FINAL PROGRESS REPORT

Project Title: Molecular Analysis of Mycobacteria in Cutting Fluids

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1. List of Abbreviations

MWF = Metalworking fluid

HP = Hypersensitivity pneumonitis

NTM = Nontuberculous mycobacteria

ATCC = American Type Culture Collection

DNA = Deoxyribonucleic acid

PCR = Polymerase Chain Reaction

QPCR = Quantitative Polymerase Chain Reaction

RFLP = Restriction Fragment Length Polymorphism

16S rRNA gene = 16S ribosomal Ribonucleic Acid gene

hsp = Heat Shock Protein gene

KDa = Kilo Dalton

ITS = 16S-23S Internal Transcribed Spacer Region

AHSPRA = Amplified Heat Shock Protein Gene Restriction Analysis

ITSPRA = Internal Transcribed Spacer PCR Restriction Analysis

RAPD = Random Amplified Polymorphic DNA

PFGE = Pulse Field Gel Electrophoresis

MRFPA = Macrorestriction fragment pattern analysis

MIC = Minimum Inhibitory Concentration

2. Abstract

Microbial contaminants particularly nontuberculous mycobacteria (NTM) in metalworking fluids (MWFs) have been implicated in occupational respiratory illnesses such as hypersensitivity pneumonitis (HP) and asthma in exposed machine workers. Conventional approaches for exposure assessment of mycobacteria in metalworking fluid environments often lead to ambiguous (false-negative and false-positive) results, and fail to identify the prevalent mycobacteria to species- and strain-level. Hence there is a need for development of more efficient exposure assessment methods for mycobacteria in these fluids. This project involved development and application of modern DNA-based approaches for efficient monitoring of mycobacteria (both culturable and non-culturable) in MWF. The efforts involved (i) development of PCR-based protocols for real-time detection and quantitation of mycobacteria in water-based MWF; (ii). PCR screening of field samples of different commercial formulations of water-based MWFs for presence of mycobacteria followed by speciation and strain identification of the cultured isolates using optimized molecular typing methods; (iii) investigation to understand the loss of viability of mycobacteria in MWF versus saline and dose-response relationships for different commercially used biocides. The DNA-based methods developed in this study included the following: (1). Genus-specific PCR assays for culture-independent detection of mycobacteria or pseudomonads; (2). mycobacteria-specific real-time PCR assay for culture-independent quantitation of total mycobacteria (both viable and non-viable); (3). PCR-RFLP assays for speciation of MWF mycobacteria that differentiated *M. immunogenum* from *M. chelonae*; (4). DNA-fingerprinting methods for strain typing of MWF mycobacterial species. Other related methods developed for exposure assessment and characterization of MWF mycobacteria included the following: (1). Protocol for enhanced selective recovery of mycobacteria from field MWF-in-use; (2). Differential fluorescence-based microscopic assay for viable and non-viable mycobacteria in MWF; (3). Rapid screening method for estimating relative biocidal potential of field MWF samples. Besides method development efforts, the study led to the screening of 101 metalworking fluid samples from geographically diverse regions of North America and led to the isolation of a total of 18 isolates of MWF mycobacteria. These isolates represented two species of Mycobacterium, *M. immunogenum* and *M. chelonae* and differed from the one isolated from MWF diluent water (*M. diernhoferi*); 14 of the isolates could be grouped into 5 distinct novel genotypes, 3 of *M. immunogenum* and 2 of *M. chelonae*. These findings changed the existing assumption that MWF-associated mycobacteria lack genetic diversity. Another part of the study led to an understanding of the biocide-induced loss of viability of the isolated mycobacteria in the fluid matrix versus the saline. Survivability of mycobacteria to commercial biocides depended on the MWF matrix and presence of co-contaminants in in-use fluids, and was strain-dependent. The developed state-of-the-art DNA-based methods are adaptable for an effective and early monitoring of the metalworking fluids for presence of mycobacteria and thus could be used to assess and minimize worker exposure to mycobacteria via MWF in the workplace.

3. Highlights/Significant Findings

- The study led to the development of highly-specific PCR protocols for real-time detection of mycobacteria in metalworking fluids (MWF) without culturing.

- The study led to the development of an efficient culture-independent mycobacteria-specific real time quantitative PCR protocol to quantify total mycobacteria (viable and non-viable) in MWF.

- The study led to the isolation of multiple isolates of mycobacteria based on large-scale screening of commercial in-use MWFs using the developed PCR methods followed by cultural recovery.

- The study led to the development of a rapid PCR protocol for identification of mycobacteria isolates based on direct cell lysis (without lengthy DNA extraction).

- The study led to the development of two new speciation protocols based on PCR-restriction analysis targeting *hsp* gene and ITS region to differentiate the two species of mycobacteria *M. immunogenum* and *M. chelonae* found to occur in MWF.

- The study revealed the occurrence of multiple genotypes of Mycobacterium in the in-use industrial fluids based on DNA fingerprinting by Amplicon sequencing and an optimized whole-genome macrorestriction analysis using PFGE. The identified genotypes of MWF mycobacteria will serve as a reference for future epidemiological studies and as test strains for biocide efficacy testing.

- The study revealed that the biocide type and load determine the survivability of *M. immunogenum* in MWF matrix emphasizing the need for a judicious application of commonly used biocides and better fluid management practices. Survivability depended on the MWF matrix and presence of co-contaminants in the in-use fluids and was strain-dependent.

4. Translation of Findings

The findings of this study are directly translatable into practical exposure assessment methods for mycobacteria in metalworking fluid environments. Analytical laboratories in this business have initiated efforts for adopting these PCR protocols in their MWF analysis for industrial clients to achieve more reliable results on mycobacteria particularly when they are non-culturable. Our developed protocols were well received by the user community in our conference presentations. The developed highly-specific PCR protocols could allow early detection of mycobacteria in these fluids even if these have turned non-culturable due to biocide use. The developed quantitative protocol would allow estimation of the increase in total mycobacterial load (viable + non-viable) in the fluid operation Colony isolates from fluids contaminated with mycobacteria could be speciated and strain typed using the developed protocols. These timely and specific analyses will help improve the fluid management practices by appropriate and timely use of the biocides.

5. Outcomes/Relevance/Impact

Existing approaches for exposure assessment to mycobacteria in metalworking fluid environments involve conventional culturing and microscopic testing. These approaches often lead to ambiguous results, yielding false-negative and false-positive results, and fail to identify the prevalent mycobacteria to species and strain-level. Our study led to the development of DNA-based approaches for direct detection (without culturing), quantitation, and speciation/strain typing of mycobacteria in these environments. The studies included optimization of mycobacteria-specific PCR protocols for real-time detection and quantification of mycobacteria in metalworking fluids (MWF) without culturing. Analytical laboratories in this business have initiated efforts for adopting the PCR protocols in their MWF analysis for industrial clients to achieve more reliable results on mycobacteria particularly when they are non-culturable. Our real-time PCR protocols were well received at ASTM symposium (December, 2004) on 'Recovery and Enumeration of Mycobacteria in MWF Environment' and based on our recommendation, a task force has been set up to validate this technique using blinded samples in order to adopt it as an ASTM standard protocol. Our direct cell-lysis based PCR protocol was adopted for clinical studies on TB in a Taiwan study. Besides, our work revealed the occurrence of multiple genotypes of two species of MWF mycobacteria (*M. immunogenum* and *M. chelonae*); this finding has changed the existing assumption in this field that only single genotype of only one species of Mycobacterium (*M. immunogenum*) is prevalent in all metalworking fluids. The characterized novel genotypes will serve as a reference for future epidemiological studies and as test strains for biocide efficacy testing.

