LRVRTVNNL(16)	425 269 104
A0A0N0KPF9	42	33	KALLRNAEL(12)	51	50	26	YRMMPRATF (33)	342
A0A0N0KPA6	38	12	SGPLVAAVL (12)	68	9	6	IRGDFALET (7)	102
A0A0N0KP56*	47	48	AGLALFRYL (11)	125	37	18	LRIEETLGD (12)	402
A0A0N0KNL1	27	12	TAQAKKEAL (11) KTPSETAKL (11), 284	267 284	28	15	LVGITPNNI (14)	144
A0A0N0KM55*	44	27	LSLVLSEPL	140	46	19	LVLSEPLGV(26)	141
A0A0N0KLT1	40	25	AAVDAAKEL (15)	66	50	22	IRLAATAIS (41) VVGIRVNTI (24)	122 182
A0A0N1LXH7	39	17	FLIGGLDDL(10)	456	48	25	VVNTGNTIA (6)	78

Note: *Antigens that were also detected in 2D proteomics; a HLA1 and II binders are the epitopes which bind to class I and class II HLA alleles; b Promiscuous epitopes are the ligands which bind to maximum number of alleles in query; c Frequency of the promiscuous epitope to bind different alleles.
Abbreviation: AA (Amino acid).

Table 2. Identification of metal-binding protein antigens.

Protein ID	Protein Name	Binding Metal Ion	Metal-ion Binding Position	Molecular Weight (KDa)
A0A0N0KQE7	Copper oxidase	Cu	486-497	55.0
A0A0N1CD96	Catalase	Fe	335-335	54.688
A0A0N1CG85	5-Methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	Zn	643-643; 645-645; 728-728	81.642
A0A0N1CH54	S-Adenosylmethionine synthase	Mg K	19-19; 289-289 45-45; 281-281	42.815
A0A0N1CI37	Isocitrate dehydrogenase	Mg/Mn	352-352; 551-551; 555-555	82.148
A0A0N1LV45	Alcohol dehydrogenase	Zn	62-76	38.294
A0A0N1LV55	Alcohol dehydrogenase	Zn	62-76	36.933
A0A0N1LVN8	Probable cytosol aminopeptidase	Mn	266-266; 271-271; 289-289; 348-348; 350-350	51.964
A0A0N0KP56	Enolase	Mg	241-241; 283-283; 310-310	45,038

isolated from the in-use industrial MWF in our efforts [22] including the current study. Despite knowledge of the etiological agent, there is no reliable fluid monitoring platform to assess the build-up of mycobacterial proteins in MWF

