MYCOBACTERIA IN METALWORKING FLUIDS
FINAL PROGRESS REPORT (2005-2008 NCE 2010)
(5R01 OH007364-6)

a. Specific Aims

In this funding cycle (2005-2008 NCE 2010), overall objective was to develop the next generation of adaptable DNA-based protocols for simplified detection/recovery of MWF-prevalent NTM strains (genotypes) and develop and apply tools and protocols for their phenotypic characterization in terms of biocide susceptibility and antigenicity. Our specific aims were: (1). To develop protocols for improved recovery and simplified DNA-based detection and quantification of NTM from different MWF types, (2). To characterize the relative biocide susceptibility of MWF-colonizing NTM genotypes, using the available and newly isolated NTM strains and common MWF biocides; (3). To study antigenic characteristics of the MWF-colonizing NTM, using the available and newly isolated HP-linked strains.

b. Studies and Results

Progress on all three aims has been made per the originally proposed plan. Furthermore, certain additional but directly related efforts warranted to accomplish the proposed plan more effectively were also pursued as we went along in the study. During this funding period (2005-2008 NCE 2010), the generated data lead to a total of 7 research papers/manuscripts and 10 abstracts/presentations (oral/poster) (see **Publications list below**). A summarized report is presented in the following paragraphs.

Aim 1. Development of protocols for improved recovery and simplified DNA-based detection and quantification of mycobacteria from different MWF types:

1A. Development of cell recovery methods for improved detection of mycobacteria in MWF:

a). *Optimization of selective recovery/enrichment for enhanced cultural detection*: Low numbers of mycobacteria are hard to efficiently detect/quantify in time by both conventional and DNA-based methods. Our efforts on cell recovery optimization included strategies for selective suppression of gram-positive and gram-negative co-contaminants using antibiotic mixture PANTA plus (a combination of 5 antibiotics; BD Biosciences, Sparks, MD) and revival of the injured cells (viable-but-non-culturable, VBNC) by pre-incubation in its presence (selective enrichment). The results showed that this selective recovery approach facilitated isolation of mycobacteria even when present at low levels (1 to 200 cells), which otherwise is a challenging task when conventional culturing method is used. The results showed that a combination of antibiotic concentration and treatment temperature is critical for attaining optimal selection and revival of *M. immunogenum* from MWF. The effect was then evaluated on in-use field MWF sample contaminated with mixed microflora (consisting of 10^7 CFU/ml of microorganisms other than mycobacteria). In field MWF, the effect was relatively more pronounced in tubes containing the lowest cell numbers (10 and 1 CFU/ml). An extended (6-day) incubation in presence of PANTA plus, caused an enrichment of *M. immunogenum* cells to the extent of ≤1 log and ≤1.5 log in pristine versus in-use MWF, respectively possibly due to induced revival and/or multiplication of cells albeit in a differential manner in the two fluids. This part of the work has been published (see ***paper # 1*** *in the Publication list*).

b). *Optimization of Immunomagnetic separation (IMS) based recovery and detection*: Here we undertook optimization of antibody-based recovery of mycobacteria cells from complex MWF matrix.
i. *Anti-Mycobacterium antibody-- Custom preparation and evaluation for specificity*: We prepared the proposed anti-Mycobacterium custom antibody (polyclonal) using *M. immunogenum* (ProteinTech, Inc., Chicago, IL). Specificity of this polyclonal antibody was then evaluated using multiple lines of testing, viz. agglutination assays (Slide- and Tube-), immunofluorescence microscopy, and ELISA. As desired for the proposed work, it showed reactivity for both species of MWF-Mycobacteria *M. immunogenum* and *M. chelonae*. This confirmed the usefulness of the raised antibody for the proposed immunomagnetic application (Aim 1). Further, in comparative ELISA

analysis about half the amount of antibody was needed for *M. immunogenum* as compared to *M. chelonae* for agglutinating equal number of cells, indicating additional components in the polyclonal antibody preparation for certain epitopes specific to *M. immunogenum*; this in turn indicated its further usefulness to identify MI-specific antigens (Aim 3).