6. Scientific Report

A. Background (brief)**:** About 1.2 million machine workers are exposed to metalworking fluids (MWF). Lately, studies by NIOSH and other groups have provided increasingly convincing evidence that nontuberculous mycobacteria (NTM) occurring in the MWF are likely causal antigens for hypersensitivity pneumonitis (HP) in MWF-exposed machine workers. Conventional culture-based methods used for analysis of mycobacteria in MWFs are time-consuming and often ambiguous as they may give false positives and false-negatives (due to their failure to detect non-culturable/non-viable cells). Therefore, development of modern DNA-based methods for their detection, identification, and quantitation in MWF and understanding of their survivability dynamics in presence of biocides will be critical in early recognition and elimination of these occupational hazards. This study related to these aspects of development of exposure assessment methods for mycobacteria in machining fluids.

B. Specific Aims: Overall objective of this research grant was to develop and apply practical DNA-based approaches for detection, quantitation, and identification of mycobacteria in MWF for understanding the prevalent strains and their survivability in commercial MWFs. The specific aims were (i) to develop polymerase chain reaction (PCR)-based protocols for detection and quantitation of NTM in water-based MWF; (ii). to PCR screen field samples of different commercial formulations of water-based MWFs for NTM followed by strain-specific identification of the NTM isolates using molecular typing methods; (iii) to investigate viability of the identified NTM strains in MWF containing biocides.

C. Progress: *Progress on all aims has been made per the proposed plan and schedule, as described below*. Furthermore, some cultural cell recovery enhancement efforts and fluorescence-based detection efforts warranted to accomplish the proposed plan more effectively were also pursued as we went along in the study.

Aim 1: Development of PCR-based protocols for NTM detection and quantification in MWF:

i). PCR methods for detection of mycobacteria (NTM) in MWF:
A. Genus-specific PCR protocol for detection of NTM isolates from MWF (Method improvement and validation): Our initial efforts included development of genus-specific PCR methods based on 16S rRNA gene and heat shock protein (*hsp*65kDa) gene for detection of non-tuberculous Mycobacteria, using *M. smegmatis* ATCC 19420 and *M. chelonae* ATCC 35752 as reference strains (**Figure 1**) (see **Reprint 1**). We then adapted the protocol for *M. immunogenum*, a frequently occurring NTM species prevalent in Metalworking fluids that has been linked with occupational hypersensitivity pneumonitis (HP). However, the developed protocol did not amplify the target as efficiently for this NTM species as for *M. chelonae* or *M. smegmatis*. Hence, we undertook further optimization of the hsp-based protocol by designing new set of primers targeting more conserved sequences spanning a 228 bp region (instead of 439 bp region) of the *hsp* gene (**Table 1**) and by selecting optimum amplification conditions compatible with the Tm of the new primers (see **Reprint 2**). Both the end product (amplicon) detection and the real-time detection formats were optimized for this new protocol. Template preparation for rapid detection was also optimized (see below). The method was successfully

evaluated on 8 different NTM isolates (colonies) including *M. immunogenum* and *M. chelonae* from MWF and several reference strains (see **Reprint 2**). We also developed a Pseudomonas-specific PCR (**Figure 2**) for detection of MWF Pseudomonads and multiplex PCR assay for simultaneous detection of both mycobacteria and pseudomonads in MWF (**Unpublished data**)

Direct cell lysis-based rapid single-tube PCR for screening the NTM isolates: The DNA isolation step consumes most of the time required in such PCR-based analysis. The lysozyme-based chemical lysis method protocol previously optimized for maximum yield of genomic DNA from mycobacteria requires culturing of the isolate, which is time consuming. Hence we optimized a protocol for direct cell lysis in the PCR tube to develop a rapid and reliable single-tube PCR method for detection of NTM. A simple, 6-min-laboratory based cell lysis method was developed (**Figure 3**) as a pre-step for NTM detection PCR without conventional DNA extraction (see **Reprint 2**). The lysis protocol was evaluated on different strains and isolates using newly developed hsp-PCR based on 228 bp amplicon discussed above (**Figure 4**). The developed direct lysis based PCR method offers a rapid and effective alternative for the detection/diagnosis of NTM isolates in less than two hours time in end product-based PCR and in 30 min time using a real-time PCR. For conclusive identification of the mycobacterial origin of the amplicon in field isolates, we linked the PCR amplification with amplicon restriction analysis using an optimized protocol **(**see **Reprint 2)**

B. **Direct PCR on MWF samples for NTM detection without culturing (Application for screening MWF samples for NTM):** We developed our MWF screening PCR by optimizing the method of DNA isolation from the fluids and using the above optimized hsp-based amplification protocol (see **Reprint 1**). Major part of our effort in this section related to optimization of DNA extraction. We compared physical methods (filtration versus centrifugation) for recovery of NTM cells from the MWF. Centrifugation of 20 ml fluid at 10,000 rpm for three consecutive spins (10 min each) yielded desirable cell pellets. Subsequently, for different consistency fluids, use of an ultra high speed (40,000 rpm for 30 min) has been found to be more reliable for cell recovery. DNA was extracted from each pellet using an optimized protocol (see **Reprints 1 and 3**). Several MWF samples obtained from Milacron were screened for NTM detection using the optimized DNA extraction protocol and mycobacterium-specific PCRs. Relative recovery and amplifiable quality of DNA from a given sample was estimated by comparative amplicon signal intensity in a diagnostic PCR. (A eubacterial PCR based on universal primers derived from conserved regions of *E. coli* 16S rRNA gene served as the diagnostic PCR based on amplification of an expected 1039 bp fragment). Use of centrifugation pre-step in the procedure yielded higher DNA recovery in comparison to filtration, indicating the former to be the method of choice for cell recovery (**Figure 5**). In an initial evaluation of the developed methods, 20 samples were screened using PCR approaches (**Table 2**). Eleven of the 16 DNA samples (M001 to M016) extracted showed positive eubacterial PCR reactions indicating their amplifiable quantity and quality. *Mycobacterium*-specific 16S rRNA gene PCR allowed an amplification of the expected 924 bp fragment from 11 of the first twenty samples (**Table 2; Figure 6**). However, only five samples exhibited positive amplification reaction with the *hsp*-gene based protocol by yielding the expected 439 bp amplicon, indicating greater specificity of the hsp-based method (**Figure 6**). Not all samples found positive based on direct PCR screening yielded colonies on the Mycobacterium growth media indicating the potential of the developed PCR-based screening method (**Table 3**) to detect non-culturable mycobacteria in MWF samples (see **Reprint 1**).

This showed reliability of detecting mycobacteria by our genus-specific PCR screening technique over the conventional culture technique. Even the non-culturable mycobacteria were detectable by the PCR approach. The developed Mycobacterium-specific PCR screening protocol could therefore serve as an efficient tool for rapid screening and analysis of MWF samples from plants with cases of hypersensitivity pneumonitis in occupational patients.

ii). PCR quantification of culturable and non-culturable mycobacteria: Conventionally, agar plating using media such as Middlebrook agar or Lowenstein Jensen agar is performed to yield an estimate of total culturable mycobacteria. However, other bacteria usually show up on these agar media thereby yielding misleading counts. Moreover, this method only applies to culturable fraction and does not account for non-culturable fraction, which may equally contribute to antigenicity and respiratory disorders. Hence, our efforts focused on development of quantitative PCR to estimate total (culturable and non-culturable) mycobacteria in MWF. In our initial efforts, a 'quantitative competitive PCR' protocol based on end product quantitation was successfully developed using *Pseudomonas fluorescens* ATCC 13525 as a reference organism and we applied this method for quantitation of total *Pseudomonas* in metalworking fluid and aerosol samples (**Figure 7**) (see **Reprint 1**). In order to make it a rapid and efficient protocol, we focused on development of 'Real-time format' of the quantitative PCR.