and there is a pressing need to identify the offending antigens of this etiological agent. These knowledge gaps have hampered the development of exposure assessment and early intervention strategies and diagnostic tools for machinist's HP. While some information has been generated on mycobacterial antigens using laboratory media-grown cultures of *M. immunogenum* [25, 26], it is unclear whether the same antigens are secreted in the machining fluid matrix and what their abundance level is, as there are no reports yet on direct identification of mycobacterial antigens from these industrial fluids. Here, we report first identification of such MWF-associated immunoreactive proteins directly from the field samples of MWF containing *M. immunogenum*. We believe that under prevailing harsh fluid conditions in MWF, specific cell-free proteins accumulate in the fluid, contributing to the antigen pool in the circulating MWF as well as the resulting aerosols, something that is critical for the onset of HP or other respiratory conditions. Identification of the most abundant seroreactive members of this antigen repertoire could therefore, provide a key to understanding the etiological and personal exposure aspects of HP development and can be exploited for developing fluid monitoring and intervention strategies. The use of immunoproteomic fractionation coupled with LC-MS/MS analysis allowed the identification of dominant putative immunoreactive proteins of MI from the in-use field MWF. A total of 71 MI proteins were identified (Fig. **3** and Tables **S1** & **S2**) in 1D- immunoproteomics, whereas 30 were identified in 2D-immunoproteomic analysis. Of these, 29 proteins were adjudged to be the most abundant based on the number of peptides appearing in the LC-MS/MS spectra. HP is a T-cell mediated disorder [16, 31, 32], so the identification of T-cell antigens is critical in understanding the pathology and diagnosis of the disease. In order to understand whether the identified 29 abundant proteins have T-cell antigen characteristics, we employed the Propred analysis which has been widely used to predict T-cell epitopes/antigens and the corresponding human HLA alleles [29, 33, 34] that these can bind. Based on this *in-silico* analysis for T-cell epitopes, all 29 candidates showed epitopes like CD^+ T cell antigens. Nearly half (thirteen) of these were strong (90- 100%) HLA I and II allele binders when queried against 47 and 51 known alleles of HLA I and II available on the server; the other half had lower binding frequencies ranging 20 to 90% of the known HLA alleles. Analysis for antigenicity showed the presence of multiple antigenic regions (antigenic determinants) in the identified abundant proteins revealing their highly antigenic nature. The identification of metal-binding T-cell antigenic proteins in the current study is significant as it may be relevant to the development of immune-mediated diseases such as HP. The role of metals in antigen presentation and activation of T cells by superantigens containing zinc binding sites such as staphylococcal enterotoxin A (SEA) and other superantigens has been well demonstrated [35]. Furthermore, zinc has been shown to regulate the activation of T cells by acting as an ionic signaling messenger [36, 37]. In light of the above observations, the identification of metal-binding T cell antigen candidates in our study from MWF is significant and may lead to new research questions for understanding the machinist HP pathology. Notably, one of the predicted T--cell antigen candidates identified in the study resembled a T-cell antigen (Ag D) experimentally validated in our prior efforts on laboratory media-grown MI cultures [20, 24]. This indicated the functional relevance of the predicted T-cell antigen candidates in this study. Further validation research will focus on specific epitopes of these candidate antigens based on their ability to activate T cells and their relevance in HP pathology. The best peptides to use as immunogens that can trigger an antibody response can be discovered by the prediction of antigenic determinants by Kolaskar and Tongaonkar (1990) method [30], which is based on the occurrence of amino acid residues in experimentally determined epitopes. Using this analysis, we found the most antigenic proteins among the 29 abundant proteins. Five of the most abundant proteins were also among the top ten antigens. The functionally validated T- cell antigen (Ag D) [20, 24] was also among the top 10 antigens. Further most of the top antigens were also among the metal-binding proteins. Interestingly, the metal binder protein A0A0N1CI37 was the top scorer for antigenic determinants [36]. Therefore, these highly antigenic protein candidates identified based on immunoproteomics and antigenic mapping methods will be utilized to functionally validate their best epitopes for the development of fluid monitoring immunoassays and platforms.

CONCLUSION

In conclusion, this is the first report on identifying dominant mycobacterial proteins directly from the industrial in-use metalworking fluid and ascertaining their antigenic potential. Among the 29 dominant proteins identified, several showed strong HLA-binding potential, implying them as candidate T-cell antigens, and the presence of high number of antigenic determinants (6 to 37), showing their potential to trigger antibody response. In addition, metal-binding T-cell antigens were identified for the first time from this source, implying their potential role in machinist HP pathology. Identified dominant proteins could be directly useful for monitoring mycobacterial antigen load in the in-use MWF fluids in occupational settings and may prove critical in the development of novel point-of-use tools/platforms for routine fluid monitoring and personal exposure/risk assessment in machinists. Furthermore, this study will open up new avenues to design larger epidemiological studies to understand antigen exposure routes and intervention measures in industrial settings prone to machinist lung disease.