ii. ***Optimization of IMS-PCR for enhanced recovery and quantification of MWF mycobacteria***: To optimize the recovery of cells from the liquid matrix, both direct and indirect approaches for immunomagnetic separation (IMS) were tested using *M. immunogenum* (ATCC 700506) suspension (10^7 through 10^0 cells/ml) in either PBS or synthetic MWF. The custom-raised MI-polyclonal antibodies (described above) and immunomagnetic Dynabeads M-280 (Dynal Biotech LLC, Wisconsin) coated with secondary antibodies (sheep anti-rabbit antibodies) were used. In the direct IMS method, primary antibody-reacted immunobeads were mixed with the cell suspensions for the immunocapture of the cells, whereas in the indirect method, primary antibody was first mixed with the cell suspension followed by use of the immunobeads for the capture of antibody-cell complexes. The two Immunocapture protocols were optimized and compared using PBS and the superior protocol was then extended to MWF matrix. The results showed relatively higher cell recovery with indirect method as compared to direct method. Using the indirect immunocapture method to recover cells, we then optimized the PCR step of the IMS-PCR analysis of the cell concentration. We investigated the effect of matrix pH on the IMS-based cell recovery efficiency. Detection efficiency in the three matrices was in the following order: in-use synthetic MWF > PBS pH 7.4 > neutralized in-use MWF, showing the applicability of the IMS-PCR protocol to the in-use MWF w/o any pH modifications. Subsequent improvements included optimization of antigen:antibody ratios.

1B. Development of simplified DNA-based detection/quantification of NTM in MWF

a. Development of Fluorescence in situ Hybridization (FISH) method for MWF mycobacteria: Fluorescence *in situ* hybridization (FISH) protocols were optimized for specific detection and quantification of *M. immunogenum* and its common co-contaminant *Pseudomonas*, in MWF. For this, we developed species-specific peptide nucleic acid (PNA) probe for *M. immunogenum* and a DNA-based probe for Pseudomonas based on their 16S rRNA gene. The results showed that the designed probe was specific to *M. immunogenum* as it did not show cross-reaction with other closely related MCC or NTM mycobacteria or other species. Additionally, we have designed specific PNA probe for *M. chelonae* (Mc) and *M. abscessus* for the specific detection of these MWF-prevalent mycobacteria species. The FISH protocols were optimized in terms of sample preparation, hybridization and washing conditions, and epifluorescence analysis. The developed FISH assays were capable of detecting both the non-culturable as well as the culturable/viable population in field MWF samples. The developed PNA-FISH/FISH assays also successfully detected both the test organisms in human sputum. This work is now published (see ***paper # 4*** *in the Publication list*).

b. Development of a Mycobacterium-specific colorimetric PCR assay (PCR-ELISA): A *Mycobacterium*-specific colorimetric-PCR assay involving PCR amplification coupled with ELISA-like amplicon detection step was developed for species-specific detection and quantification of MWF Mycobacteria. A 26 base biotin-labeled capture probe able to bind to the amplified *hsp* product of both *M. immunogenum* and *M. chelonae* was designed. Additionally, we have designed individual species-specific probes (*M. immunogenum* (Mi)- and *M. chelonae* (Mc)- specific probes) for the their specific detection in MWF. Minimal detection limit (MDL) of the assay was optimized and the developed protocol was extended to the field MWF samples contaminated with mycobacteria and a mixed background microflora and was found comparable to the real-time QPCR developed in previous funding cycle. A manuscript on these studies is now communicated (see ***paper # 5*** *in the Publication list*).

Aim 2. Characterization of relative biocide susceptibility of MWF-colonizing mycobacterial genotypes

2A. *Isolation of new NTM strains (genotypes) from HP-linked and non-linked industrial MWF samples*

During the entire study, we routinely screened industrial in-use MWF samples obtained through our collaborator. The samples confirmed positive for mycobacteria based on our optimized hsp228PCR screening were plated out on Middlebrook 7H10 agar to recover the isolates. Several NTM isolates were recovered. The isolates were first speciated using DNA-based speciation methods (AHSPRA and ITSPRA) optimized in our past funding cycle. These novel isolates belonged to the two expected species of M. chelonae complex i.e. *M. immunogenum* (MJY-22 and MJY-24) and *M. chelonae* (MJY-20, MJY-21). However, to our surprise, we also identified (first time) the third species *M. abscessus* (genotype MJY-23) of this complex from one of the MWF samples. Multiple isolates of each species were segregated as individual strains based on morphotype (smooth versus rough) and genotype.