<u>Quantitative real-time PCR (Method optimization)</u>: Our initial efforts on development of PCR-based quantification protocol included real-time quantification using Smart Cycler (Cepheid, Inc.) machine. Subsequently, we further optimized our protocol using a more sensitive real-time PCR equipment the ABI HT7900 system (see **Reprint 3**) and our optimized *hsp*-based PCR protocol (228 bp amplicon). A standard curve was prepared based on increasing number of *M. immunogenum* cells (10^1, 10^2, 10^3, 10^4, and 10^5 cells, respectively). As few as 10 cells could yield quantifiable signal in this protocol although even a single cell could yield a detectable signal (**Figure 8**). Total DNA isolated directly from different MWF samples was then analyzed to estimate the number of mycobacteria in each sample based on the developed standard curve (**Table 4**).

<u>Further development and validation of the real-time QPCR</u>: Subsequently, the mycobacterium-specific quantitative real-time PCR protocol was further developed (to improve the minimum detection/quantification limit from 10 CFU/ml to 1 CFU/ml while using the simpler equipment, the SmartCycler) and validated for different metalworking fluid types with varying consistency. Particularly, attention was paid towards optimizing the cell/DNA recovery using simulated samples (high count as well as low count) and method validation and application of the method to field in-use samples (see **Reprint 8**).

<u>Aim 2:</u> <u>Development and application of DNA-based identification methods for non-tuberculous Mycobacteria (NTM) in used MWF</u>:

i). NTM isolates from MWF: Eighty four field samples of MWF drawn from different industrial plants were obtained through local Milacron, Inc. After initial analysis of all samples for pH, total bacterial count, and acid fast staining, these were screened by mycobacterium-specific PCR for the presence of NTM. The 46 samples yielding positive signal for presence of NTM were directly plated to isolate culturable mycobacteria on Middlebrook 7H10 (M7H10) agar and Lowenstein Jensen (LJ) agar by pour plate and spread plate methods. Alternately, each sample (10 ml) was centrifuged and the bacterial pellet suspension was plated on mycobacterium-specific media to isolate putative non-tuberculous mycobacteria (NTM) colonies. Plates were incubated at both 35^0-37^oC and 45^0C for 7 days and longer to allow for mesophilic and thermophilic strains of NTM. Candidate strains were picked based on colony types and their identity as mycobacteria was tested by conventional acid-fast staining. Several putative mycobacterial isolates picked from 35-37^0C M7H10 and LJ plates based on morphological criteria (colony features and staining characteristics) were subjected to confirmation and further identification by optimization and use of the DNA-based methods as described below (**Reprint 6**).

Further Developments in NTM cultural recovery and collection of additional isolates: Considering the problem of efficient recovery of mycobacteria from the MWF matrix, we focused on optimizing the selective cultural recovery of mycobacteria from MWF (to enhance their cultural isolation) and the factors affecting this recovery. This is important not only for their more efficient cultural isolation but also for an efficient detection/quantification by PCR/QPCR. A decontamination-based recovery strategy (to suppress non-mycobacterial flora in isolations) was developed (see **Reprint 8**). So far, a total of 101 MWF samples have been screened and 19 NTM isolates have been obtained. Identification and characterization of these isolates involved optimization and use of the following DNA-based methodologies.

ii). DNA (PCR- and genomic fingerprinting)-based identification of the NTM isolates:
A. Genus-level identification (Rapid Mycobacterium-specific PCRs): PCR identification conventionally requires DNA isolation from liquid cultures using lysozyme-based lysis method as optimized previously under Aim 1. Since our goal was to develop a rapid PCR method for identification of mycobacterial isolates from MWF, we optimized a direct cell-lysis based PCR to circumvent the time-consuming DNA isolation step; the developed method allowed cell lysis directly in the same PCR tube before amplification (see under Aim 1). This improved single tube, direct lysis-based protocol helped us rapidly screen the colony isolates for the genus-level identification (see **Reprint 2**).

Genus-level identification was performed using the appropriate genus-specific PCR (based on 16S rRNA gene and *hsp* gene) on the isolates (**Table 3**). Appearance of the expected size amplicon (924 bp for 16S PCR and 439 bp for hsp-PCR) from a given isolate confirmed its mycobacterial identity (**Figure 6**). In one instance, of the putative 14 colony isolates from M7H10 agar plates, only three were confirmed as Mycobacterium by genus-specific PCRs, indicating the potential of the developed PCR protocols. Reference strains of Mycobacterium served as positive controls for comparison in these amplifications.

B. Species-level identification: (i). Amplicon restriction pattern-based assays: A modified hsp-PCR was developed based on a larger amplicon (667 bp as against 439 bp) for more reliable restriction pattern analysis for species differentiation of mycobacteria (**Figure 9A**). This method, that we named as "AHSPRA" (Amplified HSP Restriction Analysis), based on the restriction pattern analysis of the 667 bp hsp gene amplicon differentiated between *M. immunogenum* and *M. chelonae* (see **Reprint 4**). Another protocol developed in parallel based on the 16S-23S Internal Transcribed Spacer (ITS) region (see **Reprint 5**) could also differentiate between the two species and was simpler (**Figure 9B**) for product profiling as it required agarose gel electrophoresis instead of polyacrylamide gel electrophoresis needed for AHSPRA. This method, that we named as "ITSPRA" (Internal Transcribed Spacer Restriction Analysis) involved amplification of ~260 bp ITS using the newly designed Mycobacterium-specific primers followed by optimized restriction analysis. AHSPRA and ITSPRA comparison on the 11 MWF isolates yielded distinct patterns matching either of the two closely related species *M. chelonae* and *M. immunogenum*, indicating the prevalence of only these two NTM species in MWF. Seven isolates (M-JY1, 2, 6, 7, 8, 9, and 11) belonged to *M. chelonae*, whereas four isolates (M-JY3, 4, 10, and 12) belonged to *M. immunogenum*. The remaining isolate (M-JY5), isolated from MWF diluent water, however, showed distinctly different patterns as it belonged to a different species *M. diernhoferi* as determined based on amplicon sequencing (see below).This showed that AHSPRA and ITSPRA patterns would enable us to distinguish MWF prevalent species of Mycobacteria without sequencing. Lately isolated additional 5 NTM isolates from MWF identified using ITSPRA also belonged to these two species *M. immunogenum* and *M. chelonae*.

(ii). Development of species-specific multiplex PCR assay for MWF mycobacteria: Lately (since the previous submission), we have optimized a multiplex PCR protocol based on species-specific primers that distinguishes between the two MWF species *M. immunogenum* and *M. chelonae* in one PCR reaction instead of using restriction analysis (**Unpublished data**). This species-specific assay is expected to serve as useful more rapid tool (than those based on restriction analysis) for larger epidemiological applications for screening a large number of NTM isolates from MWF.