LIST OF ABBREVIATIONS

MWF	=	Metalworking Fluid
MI	=	Mycobacterium Immunogenum
MJY-27	=	Mycobacterium Immunogenum Strain MJY-27
CCF	=	Circulating Cell-free
LC-MS/MS	=	Liquid Chromatography–mass Spectrometry

AgD	= Antigen D
HP	= Hypersensitivity Pneumonitis
MOL	= Machine operator's Lung
AHSPRA	= Amplified hsp65 Restriction Analysis Method
1D	= One-dimensional
2D	= Two-dimensional
BLAST	= Basic Local Alignment Search Tool
HSP65	= 65 Kilodalton Heat Shock Protein
DC assay	= Detergent Compatible Protein Assay
HLA	= Human Leukocyte Antigen
LTQ	= Linear Ion Trap
HS	= Human Serum
RS	= Rabbit Serum

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings are revealed in this manuscript.

FUNDING

This study was supported by grant 1R21OH011826 (to JSY) from the National Institute for Occupational Safety and Health (NIOSH), and the Centers for Disease Control and Prevention (CDC). We also acknowledge the Pilot Research Project training award (to HC and JSY) from the University of Cincinnati's NIOSH Education and Research Center Grant T42/OH008432-10. The Vermont Biomedical Research Network Proteomics Facility (RRID:SCR_018667) provided support through NIH grant P20GM103449 from the IDeA (Institutional Development Award) Networks of Biomedical Research Excellence (INBRE) Program of the NIH's National Institute of General Medical Sciences.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

JSY and HC contributed to the study design, performance, analysis, and manuscript draft. BA and YWL contributed to the identification and analysis of protein spots by liquid chromatography-tandem mass spectrometry (LC-MS/MS at Vermont Biomedical Research Network Proteomics Facility, University of Vermont, Burlington.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

REFERENCES

[1] Rosenman, K.D. Asthma, hypersensitivity pneumonitis and other respiratory diseases caused by metalworking fluids. *Curr. Opin. Allergy Clin. Immunol.,* **2009**, *9*(2), 97-102. http://dx.doi.org/10.1097/ACI.0b013e3283229f96 PMID: 19307882

[2] Rosenman, K. Occupational diseases in individuals exposed to metal working fluids. *Curr. Opin. Allergy Clin. Immunol.,* **2015**, *15*(2), 131-136. http://dx.doi.org/10.1097/ACI.0000000000000140 PMID: 25564768

[3] Respiratory illness in workers exposed to metalworking fluid contaminated with nontuberculous mycobacteria--Ohio, 2001. *MMWR Morb. Mortal. Wkly. Rep.,* **2002**, *51*(16), 349-352. PMID: 12004986

[4] O'Brien, D.M. Aerosol mapping of a facility with multiple cases of hypersensitivity pneumonitis: Demonstration of mist reduction and a possible dose/response relationship. *Appl. Occup. Environ. Hyg.,* **2003**, *18*(11), 947-952. http://dx.doi.org/10.1080/10473220390237656 PMID: 14555448

[5] Raghu, G.; Remy-Jardin, M.; Ryerson, C.J.; Myers, J.L.; Kreuter, M.; Vasakova, M.; Bargagli, E.; Chung, J.H.; Collins, B.F.; Bendstrup, E.; Chami, H.A.; Chua, A.T.; Corte, T.J.; Dalphin, J.C.; Danoff, S.K.; Diaz-Mendoza, J.; Duggal, A.; Egashira, R.; Ewing, T.; Gulati, M.; Inoue, Y.; Jenkins, A.R.; Johannson, K.A.; Johkoh, T.; Tamae-Kakazu, M.; Kitaichi, M.; Knight, S.L.; Koschel, D.; Lederer, D.J.; Mageto, Y.; Maier, L.A.; Matiz, C.; Morell, F.; Nicholson, A.G.; Patolia, S.; Pereira, C.A.; Renzoni, E.A.; Salisbury, M.L.; Selman, M.; Walsh, S.L.F.; Wuyts, W.A.; Wilson, K.C. Diagnosis of hypersensitivity pneumonitis in adults: An official ATS/JRS/ALAT clinical practice guideline. *Am. J. Respir. Crit. Care Med.,* **2020**, *202*(3), e36-e69. http://dx.doi.org/10.1164/rccm.202005-2032ST PMID: 32706311