2B&C. *Optimization of rapid protocols to measure biocide susceptibility of MWF mycobacteria strains/genotypes*:

i). Comparative biocide susceptibility of MWF mycobacteria genotypes based on culturing assay (viability analysis): Four *M. immunogenum* genotypes, MJY-3, MJY-4, MJY-10, and MJY-12 isolated in our studies and a reference strain (ATCC 700506) originally isolated from MWF, were compared for their relative biocide susceptibility based on dose-response analysis using suspension assay coupled with culturing approach. Two biocides, one formaldehyde-releasing (Grotan containing Triazine as active ingredient) and one non-formaldehyde-releasing (Kathon 886MW containing Isothiazoline as active ingredient), and two fluid types, synthetic and semi-synthetic, were used to investigate the dose-response. Interestingly, other results showed that *M. immunogenum* reference strain offered higher resistance of 6-fold and 3.5-fold than MJY-4 and MJY-12 isolates for Groton biocide in synthetic MWF. Similarly, MJY-4 and MJY-10 showed less MIC values than *M. immunogenum* reference strain for Kathon biocide in semi-synthetic MWF. Taken together, these results showed that individual genotypes differ in their sensitivity to the common MWF biocides; this work is now published (see ***paper # 2*** *in the Publication list*).

ii. Differential Fluorescence-based assay for biocide susceptibility analysis of MWF mycobacteria: In order to study loss of viability of mycobacteria in presence of biocides in MWF with mixed flora, we developed a two-step fluorescence microscopy-based protocol to specifically measure viable versus non-viable cells of mycobacteria. The technique was then further developed to adapt for real world contaminated MWF samples to determine viable versus non-viable mycobacteria in the mixed microbial population. This non-DNA based fluorescence method could be useful in routine monitoring of biocidal activity toward mycobacteria in MWF. A manuscript on this part of the work is now published (see ***paper # 3*** *in the Publication list*).

iii). ATP-based high throughput assay for biocide susceptibility analysis of NTM genotypes: We optimized a rapid ATP-based assay for testing the biocide susceptibility of *Mycobacterium immunogenum*. The assay is based on the quantitation of intracellular ATP in the cell and reflects the viable cell count. The assay was developed by optimization of mycobacterial cell lysis protocol for extraction of ATP using different chemical and/or mechanical treatments and standardization of growth conditions and inoculum size for biocide susceptibility testing of MWF mycobacteria. BacTiter-Glo kit (Promega, Madison, WI) was used for quantification of the released cellular ATP (the reagent relies on the properties of luciferase enzyme and generates a luminescent signal which is proportional to the amount of ATP present). Lysozyme (final concentration 3mg/ml) treatment for 1 hour followed by bead beating (0.1 mm glass beads) was found to be the most efficient lysis method. Sauton's medium was found to give better growth giving higher cell counts as compared to MB7H9 broth, and lower inoculum was found to give more steady growth. The developed mycobacterial ATP

assay was evaluated for Kathon biocide susceptibility testing using the "growth inhibition assay". The MIC of Kathon, defined as the minimal concentration of biocide required for complete inhibition of growth (absence of any visible growth on the plates and luminescence values ≤ 100), was determined to be 25 ppm, 40 ppm and 20 ppm respectively, for *M. immunogenum* genotypes 700506, MJY-4, and MJY-13 and 10 ppm for both *M. chelonae* ATCC 35752 and *M. abscessus* ATCC 19977. This part of the work has led to a manuscript (see ***paper # 6*** *in the Publication list*).

Aim 3. Study antigenic characteristics of the MWF-colonizing nontuberculous mycobacteria (NTM) genotypes.

3A. Compare antigenic potential of MWF-isolated NTM strains/genotypes

a). *Macrophage immunomodulation by M. immunogenum genotypes*: Three different MI genotypes (700506, MJY-3, and MJY-13) were interacted with the macrophage cells (MH-S cell line) to understand their relative immunomodulating potential in terms of regulation of expression of pro-inflammatory cytokines (TNFα, IL-1β, IL-1α, IL-5, IL-6, IL-18) and anti-inflammatory cytokine (IL-10) and cellular response in terms of NO production, cytotoxicity and cell viability. The three MI genotypes showed the macrophage responses in the following order: Immunological response: 700506 > MJY-3 ≥ MJY-13 and Cellular damage: MJY-3 > 700506 ≥ MJY-13. Overall, the studies showed highest immunomodulation activity for 70056 and the lowest for MJY-13. This part of the work will be continued in the next cycle.