C. Strain-level identification: Two strategies, amplicon sequencing and genome fingerprinting, meant for strain-specific characterization of bacteria, were optimized and applied for identification of NTM strains isolated from field MWF (see **Reprints 1 and 6**).

i). Amplicon sequencing: The basis of this approach was to amplify and sequence the hypervariable region of DNA from closely related strains. We targeted the hypervariable sequences of *hsp* gene and ITS region for the purpose to differentiate among strains of mycobacteria. Amplicons from the newly developed hsp-PCR (667 bp) and ITS-PCR (~260 bp), for each Mycobacterial isolate were purified (Gene Clean method) and sequenced by automated sequencing using big-dye terminator kit. Sequence comparison among the 11 isolates helped distinguish between closely related strains of a species (see **Reprint 6)**. The hsp sequence comparison (**Figure 10**) on the 7 *M. chelonae* isolates showed two strains, one including the isolates M-JY1, 2, 6, 7, 8, and 9, whereas the other including the isolate M-JY11; the latter had merely one base difference from the remaining six isolates. On the other hand, the four *M. immunogenum* isolates (M-JY3, 4, 10 and 12) showed an identical hsp sequence indicating one common strain. This strain, however, showed one base difference from a reference strain (ATCC 700506) already isolated from MWF by others. This shows the prevalence of more than one strain of this species as well in MWF. Comparison of the ITS sequence (~260 bp), however, revealed (**Figure 11**) 3 strains for *M. chelonae* isolates; strain 1 included M-JY1, 2, 7, 8, and 9 which had identical sequence. Strain 2 included M-JY6, which had 3 base differences from strain 1, and strain 3 included M-JY11, which differed by 3 bases from strain 1 and by 2 bases from strain 2. On the other hand, the four isolates of *M. immunogenum* (M-JY3, 4, 10 and 12) showed identical sequence representing one common strain. Like with hsp sequence, this isolated *M. immunogenum* strain differed by one base from the reference strain based on ITS sequence. These observations indicate usefulness of the *hsp* gene- and ITS region- sequencing in strain identification, albeit on a limited sequence difference basis. Amplicon sequences (hsp and ITS) for the water isolate M-JY5 showed significant base differences from the eleven MWF isolates and was identified as *M. diernhoferi* based on Blast homology in the gene database. Of the two Pseudomonas isolates sequenced for their 16S amplicon (440 bp), one showed closest Blast homology to *P. nitroreducens* whereas the other isolate matched an undefined *Pseudomonas* species (**Unpublished data**).

ii). <u>Genome fingerprinting</u>: Genomic DNA fingerprinting using Pulsed-field gel electrophoresis (PFGE) was optimized for mycobacteria using reference strains (*M. immunogenum*, *M. chelonae* and *M. smegmatis*) and the optimized conditions were applied to selected field isolates (see **Reprints 1 and 6**). The field isolates offered especially difficult challenges because of problems such as incomplete and variable cell wall lysis and DNA release, broken DNA, etc. Optimization of PFGE was undertaken using BioRad's CHEF DR III system (**Figure 12**). Variables were optimized for different steps including preparation of optimal cell suspension and agarose plugs, DNA release and restriction digestion in the plugs and CHEF DR III gel electrophoresis conditions.

PFGE based Macrorestriction fragment pattern analysis (MRFPA) of the 14-mycobacterial isolates was determined using *Xba*I restriction digestion (**Figure 12**; see **Reprint 6**). The 7 *M. chelonae* isolates M-JY1, 2, 6, 7, 8, 9 and 11 belonged to three different clones (genotypes). However, based on the widely accepted criteria described by Tenover et al (1993), the isolates were groupable into 2 clones; the five isolates (M-JY1, 6, 7, 8, 9) with identical MRFPA patterns were groupable under clone 1, which also included M-JY2, that differed only by absence of one band, and M-JY11 with a different MRFPA pattern represented clone 2 (which was identical to *M. chelonae* reference strain). On the other hand, the six *M. immunogenum* isolates belonged to three different clones, clone 1 included M-JY3 and M-JY4, clone 2 included M-JY10 and 12, and clone 3 included M-JY13 and 14, each clone represented by identical MRFPA patterns. Interestingly, neither of these clones showed similarity to *M. immunogenum* reference strain. The *M. diernhoferi* water isolate M-JY5 represented a unique clone with MRFPA non-identical to all eleven MWF isolates as well as the reference strains. The MRFPA patterns generated by another restriction enzyme *Spe*I differed to some extent (**Table 5**) and were not as discriminatory for strain typing as the *Xba*I patterns. Dendogram analysis on both MRFPA patterns yielded additional information that facilitated the genotype grouping.

On comparison of the two methods optimized, the amplicon sequencing and the PFGE, the latter showed a better resolving power to differentiate among strains for the two MWF mycobacterial species. In parallel, optimization of a PCR-based genotyping method (RAPD-PCR fingerprinting) was attempted in terms of primers and conditions. The method yielded promising patterns that seemed to distinguish strains for the MWF isolates; however, the method was prone to ambiguity due to generation of non-specific bands. Taken together, the PFGE-based method proved most efficient for the strain genotyping.

<u>Aim 3</u>: <u>Viability of Mycobacterial strains in MWF containing biocides:</u>

i. <u>Agar diffusion assay for biocidal activity of MWF</u>: Initial efforts under this aim included development of rapid biocidal activity assays (well diffusion- and disk diffusion- assays) for MWF using different test organisms (*Mycobacterium smegmatis, Ps. fluorescens,* or *E. coli*) and their application to field samples of MWF. Relative biocidal load in each sample was tested based on these assay methods. Zone of clearance (mm diameter) was used as a relative measure of biocidal activity against the tested strain (**Table 6**) (**Unpublished data**). The results showed that the MWF samples yielding mycobacterial isolates had considerable biocidal potential, indicating survivability of mycobacteria in presence of the added commercial biocides.

ii. <u>Fluorescence-based assay for viable versus non-viable mycobacteria in MWF</u>: In order to study viability of mycobacteria in presence of biocides in MWF, we developed a two-step fluorescence-based protocol to specifically measure viable versus non-viable cells of mycobacteria. The first step was meant to selectively detect total mycobacteria (viable+non-viable) in mixed flora of MWF using auramine-rhodamine fluorescent staining (BD Biosciences, Sparks, MD) and the second step to detect viable mycobacteria using suitable pretreatment of the sample followed by Baclight fluorescent staining (Mol. Probes, Inc., Eugene, OR) of pretreated sample; a combination of the two allowed differentiation between viable and non-viable mycobacteria cells. In the former step, NTM cells stained red whereas the other cells remained unstained. In the latter, when a simulated sample (based on a defined mixture of the viable and non-viable cells prepared from the logarithmically growing NTM cells) was examined, viable cells and heat-killed (non-viable) cells were shown to exhibit green and red fluorescence, respectively and the results on viable count were comparable to those obtained by the cultural method, indicating the validity of the developed protocol. The technique (**Figure 13**) was then further developed to adapt for real world contaminated MWF sample to determine viable versus non-viable mycobacteria in the mixed microbial population. The first step involved direct staining of the sample for specific detection of total mycobacteria (viable + non-viable) by AO staining, whereas the second step involved pretreatment of the sample with an antibiotic mixture PANTA plus to kill non-mycobacterial population, before staining for viable mycobacteria. This non-DNA based fluorescence method may be useful in biocide studies on mycobacteria in MWF.

iii). <u>Dose-response analysis for commercial biocides towards MWF *Mycobacteria*</u>: Biocidal resistance was compared based on Dose response analysis for two *M. immunogenum* strains, MJY-3 strain isolated in this study and the reference strain (ATCC 700506) isolated previously from MWF. 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. These dose-response data are shown in **Figure 14**. The minimum inhibitory concentration (MIC) value (corresponding to 100% kill) for *M. immunogenum*-MJY-3 strain was observed to be 50,000 ppm in both synthetic and semi-synthetic fluids for Grotan, and 4000 ppm in synthetic fluid and 7000 ppm in semi-synthetic fluid for Kathon.