[6] Alberti, M.L.; Rincon-Alvarez, E.; Buendia-Roldan, I.; Selman, M. Hypersensitivity pneumonitis: Diagnostic and therapeutic challenges. *Front. Med.,* **2021**, *8*, 718299. http://dx.doi.org/10.3389/fmed.2021.718299 PMID: 34631740

[7] Hamblin, M.; Prosch, H.; Vašáková, M. Diagnosis, course and management of hypersensitivity pneumonitis. *Eur. Respir. Rev.,* **2022**, *31*(163), 210169. http://dx.doi.org/10.1183/16000617.0169-2021 PMID: 35140104

[8] Burge, P.S. Hypersensitivity pneumonitis due to metalworking fluid aerosols. *Curr. Allergy Asthma Rep.,* **2016**, *16*(8), 59. http://dx.doi.org/10.1007/s11882-016-0639-0 PMID: 27473679

[9] Nett, R.J.; Stanton, M.; Grimes, G.R. Occupational respiratory and skin diseases among workers exposed to metalworking fluids. *Curr. Opin. Allergy Clin. Immunol.,* **2021**, *21*(2), 121-127. http://dx.doi.org/10.1097/ACI.0000000000000717 PMID: 33394701

[10] Cohen, H.; White, E.M. Metalworking fluid mist occupational exposure limits: A discussion of alternative methods. *J. Occup. Environ. Hyg.,* **2006**, *3*(9), 501-507. http://dx.doi.org/10.1080/15459620600867872 PMID: 16857649

[11] Shelton, B.G.; Flanders, W.D.; Morris, G.K. *Mycobacterium* sp. as a possible cause of hypersensitivity pneumonitis in machine workers. *Emerg. Infect. Dis.,* **1999**, *5*(2), 270-273. http://dx.doi.org/10.3201/eid0502.990213 PMID: 10221881

[12] Tillie-Leblond, I.; Grenouillet, F.; Reboux, G.; Roussel, S.; Chouraki, B.; Lorthois, C.; Dalphin, J.C.; Wallaert, B.; Millon, L. Hypersensitivity pneumonitis and metalworking fluids contaminated by mycobacteria. *Eur. Respir. J.,* **2011**, *37*(3), 640-647.

http://dx.doi.org/10.1183/09031936.00195009 PMID: 20693254

[13] Trout, D.; Weissman, D.N.; Lewis, D.; Brundage, R.A.; Franzblau, A.; Remick, D. Evaluation of hypersensitivity pneumonitis among workers exposed to metal removal fluids. *Appl. Occup. Environ. Hyg.,* **2003**, *18*(11), 953-960. http://dx.doi.org/10.1080/10473220390237683 PMID: 14555449

[14] Wilson, R.W.; Steingrube, V.A.; Böttger, E.C.; Springer, B.; Brown-Elliott, B.A.; Vincent, V.; Jost, K.C.; Zhang, Y.; Garcia, M.J.; Chiu, S.H.; Onyi, G.O.; Rossmoore, H.; Nash, D.R.; Wallace, R.J. *Mycobacterium immunogenum* sp. nov., a novel species related to *Mycobacterium abscessus* and associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy. *Int. J. Syst. Evol. Microbiol.,* **2001**, *51*(5), 1751-1764. http://dx.doi.org/10.1099/00207713-51-5-1751 PMID: 11594606

[15] Watt, W.D. Observations on the relationship between triazines and mycobacteria in metal removal fluids. *Appl. Occup. Environ. Hyg.,* **2003**, *18*(11), 961-965. http://dx.doi.org/10.1080/10473220390237692 PMID: 14555450

[16] Johansson, E.; Yadav, J.S. Differential immunogenicity and lung disease-inducing potential of mycobacterium immunogenum genotypes and impact of co-exposure with pseudomonas: Optimizing a mouse model of chronic hypersensitivity pneumonitis. *Int. J. Mol. Sci.,* **2024**, *25*(4), 2058. http://dx.doi.org/10.3390/ijms25042058