***b). Antibody response to MWF-mycobacteria in HP patients' sera (ELISA)*:** Serum samples collected from three MWF-associated hypersensitivity pneumonitis (HP) patients were tested for the presence of antibodies against *M. immunogenum* (MI) and *M. chelonae* (MC) genotypes using our optimized ELISA method. The serum (as a source of the primary antibody) was reacted with the defined antigen (cells) of MI adsorbed in a microtiter plate. The results showed that all the six serum samples (two samples from each patient) tested were positive for the presence of antibodies for the individual test genotypes of *M. immunogenum*, albeit at low and varying intensities, which indicates that the HP patients were exposed to the MWF mycobacteria, but not necessarily to these specific strains. We then screened the seroreactivity of other MWF-isolated mycobacterial genotypes of *M. chelonae* against these patient sera. The results showed that each patient serum showed variable antibody response to different genotypes of a given species and between species (**Fig. 1**). This indicates that individual specific antigens differentially distributed across the MWF-associated mycobacteria genotypes may be responsible for the observed variability in antibody response and that isolation of a panel of such key immunodominant antigens will help develop more efficient assay to monitor Ab response to mycobacteria in HP sera (yet to publish alongwith more data).

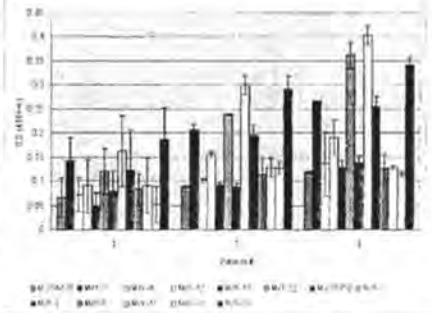

Figure 1. Antibody response to MWF-mycobacteria in HP patient sera, as measured by ELISA. The test species/strains included *M. immunogenum* (genotypes MJY-3, MJY-4, MJY-12, MJY-13, MJY-22), and *M. chelonae* (genotypes MJY-1, MJY-2, MJY-6, MJY-11, MJY-20, MJY-21). The results represented are the average of two serum samples from each patient.

3B. Identify specific antigen(s) of HP-linked *M. immunogenum* genotypes using polyclonal antibodies: For the patient serum to be useful as antibody probe, the target NTM strain (antigen source) should be, either the corresponding isolate from the MWF associated with the same HP case (which was not available in our case and is usually impractical in this industry) or should be a strain with high immuno-crossreactivity. Considering this problem, we used the custom-raised MI strain-specific antibody as probe (instead of the HP sera) for the following antigen identification studies on MI genotypes under this aim, using immunoproteomic approach.

3B-a. Identification of specific antigen(s) using polyclonal antibody (immunoproteomic approach): *i). Optimization of preparation and analysis of protein fractions*: Extracellular (supernatant) and cellular (cell wall and cytosolic) protein fractions of *M. immunogenum* 70056 cultures grown in Middlebrook broth were prepared for probing for immunogenic proteins. Extracellular proteins were obtained by concentration and precipitation of the culture supernatant. Cellular protein extraction was first optimized using bactozyme digestion and sonication.

ii). Optimization and use of immunoproteomic profiling: We optimized fractional isolation of the individual sub-cellular fractions (cell wall-, cytosolic-, membrane-) from the mycobacterial cells grown in broth cultures. Proteins in the whole extract and sub-cellular fractions were fractionated by 2D gel electrophoresis, by optimization of the IEF conditions. Since most of the proteins were found to be in the acidic range (< pH 7.0), they were focused using pH 4-7 IEF strip. The immunoreactive protein spots were identified using the anti- MI antibody probe in the Western Blot analysis. Identity of these bands was determined using MALDI-TOF analysis. This way, immunoproteomic profiling of whole extract and different subcellular protein fractions of *M. immunogenum* was performed using a combination of 2D-Gel electrophoresis, immunoblotting, and MALDI-TOF (Fig 2).