Comparatively less MIC values for the *M. immunogenum* reference strain were observed, 30,000 ppm in synthetic and 20,000 ppm in semi-synthetic fluid for Grotan and 600 ppm (not plotted in the figure due to scale-reasons) and 5000 ppm for Kathon in synthetic and semi-synthetic fluids, respectively. The results showed that *M. immunogenum* strain M-JY3 has 1.6-fold and 2.5-fold higher resistance than ATCC strain towards Grotan in synthetic and semi-synthetic fluids and increased resistance of 6.6-fold in synthetic and 1.4-fold in semi-synthetic fluids towards Kathon. Furthermore, the non-formaldehyde (HCHO) biocide Kathon was observed to be effective at much lower concentration as compared to HCHO-releasing Grotan biocide for both strains (**Unpublished data**).

Our comparative studies on *Mycobacterium* and *Pseudomonas* have shown that, mycobacteria are more resistant than Pseudomonads to all 4 tested biocides, 2 formaldehyde-releasing (Grotan and Bioban) and 2 non-formaldehyde-releasing (Kathon and Preventol) biocides and in both fluid types (synthetic and semi-synthetic) and that Mycobacteria show more resistance towards these biocides in mixed cell population with co-occurring contaminants such as Pseudomonas. Detailed Dose-response data were generated (see **Reprint 7**).

D. Conclusions

- The study led to the optimization of mycobacteria-specific PCR protocols for real-time detection of mycobacteria in metalworking fluids (MWF) without culturing.
- The study led to the development of an efficient culture-independent mycobacteria-specific real time quantitative PCR protocol to quantify total mycobacteria (viable and non-viable) in MWF.
- The study led to the isolation of multiple isolates of mycobacteria based on large-scale screening of commercial in-use MWFs using the developed PCR methods followed by cultural recovery.
- The study led to the development of a rapid PCR protocol for identification of mycobacteria isolates based on direct cell lysis (without lengthy DNA extraction).
- The study led to the development of two new speciation protocols based on PCR-restriction analysis targeting *hsp* gene and ITS region to differentiate the two species of mycobacteria *M. immunogenum* and *M. chelonae* found to occur in MWF.
- The study revealed the occurrence of multiple genotypes of Mycobacterium in the in-use industrial fluids based on DNA fingerprinting by Amplicon sequencing and an optimized whole-genome macrorestriction analysis using PFGE. The identified genotypes of MWF mycobacteria will serve as a reference for future epidemiological studies and as test strains for biocide efficacy testing.
- The study revealed that the biocide type and load determine the survivability of *M. immunogenum* in MWF matrix emphasizing the need for a judicious application of commonly used biocides and better fluid management practices. Survivability depended on the MWF matrix and presence of co-contaminants in the in-use fluids and was strain-dependent

7. Publications

Research Papers

1. Yadav, J. S., I. U. H. Khan, F. Fakhari, and M. B. Soellner. 2003. 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.* **18:**966-975.
2. Khan, I. U. H., and J. S. Yadav. 2004a. Development of a single-tube, cell lysis-based, genus-specific PCR method for rapid identification of mycobacteria: optimization of cell lysis, PCR primers and conditions, and restriction pattern analysis. *J. Clin. Microbiol.* **42:**453-457.
3. Khan, I. U. H., and J. S. Yadav. 2004b. Real-time PCR assays for genus-specific detection and quantification of culturable and non-culturable mycobacteria and pseudomonads in metalworking fluids. *Mol. Cell. Probes*. **18**: 67-73.
4. Selvaraju, S. B., I. U. H. Khan, and J. S. Yadav. 2005. A new method for species identification and differentiation of *Mycobacterium chelonae* complex based on amplified *hsp*65 restriction analysis (AHSPRA). *Mol. Cell. Probes*. **19**:93-99.
5. Khan, I. U. H., S. B. Selvaraju, and J. S. Yadav. 2005. Method for rapid identification and species differentiation for *Mycobacterium chelonae* complex based on 16S-23S Ribosomal DNA internal transcribed spacer (ITS) PCR-restriction analysis (ITSPRA). *J. Clin. Microbiol*. **43**(9): 4466-4472.
6. Khan, I. U. H., S. B. Selvaraju and J. S. Yadav. 2005. Occurrence and characterization of multiple novel genotypes of *Mycobacterium immunogenum* and *Mycobacterium chelonae* in metalworking fluids. *FEMS Microbiol. Ecol*. **54**:329-338.
7. Selvaraju, S. B., I. U. H. Khan, and J. S. Yadav. 2005. Biocidal activity of formaldehyde and nonformaldehyde biocides toward *Mycobacterium immunogenum* and *Pseudomonas fluorescens* in pure and mixed suspensions in simulated synthetic metalworking fluid and saline. *Appl. Environ. Microbiol*. **71**(1):542-546.
8. 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).
9. Selvaraju, S., I.U.H. Khan, and J.S. Yadav. Fluorescence-based methods for specific detection and quantification of culturable versus non-culturable mycobacteria in the mixed microflora in metalworking fluids. (in preparation).

Abstracts

1. Yadav, J. S. and I. Khan. 2002. Respiratory pathogens in metalworking fluids: DNA-based methodologies for rapid detection, quantification, and species- or strain-level identification of *Mycobacteria* and *Pseudomonas*. Oral presentation; Proc. Amer. Conf. of Government Indus. Hygienists (ACGIH), Oct.2-4, 2002, Cincinnati, OH, p27.
2. Khan, I.U.H., V. Subramanian, and J.S. Yadav. 2003. Real-time PCR detection and quantification of culturable and non-culturable mycobacteria from occupational

environments. Abstract book (Abs # U042-608), 103rd American Society of Microbiology, May 18-222, 2003, Washington DC.

3. Yadav, J.S, Khan, IUH, and S.B. Selvaraju. 2004. Enhanced Recovery and Real-Time PCR Based Quantification of Mycobacteria from Metalworking Fluids. Abstract for Invited presentation at ASTM Joint Symposium on ‘Recovery and Enumeration of Mycobacteria from the Metalworking Fluid Environment’, Dec. 5, 2004, Tampa, Florida.
4. Khan, I.U.H., S.B.S. Selvaraju, and J.S. Yadav.2004. Multiple Genotypes of *Mycobacterium immunogenum* and *Mycobacterium chelonae* Isolated from Occupational Environment, Accepted for 104th ASM Meeting, New Orleans, LA (May 23 - 27, 2004).
5. Selvaraju S.B.S., I.U.H. Khan, and J.S. Yadav.2004. Biocide resistance of *mycobacterium immunogenum* and *pseudomonas fluorescens* in synthetic metalworking fluid. Accepted for 104th American Society of Microbiology (ASM) Meeting, New Orleans, LA (May 23 - 27, 2004).
6. Khan, I. U. H., S. B. Selvaraju and J. S. Yadav. 2004. Genotypic identification and characterization of species of nontuberculous mycobacteria implicated in occupational respiratory illnesses. Abstract book (Abs # 14); University of Cincinnati Postdoctoral Scholars Research Forum, June 4, 2004, Cincinnati, OH.
7. Selvaraju, S. B., I. U. H. Khan, and J. S. Yadav. 2004. Antimicrobial activity of formaldehyde- and nonformaldehyde- type biocides against the potential respiratory pathogen *Mycobacterium immunogenum*. Abstract book (Abs # 21); University of Cincinnati Postdoctoral Scholars Research Forum, June 4, 2004, Cincinnati, OH.
8. Yadav, J.S., S. B. Selvaraju, and I. U. H. Khan. 2005. Enhanced recovery and real-time PCR based quantification of mycobacteria from metalworking fluids. ASTM symposium E-34 on ‘Recovery and Enumeration of Mycobacteria from metalworking fluid environment’, Dec. 5, 2004, Tampa, FL