[17] Johansson, E.; Boivin, G.P.; Yadav, J.S. Early immunopathological events in acute model of mycobacterial hypersensitivity pneumonitis in mice. *J. Immunotoxicol.,* **2017**, *14*(1), 77-88. http://dx.doi.org/10.1080/1547691X.2016.1273284 PMID: 28094581

[18] Gordon, T.; Nadziejko, C.; Galdanes, K.; Lewis, D.; Donnelly, K. *Mycobacterium immunogenum* causes hypersensitivity pneumonitis-like pathology in mice. *Inhal. Toxicol.,* **2006**, *18*(6), 449-456. http://dx.doi.org/10.1080/08958370600563904 PMID: 16556584

[19] Chandra, H.; Yadav, E.; Yadav, J.S. Alveolar macrophage innate response to *Mycobacterium immunogenum*, the etiological agent of hypersensitivity pneumonitis: Role of JNK and p38 MAPK pathways. *PLoS One,* **2013**, *8*(12), e83172. http://dx.doi.org/10.1371/journal.pone.0083172 PMID: 24349452

[20] Chandra, H.; Yadav, J.S. T-cell antigens of *Mycobacterium immunogenum*, an etiological agent of occupational hypersensitivity pneumonitis. *Mol. Immunol.,* **2016**, *75*, 168-177. http://dx.doi.org/10.1016/j.molimm.2016.05.020 PMID: 27294559

[21] Yadav, J.S.; Khan, I.U.H.; Fakhari, F.; Soellner, M.B. DNA-based methodologies for rapid detection, quantification, and species- or strain-level identification of respiratory pathogens (Mycobacteria and Pseudomonads) in metalworking fluids. *Appl. Occup. Environ. Hyg.,* **2003**, *18*(11), 966-975. http://dx.doi.org/10.1080/10473220390237700 PMID: 14555451

[22] Khan, I.U.H.; Selvaraju, S.B.; Yadav, J.S. Occurrence and characterization of multiple novel genotypes of *Mycobacterium immunogenum* and *Mycobacterium chelonae* in metalworking fluids. *FEMS Microbiol. Ecol.,* **2005**, *54*(3), 329-338. http://dx.doi.org/10.1016/j.femsec.2005.04.009 PMID: 16332331

[23] Chandra, H.; Yadav, J.S. Human leukocyte antigen (HLA)-binding epitopes dataset for the newly identified T-cell antigens of *Mycobacterium immunogenum. Data Brief,* **2016**, *8*, 1069-1071. http://dx.doi.org/10.1016/j.dib.2016.06.045 PMID: 27508266

[24] Chandra, H.; Lockey, J.; Yadav, J.S. Novel antigens of *Mycobacterium immunogenum* relevant in serodiagnosis of occupational hypersensitivity pneumonitis in machinists. *Ann. Allergy Asthma Immunol.,* **2015**, *114*(6), 525-526.e4. http://dx.doi.org/10.1016/j.anai.2015.03.005 PMID: 25863449

[25] Gupta, M.K.; Subramanian, V.; Yadav, J.S. Immunoproteomic identification of secretory and subcellular protein antigens and functional evaluation of the secretome fraction of *Mycobacterium immunogenum*, a newly recognized species of the *Mycobacterium chelonae-Mycobacterium abscessus* group. *J. Proteome Res.,* **2009**, *8*(5), 2319-2330. http://dx.doi.org/10.1021/pr8009462 PMID: 19209886

[26] Roussel, S.; Rognon, B.; Barrera, C.; Reboux, G.; Salamin, K.; Grenouillet, F.; Thaon, I.; Dalphin, J.C.; Tillie-Leblond, I.; Quadroni, M.; Monod, M.; Millon, L. Immuno-reactive proteins from *Mycobacterium immunogenum* useful for serodiagnosis of metalworking fluid hypersensitivity pneumonitis. *Int. J. Med. Microbiol.,* **2011**, *301*(2), 150-156. http://dx.doi.org/10.1016/j.ijmm.2010.07.002 PMID: 20850379