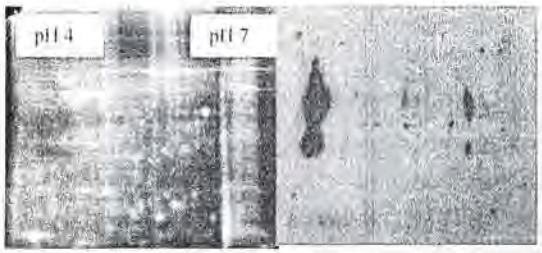

Figure 2: 2D analysis of the whole cell proteins of *M. immunogenum* 700506. Left panel: 2-Gel separation using the 1st dimension with IEF of pH 4-7 and 2nd dimension with 10 % SDS –PAGE. Right panel: Western Blot of the 2D gel to detect multiple immunogenic protein candidates.

iii). Antigen identification and immunogenicity in M. immunogenum: In all, we have identified 33 immunoreactive proteins, comprising of 4 secretory, 6 cell wall-associated, 11 membranous, and 12 cytosolic proteins (27). Of these, five immunogens (antigens) namely HSP65 (groEL), PE-PGRS, PPE, Antigen 85A, and SOD matched the known antigens in mycobacteria and seemed to represent Mycobacterium genus-specific antigens. Others represented novel antigens hitherto unidentified in mycobacteria and/or in any group of bacteria. In order to evaluate whether the antigens identified have the expected immunogenic potential, a selected immunodominant spot (containing multiple co-migrating secretory antigens) was eluted and subjected to functional characterization using murine alveolar macrophages. This 'test antigens mixture' caused upregulation of the proinflammatory cytokines (IL-1ß and TNF-α) and down-regulation of anti-inflammatory cytokine IL-10 in the macrophages, an observation consistent with that for the whole MI cells observed in the preceding subsection. This part of the work has now been published (see ***paper # 7*** *in the Publication list*).

iv). Antigen identification in *M. chelonae*: We have also optimized extraction and proteomic analysis of the secretome (cell-free secreted proteins) of *M. chelonae* and other member species of M. chelonae complex colonizing MWF. *M. chelonae* cultures grown in Middlebrook 7H9 broth showed the presence of 53 secreted proteins of which 29 were differentially induced in *M. chelonae* as against *M. abscessus*, as identified by 2DE and MALDI-TOF analysis. These preliminary studies will directly help us pursue further proposed work on *M. chelonae* and its genotypes isolated from MWF.

v). Comparative antigen profiling of *M. immunogenum* genotypes: Immunoprotein profiles were compared for the other genotype/strain (MJY-13) of *M. immunogenum* grown in broth culture, by probing with the same polyclonal antibody as raised against genotype 700506 and using the whole cell protein extract. Likewise, the whole cell protein extracts from *M. chelonae* and *M. abscessus* were probed using this *M. immunogenum* antibody. Putative genotype-specific and species-specific distribution of MI antigen homologs was generated from the test species/genotypes.

3B-b. Evaluation of the identified antigen(s): i). Using *HP sera*: We tested reactivity of the 2D-gel separated immunoreactive band(s) of *M. immunogenum* against the available HP sera from 3 different HP patients as probe. However, because of the random and non-specific nature of the sera

vis-à-vis the antigen source strain, the diagnostic potential of *M. immunogenum* immunoreactive proteins could not be ascertained in a discernible manner. This pointed to the need to use a controlled in vivo model to generate specific sera from Mycobacteria-induced HP and use them to identify the desired panel of hyperimmunogenic antigens distributed across genotypes as putative diagnostic markers for HP. To address this problem in our future studies (competitive renewal), we therefore developed an in vivo mouse model system.

ii). Using *In vivo model of mycobacteria-induced HP*: In combination with other funding resources, we have optimized an *M. immunogenum* (MI)-challenge mouse model in our hands with a long-term goal to utilize it for understanding the MI immunogenic factors and MI-induced HP diagnosis and pathogenesis (this work continues in the next funding cycle). The HP-induction potential of MI strain (ATCC 700506) originally isolated from HP-linked MWF was compared to that of the known HP-inducing thermoactinomycete *Saccharopolyspora rectivirgula* in an established farmer's lung (HP) mouse model. We have also developed an in vivo model for *M. chelonae* (MC) chronic lung exposure on the lines of *M. immunogenum* (MI), using the same mouse strain. The MC-challenge model induced pathological changes (histopathological and pathophysiological) analogous to those induced by MI, albeit to a variable extent. This model will help us pursue research on *M. chelonae* genotypes.