8. RESEARCH DATA (Figures and Tables)

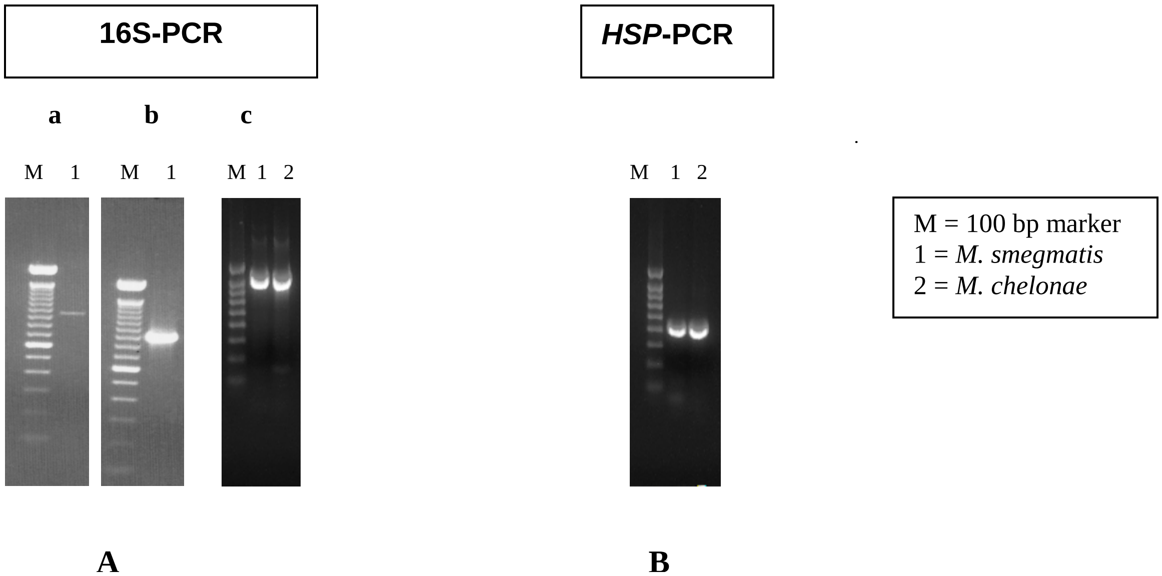


FIGURE 1. Development of genus-specific PCRs for *Mycobacterium* using the reference strains of *M. smegmatis and M. chelonae*:
(A). 16S RNA gene-based PCR yielding a 924 bp amplicon using (a). regular AmpliTaq DNA polymerase; (b). AmpliTaq Gold Taq;(c). LA-Taq (TaKaRa).
(B). Heat shock protein (HSP) gene-based PCR yielding a 439 bp amplicon

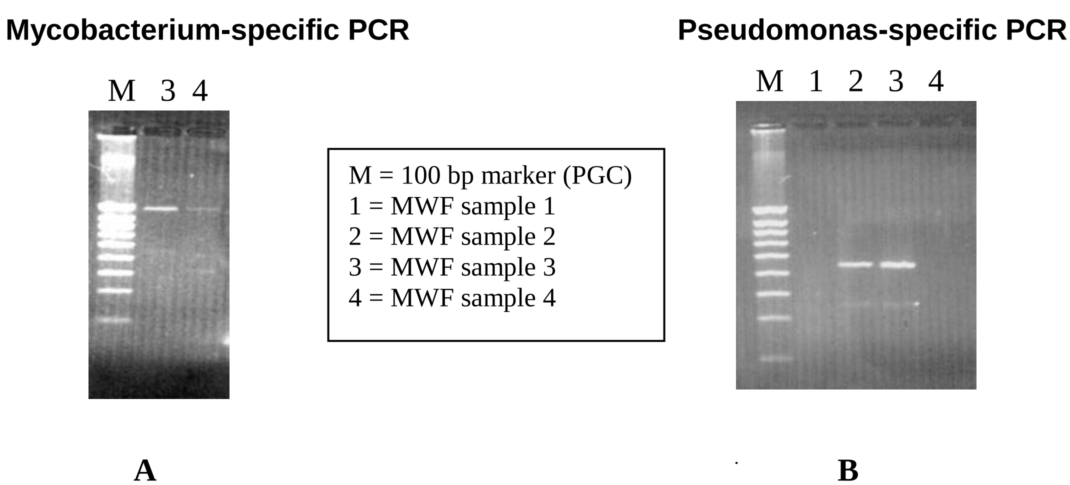


FIGURE 2: Application of genus-specific PCRs to commercial MWF samples for culture-independent detection of mycobacteria and Pseudomonads.

- **A.** Mycobacterium-specific 16S-PCR based detection of mycobacteria in commercial MWF samples (3 and 4).The expected 924 bp amplicon was observed
- **B.** Pseudomonas-specific 16S PCR based detection of Pseudomonads in commercial MWF samples (1 through 4). The expected 439 bp amplicon was observed for samples 2 and 3.

TABLE 1. Oligonucleotide Primers for *hsp*-based PCR protocols for mycobacteria

Amplicon (bp)	Forward (5´-3´)	Reverse (5´-3´)
439	ACCAACGATGGTGTGTCCAT	CTTGTCGAACCGCATACCCT
228	CTGGTCAAGGAAGGTCTGCG	GATGACACCCTCGTTGCCAAC

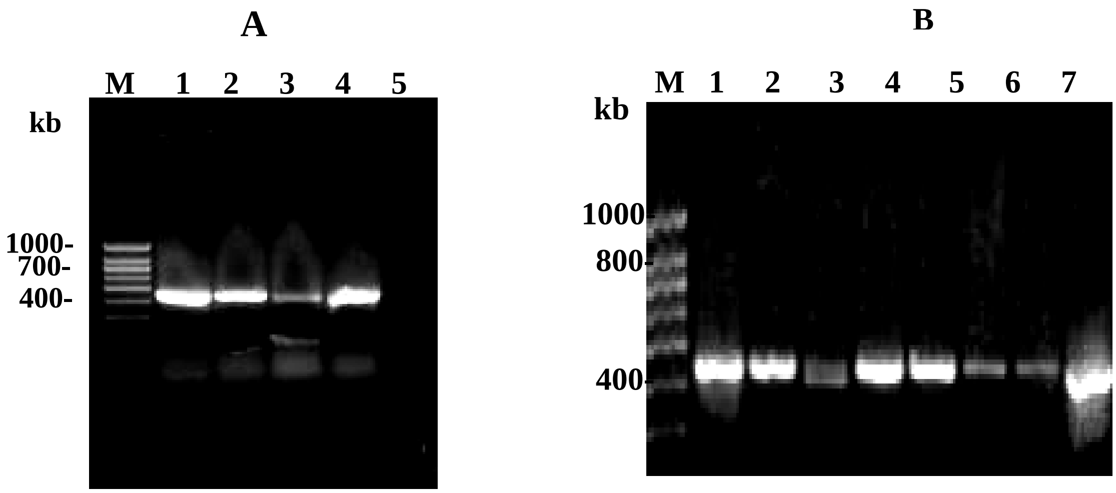


FIGURE 3. Development and evaluation of a direct cell lysis protocol as a pre-step for *hsp*-based PCR-amplification of different reference species and isolates of mycobacteria. Panel A. Lanes 1-4: *M. smegmatis, M. chelonae, M. immunogenum* and *M. bovis*; **Panel B.** Lanes 1-3: *M. smegmatis*, *M. chelonae*, *M. immunogenum*; Lanes 4-8: *Mycobacterium* isolates M-JY1, M-JY2, M-JY3, M-JY4 and M-JY5; Lane M: 100 DNA bp size marker (PGC Scientifics, USA). The optimized lysis protocol consisted of the use of lysis reagent 5 in combination with heating regime V. PCR amplification was based on 439 bp region of the mycobacterium-specific hsp gene.