[27] Selvaraju, S.B.; Khan, I.U.H.; Yadav, J.S. A new method for species identification and differentiation of Mycobacterium chelonae complex based on amplified hsp65 restriction analysis (AH-SPRA). *Mol. Cell. Probes,* **2005**, *19*(2), 93-99. http://dx.doi.org/10.1016/j.mcp.2004.09.007 PMID: 15680210

[28] Kapoor, R.; Yadav, J.S. Expanding the mycobacterial diversity of metalworking fluids (MWFs): Evidence showing MWF colonization by *Mycobacterium abscessus. FEMS Microbiol. Ecol.,* **2012**, *79*(2), 392-399. http://dx.doi.org/10.1111/j.1574-6941.2011.01227.x PMID: 22092754

[29] Singh, H.; Raghava, G.P.S. ProPred: Prediction of HLA-DR binding sites. *Bioinformatics,* **2001**, *17*(12), 1236-1237. http://dx.doi.org/10.1093/bioinformatics/17.12.1236 PMID: 11751237

[30] Kolaskar, A.S.; Tongaonkar, P.C. A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Lett.,* **1990**, *276*(1-2), 172-174. http://dx.doi.org/10.1016/0014-5793(90)80535-Q PMID: 1702393

[31] Selman, M.; Pardo, A.; King, T.E., Jr Hypersensitivity pneumonitis. *Am. J. Respir. Crit. Care Med.,* **2012**, *186*(4), 314-324. http://dx.doi.org/10.1164/rccm.201203-0513CI PMID: 22679012

[32] Thorne, P.S.; Adamcakova-Dodd, A.; Kelly, K.M.; O'Neill, M.E.; Duchaine, C. Metalworking fluid with mycobacteria and endotoxin induces hypersensitivity pneumonitis in mice. *Am. J. Respir. Crit. Care Med.,* **2006**, *173*(7), 759-768. http://dx.doi.org/10.1164/rccm.200405-627OC PMID: 16387809

[33] Bui, H.H.; Sidney, J.; Dinh, K.; Southwood, S.; Newman, M.J.; Sette, A. Predicting population coverage of T-cell epitope-based diagnostics and vaccines. *BMC Bioinformatics,* **2006**, *7*(1), 153. http://dx.doi.org/10.1186/1471-2105-7-153 PMID: 16545123

[34] Sakib, M.S.; Islam, M.R.; Hasan, A.K.M.M.; Nabi, A.H.M.N. Prediction of epitope-based peptides for the utility of vaccine development from fusion and glycoprotein of nipah virus using *in silico* approach. *Adv. Bioinforma.,* **2014**, *2014*, 1-17. http://dx.doi.org/10.1155/2014/402492 PMID: 25147564

[35] Li, H.; Llera, A.; Malchiodi, E.L.; Mariuzza, R.A. The structural basis of T cell activation by superantigens. *Annu. Rev. Immunol.,* **1999**, *17*(1), 435-466. http://dx.doi.org/10.1146/annurev.immunol.17.1.435 PMID: 10358765

[36] Yu, M.; Lee, W.W.; Tomar, D.; Pryshchep, S.; Czesnikiewicz-Guzik, M.; Lamar, D.L.; Li, G.; Singh, K.; Tian, L.; Weyand, C.M.; Goronzy, J.J. Regulation of T cell receptor signaling by activation-induced zinc influx. *J. Exp. Med.,* **2011**, *208*(4), 775-785. http://dx.doi.org/10.1084/jem.20100031 PMID: 21422171

[37] Bordon, Y. Heavy metal rocks T cells. *Nat. Rev. Immunol.,* **2011**, *11*(5), 300-301. http://dx.doi.org/10.1038/nri2977 PMID: 21494265