(A). Antigen challenge and analysis: C57Bl/6j mice (male, 6 weeks old) were exposed to *M. immunogenum* (MI) inoculum (1x 10^6 cfu/animal) by nasal instillation (50ul) for 3 consecutive days per week for three weeks. In negative and positive control groups, mice were instilled with normal saline (50 µl) and the known HP inducing thermoactinomycete *S. rectivirgula* (SR) antigen preparation (250ug/50ul), respectively using the same dosing regime for 3 weeks. All inocula were prepared in endotoxin-free water, as detailed in Experimental Protocols section of this application. An initial experiment to confirm the consistency of dose delivery was performed. Eight mice were used in each group for the periodic dosing and analysis, except the MI group wherein 14 animals were used (the additional 6 were meant for *M. immunogenum* CFU analysis, 3 right after dosing and 3 at the end of dosing). After 4 h of the last dose instillation, mice were necropsied. In each group, lungs of 3 mice were processed for histopathological analysis and CFU analysis. Lungs from the remaining five animals were lavaged and the lung tissue homogenized. The BAL fluid and lung tissue homogenates were processed for pathophysiological analysis in terms of different parameters. BAL fluid was analyzed for nitric oxide (NO), lactate dehydrogenase (LDH), total cell count (TCC), differential cell count (DCC), total protein content, INF-gamma and other cytokines/chemokines, and CD4/CD8 cell counts. Spleens were taken out from the antigen-treated and vehicle treated animals for analysis of cell-mediated response (T-cell proliferation and IFN-γ assays).

(B). Pathophysiological and immune response changes consistent with HP: The histopathological analysis in the *M. immunogenum*-challenged lungs showed pathological changes consistent with hypersensensitivity pneumonitis. BAL fluid analysis showed cellular infiltration, induction of proinflammatory cytokines/chemokines, and change in CD4/CD8 ratio, consistent with hypersensitivity pneumonitis lung as in the positive control and typical Th1 type response consistent with HP immunopathology. Lung tissue showed increases in protein content and proinflammatory cytokine/chemokines. Splenic lymphocyctes from the MI-exposed experimental mice showed Th1 type cell-mediated immune response in terms of T-cell proliferation and IFN-gamma induction, when challenged with the same mycobacterial strain (*M. immunogenum* 70056). Such response to *M. immunogenum* challenge was specific and was not observed with the negative and positive control spleens, as expected. In CFU analysis, lack of recovery of the live mycobacteria from the challenged mice, showed no live infection implying that the changes are a result of preformed antigens. In essence, the pathophysiological and immune response changes in the MI-induced HP mice and in the established experimental HP model (positive control) showed the same trend and were almost comparable at the tested doses of the respective antigens. This shows that our MI-challenge in vivo model is useful to conduct future studies on HP induction potential of MI and role of its individual antigens.

c. Significance

Mycobacteria have been implicated in respiratory disorders in workers exposed to metalworking fluids. Therefore detection and quantitation of Mycobacteria in MWF is warranted for early assessment and elimination of these occupational hazards. The proposed study (aim 1) has yielded more efficient and simplified DNA-based tools for mycobacterial contamination monitoring in occupational MWF environments. Biocide susceptibility studies (aim 2) have provided a protocol for rapidly assessing the comparative biocide resistance of prevalent strains of mycobacteria in commercial MWF and provided solution to the need for early identification of biocide resistant mycobacteria in MWF operations. Data on relative resistance of the prevalent strains against the currently used biocides may provide direction on the judicious use of these biocides and better fluid management practices. These studies will also provide the basis for development of more effective biocides (effective against identified resistant MWF strains of mycobacteria) and formulation of a representative test inoculum (containing most resistant and sensitive strains of mycobacteria) for evaluation and testing of new biocides, which may eventually lead to the development of improved MWF and biocide formulations. Immunological characterization of MWF-prevalent NTM strains proposed under Aim 3 has helped identify the potentially immunogenic strains and their antigens that may have relevance to HP and other respiratory immune disorders in exposed workers. The information generated on antigenic potential of MWF-prevalent NTM strains will help in more informed design and interpretation of future epidemiological studies on MWF-linked illnesses. Identification of immunogenic strains and antigens will help develop future immunodiagnostic tests both for exposure assessment and clinical diagnosis of MWF-linked immune disorders such as HP in the exposed machine workers and could thus be critical for developing diagnostic and intervention strategies.