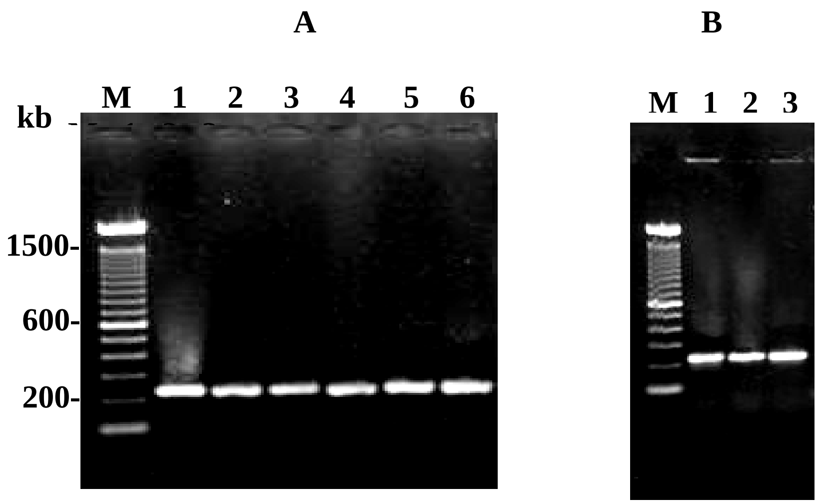


FIGURE 4. Development and evaluation of the new single tube *hsp*-based PCR-method for genus-level identification of mycobacteria isolates. Panel A. Lane 1: *M. immunogenum* strain ATCC 700506; Lanes 2-6: *Mycobacterium* isolates M-JY1, M-JY2, M-JY3, M-JY4 and M-JY5; **Panel B**. Lanes 1-3: *Mycobacterium* isolates M-JY6, M-JY7 and M-JY8, Lane M: 100 DNA bp size marker (Invitrogen, USA). Cells were lysed using optimized direct cell lysis method and the lysates were amplified using PCR primers and conditions described for amplification of a 228 bp PCR product of the *hsp* gene.

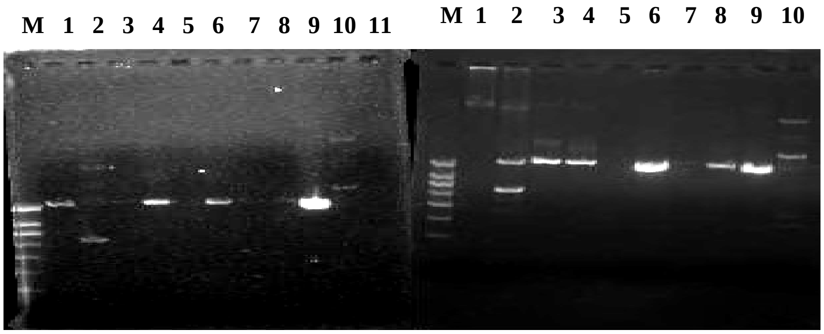


FIGURE 5: Optimization of total microbial DNA isolation from MWF matrix: comparison of filtration (panel A) versus centrifugation (panel B) as a prestep based on PCR amplifiability. The PCR amplification was based on 16S gene-based universal eubacterial primers that yielded an expected 1039 bp amplicon. Lanes 1-8: MWF samples M-004, M-005, M-006, M-010, M-011, M-012, M-015, and M-016; Lane 9: Positive control (*M. smegmatis*); Lane 10: Positive control (*P. fluorescens*); Lane 11: Negative control (no template DNA); Lane M: 100 bp DNA ladder (size marker).

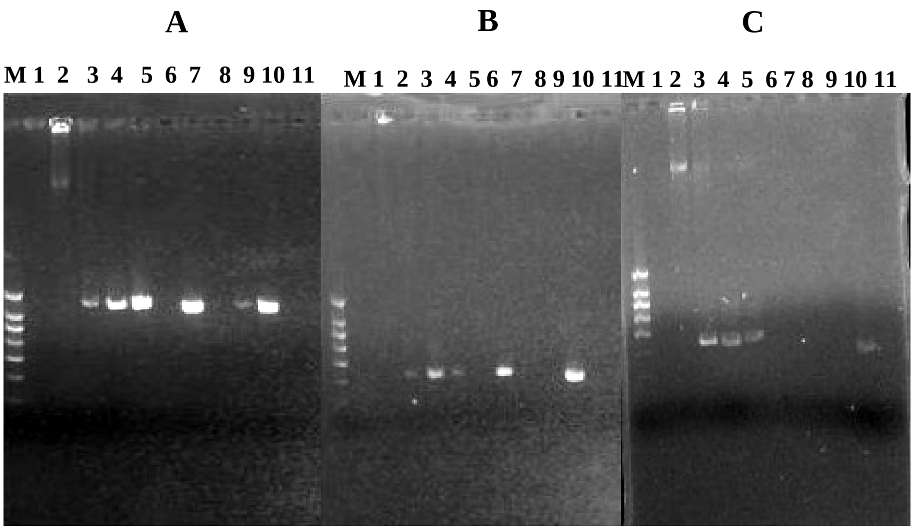


FIGURE 6. Culture-independent direct PCR detection of mycobacteria and pseudomonads in field MWF using optimized DNA isolation and Genus-specific PCR amplifications. Panel A. Mycobacterium-specific PCR based on 16S rRNA gene with an expected amplicon of 924 bp; **Panel B.** Mycobacterium-specific PCR based on heat shock protein (*hsp*) gene with an expected amplicon of 439 bp; **Panel C.** Pseudomonas-specific PCR based on 16S rRNA gene with an expected amplicon of 440 bp. Lanes 2-9: MWF DNA from samples M-004, M-005, M-006, M-010, M-011, M-012, M-015, and M-016, Lane 1: Negative control (no template DNA); Lane 10: Positive control (*M. smegmatis* in panels A&B and *P. fluorescens* in panel C); Lane 11: Negative control (*P. fluorescens* in panels A&B and *M. smegmatis* in panel C); Lane M: 100 bp DNA ladder (size marker).

Table 2: PCR-based screening of field MWF samples for bacterial contamination[B] and detection of mycobacteria[C] and pseudomonads[D]

Sample Code	Sample Type	pH	Eubacterial PCR[B]	Genus Specific-PCR	
				Mycobacterium-specific[C]	*Pseudomonas*-specific[D]
MWF 1	Synthetic	8.5	ND	-	-
MWF 2	Synthetic	7.5	ND	-	+
MWF 3	Synthetic	7.2	ND	+	+
MWF 4	Synthetic	7.2	ND	+	-
M-001	Drawing	8.3	-	-	+
M-002	Semi-Synthetic	8.7	-	-	-
M-003	Semi-Synthetic	8.1	-	-	-
M-004	Synthetic	6.8	+	-	-
M-005	Synthetic	8.2	+	+	+
M-006	Synthetic	6.6	+	+	+
M-007	Soluble Oil	8.1	-	-	-
M-008	Semi-Synthetic	6.2	+	+	+
M-009	Semi-Synthetic	7.6	+	+	+
M-010	Semi-Synthetic	7.3	+	+	+
M-011	Semi-Synthetic	8.4	-	-	-
M-012	Semi-Synthetic	8.4	+	+	-
M-013	Semi-Synthetic	8.0	+	+	+
M-014	Double Mix	8.3	+	+	-
M-015	Synthetic	8.9	+	-	-
M-016	Synthetic	8.7	+	+	-
4360	MWF	ND	-	+	+
4362	Water	ND	-	+	+
SS1	Synthetic	8.4	-	-	-
SS2	Synthetic	8.7	-	-	+
A305881	Semi-synthetic	8.6	+	-	+
B304896	Soluble oil	7.8	+	-	+
B304898	Soluble oil	7.9	+	-	+
B305805	Semi-synthetic	6.3	+	+	+
B305806	Semi-synthetic	6.6	+	+	+
B305814	Semi-synthetic	8.7	+	-	+
B306193	Synthetic	8.1	+	-	+
A306219	Soluble oil	6.7	+	+	+
A306237	Semi-synthetic	9.0	+	+	+
A306238	Semi-synthetic	8.8	+	+	+
A306241	Semi-synthetic	8.1	+	+	+
A306242	Semi-synthetic	8.7	+	+	+