e. Publications

Research Papers

1. Yadav, J.S., S. B. Selvaraju, and I. U. H. Khan. 2006. Enhanced recovery and real-time PCR based quantification of Mycobacteria from metalworking fluids. *J ASTM Internat.* 3: 1-18 (paper ID: JAI12839).
2. Selvaraju, S.B., I.U.H. Khan, and J.S. Yadav. 2008a. Differential biocidal susceptibility of multiple genotypes of *Mycobacterium immunogenum*. *J. Industrial Microbiol. Biotechnol.* 35:197-203.
3. Selvaraju, S.B., I.U.H. Khan, and J.S. Yadav. 2008b. Specific detection and quantification of culturable and non-culturable mycobacteria in metalworking fluids by fluorescence-based methods. *Lett. Appl. Microbiol* 47:451-456.
4. Selvaraju, S.B. R. Kapoor, and J.S. Yadav. 2008. Peptide Nucleic Acid-Fluorescence in-situ hybridization (PNA-FISH) assay for specific detection of *Mycobacterium immunogenum* and DNA-FISH assay for analysis of pseudomonads in metalworking fluids and sputum. *Mol. Cell. Probes* 22:273-280.
5. Kapoor R and J. S. Yadav. 2009. Development of a species-specific colorimetric-PCR assay for detection and species differentiation of *Mycobacterium immunogenum* and *M. chelonae* and its comparison with quantitative real-time PCR for metalworking fluids. *Mol. Cell. Probes* 23:75-82.
6. Kapoor, R. and J.S. Yadav. 2009. Development of a rapid ATP-based biocide susceptibility assay for Mycobacteria and its comparison with the conventional culturing method for determining the biocidal susceptibility of *M. immunogenum* and its genotypes *J. Clin. Microbiol.* (Ms. in submission).
7. Gupta, M., V. Subramanian, and J.S. Yadav. 2009. Immunoproteomic identification of secretory and subcellular antigenic proteins and functional evaluation of the secretome

fraction of *Mycobacterium immunogenum*, a newly recognized species of the *M. chelonae-M. abscessus* group. *J. Proteome Res.* 8:2319-2330.

Abstracts/Presentations:

1. Kapoor, R., S.B.Selvaraju, and, J.S.Yadav.2007. A simplified DNA amplification and colorimetry- based assay for specific detection and quantification of MWF Mycobacteria. Proc. 107th General Meeting of the American Society for Microbiology, Toronto, Canada May 21-25, 2007.
2. Selvaraju, S.B., and J.S.Yadav. 2007. Differential biocide susceptibility of the multiple genotypes of *Mycobacterium immunogenum*. Proc. 107th General Meeting of the American Society for Microbiology, Toronto, Canada May 21-25, 2007.
3. Selvaraju, S.B., and J.S.Yadav. 2007. Fluorescent in-situ hybridization assay for rapid detection of *Mycobacterium immunogenum*. Proc. 107th General Meeting of the American Society for Microbiology, Toronto, Canada May 21-25, 2007.
4. Gupta, M. K., V. Subramanian, and J.S. Yadav. 2007. Antigenic proteins in *Mycobacterium immunogenum*. Proc. 1st Microbial Pathogenesis Research Retreat, Cincinnati, OH, Sep 10, 2007.
5. Bangar, H., and J. S. Yadav. 2007. Alveolar macrophage responses to *Mycobacterium immunogenum*. Proc. 1st Microbial Pathogenesis Research Retreat, Cincinnati, OH, Sep 10, 2007.
6. Gupta, M. K., V. Subramanian, and J.S. Yadav. 2008. Immunoproteomic identification of antigenic proteins in *Mycobacterium immunogenum*, a species implicated in hypersensitivity pneumonitis. Proc. 108th General Meeting of the American Society for Microbiology, Boston, MA June 1-5, 2008.
7. Bangar, H. and J. S. Yadav. 2008. Cellular and immunological responses of alveolar macrophages to *Mycobacterium immunogenum,* a species associated with occupational hypersensitivity pneumonitis. Proc. 108th General Meeting of the American Society for Microbiology, Boston, MA June 1-5, 2008.
8. Yadav, J.S. R. Kapoor, and S. Selvaraju. 2008. Mycobacteria in metalworking fluids: advanced assays for assessment and biocide control. Invited Oral presentation, Proc. 3rd MRF Symp., Dearborn, MI, Oct.5-8, 2008.
9. Bangar, H. M.K. Gupta, and J.S. Yadav.2009. *Mycobacterium immunogenum* induces variable hypersensitivity pneumonitis pathology in mice: studies on pathological, cellular, and immunological responses. Proc. 109th ASM General Meeting, Philadelphia May 17-21, 2009.
10. Gupta, M.K. and J.S. Yadav.2009. Comparative secretome analysis for pathogenic species of the *Mycobacterium chelonae_abscessus* complex. Proc. 109th ASM General Meeting, Philadelphia May 17-21, 2009.

NEWS ITEMS (Public Media Coverage)

NEWS ITEMS on Mycobacterial antigen identification studies

1. Finding antigen culprits in an occupational disease
By Laura Cassiday, Research profile section ***J. Proteome Res.*, 2009**, *8* (5), p 2136
Publication Date (Web): March 23, 2009 (Research Profile) **DOI:** 10.1021/pr900201z
http://pubs.acs.org/doi/abs/10.1021/pr900201z

2. An advance in solving the mysterious machine-workers' disease. ***American Chemical Society's Weekly PressPac*** -- *May 20, 2009* released to 20 outlets-selected URLs given below.
http://www.sciencedaily.com/releases/2009/05/090525115310.htm
http://www.eurekalert.org/pub_releases/2009-05/acs-acs052009.php
http://esciencenews.com/sources/science.daily/2009/05/25/advance.in.solving.mysterious.machine.workers.disease
http://www.agencyscience.com/clippings_display.php?q=sc_n-09052510-an-advance-solving-the-mysterious-machine-workers-disease
http://www.vidyya.com/vol11/v11i133_7.htm
http://www.bio-medicine.org/biology-news-1/American-Chemical-Societys-Weekly-PressPac----May-20--2009-8551-1/
http://www.mctooling.com/index/images/item1306.htm

3. Q&A: University of Cincinnati Researchers ID Proteins in Mycobacterium Afflicting Machinists, June 04, 2009, By Tony Fong, **ProteoMonitor** *The global newsweekly of Proteomics Technology,* GenomeWeb Daily News—June 4, 2009

http://www.genomeweb.com/proteomics/qa-university-cincinnati-researchers-id-proteins-mycobacterium-afflicting-machin

4. UC HEALTH NEWS Daily (June 4, 2009); Q&A: University of Cincinnati Researchers ID Proteins in Mycobacterium Afflicting Machinists; GenomeWeb Daily News—June 4, 2009

Form Approved Through 08/31/2015
OMB No. 0925-0002

Department of Health and Human Services

Final Invention Statement and Certification

(For Grant or Award)

DHHS Grant or Award No.

5 RO1 OH007364

A. We hereby certify that, to the best of our knowledge and belief, all inventions are listed below which were conceived and/or first actually reduced to practice during the course of work under the above-referenced DHHS grant or award for the period

7/1/2005 through 6/30/2010 .

original effective date — *date of termination*

B. Inventions (Note: If no inventions have been made under the grant or award, insert the word *"NONE"* under Title below.)

NAME OF INVENTOR	TITLE OF INVENTION	DATE REPORTED TO DHHS
N/A	N/A	N/A
(Use continuation sheet if necessary)		

C. Signature — This block ***must*** be signed by an official authorized to sign on behalf of the institution.

Title

Typed Name ***Deborah Galloway*** ~~Associate Vice President~~ Sponsored Research Services

Signature Deborah Galloway

Date 7/24/13

Name and Mailing Address of Institution

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