[B] Eubacterial universal PCR was based on amplification of 1039 bp fragment of the 16S rRNA gene
[C] Mycobacterium-specific PCR was based on amplification of 16S rRNA gene (924 bp) or hsp gene (228 bp).
[D] Pseudomonads-specific PCR was based on amplification of 440 bp fragment of the 16S rRNA gene
ND= Not done

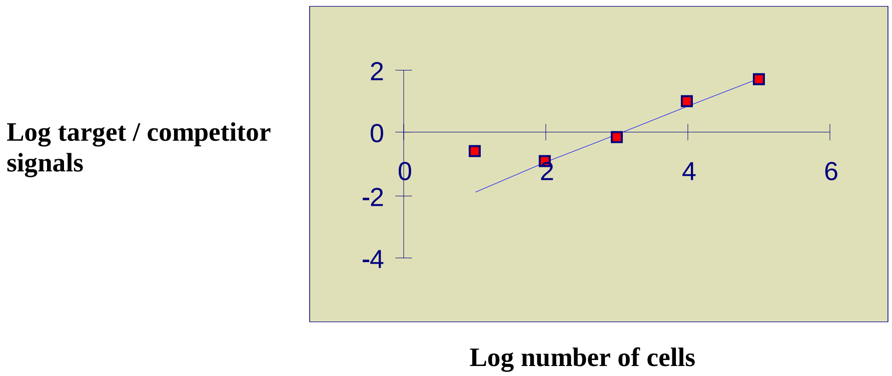
TABLE 3: Cultural isolation[A] of putative *Mycobacteria* and *Pseudomonads* from MWF and confirmation of the colony isolates by genus-specific PCRs[B]

Sample Code	***Mycobacterium***		***Pseudomonads***	
	MiddleBrook Agar (MBA)	PCR confirmation	Pseudomonas Isolation Agar (PIA)	PCR confirmation
MWF 1	-	-	-	-
MWF 2	-	-	-	-
MWF 3	+	-	-	-
MWF 4	+	-	-	-
M-001	+	-	-	-
M-002	+	-	-	-
M-003	+	-	+	-
M-004	+	-	+	+
M-005	+	-	-	-
M-006	-	-	-	-
M-007	+	-	++	-
M-008	-	-	++	-
M-009	-	-	+	-
M-010	+	-	-	+
M-011	+	+	-	-
M-012	+	+	-	-
M-013	+	+	-	-
M-014	+	-	-	-
M-015	+	-	-	-
M-016	-	-	-	-
4360	+	+	-	-
4362	+	+	-	-
SS1	++	+	-	-
SS2	+	+	-	-

[A] Plus (+) signs indicate number of colonies isolated whereas minus (–) sign indicates no growth

[B] Genus-specific PCR (for *Mycobacterium* and *Pseudomonas*) was used on colonies. Plus (+) sign indicates PCR confirmation of the genus-identity for putative isolates in each category

A



A. Standard curve for quantitative competitive PCR to measure *Pseudomonas*. Serial dilutions of a lower cell density culture (10^6/ml) were prepared and DNA extracted from the diluted cell suspensions containing 10^5, 10^4, 10^3, 10^2, and 10^1 cells/ml. DNA was coamplified with a selected amount of competitor DNA (78 fg) and the end product signals for target and competitor were quantitated using Nucleotech fluorescent gel analyzer. The x-axis gives log number of cells and the y-axis represents log ratio of target to competitor signals. y = ax + b [a=0.908; b= -2.78; r = 0.995]

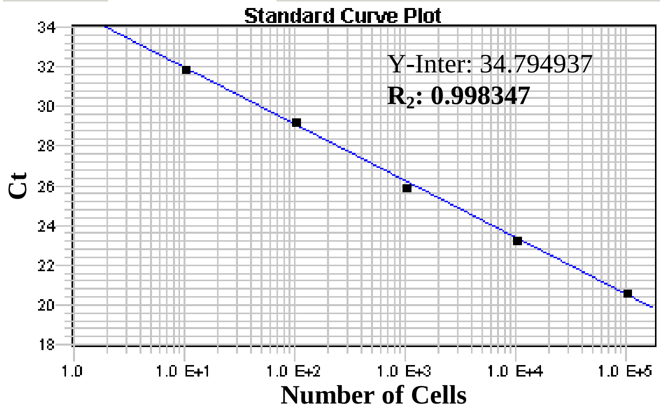
B

Quantification of *Pseudomonas* in MWF liquid and aerosols using the developed Quantitative Competitive PCR

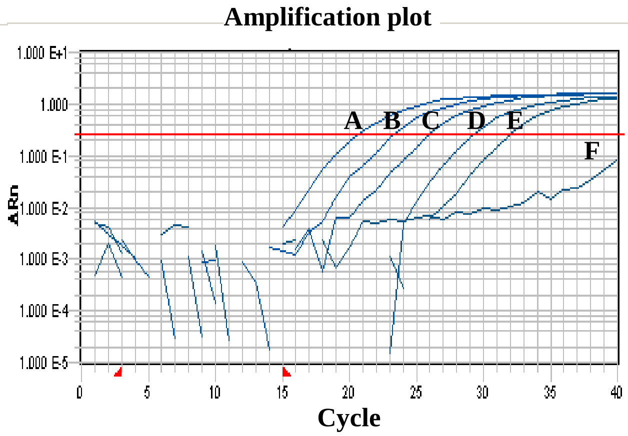
Sample	Percent Area T	Percent Area C	Ratio (T/C)	Log ratio (Y value)	Cells/ml*
MWF(liquid)	33.79	66.21	0.510	-0.292	3.648×10^3
MWF aerosol	23.21	76.79	0.302	-0.520	2.046×10^3

T = Target signal; C = Competitor signal
*Based on standard curve & dilution factor

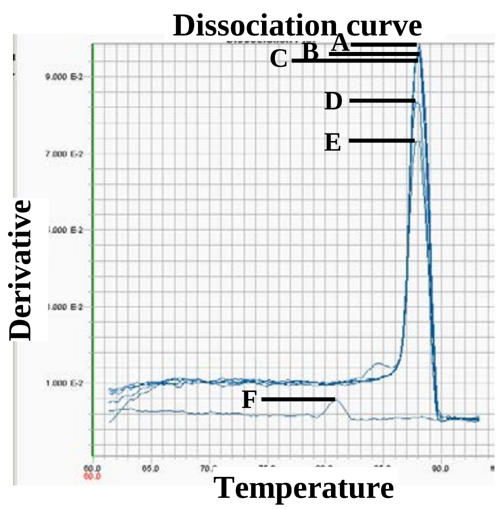
FIGURE 7. Quantitative competitive PCR to measure *Pseudomonas* in metalworking fluids and aerosols. (A) Preparation of standard curve; **(B).** Application to field samples

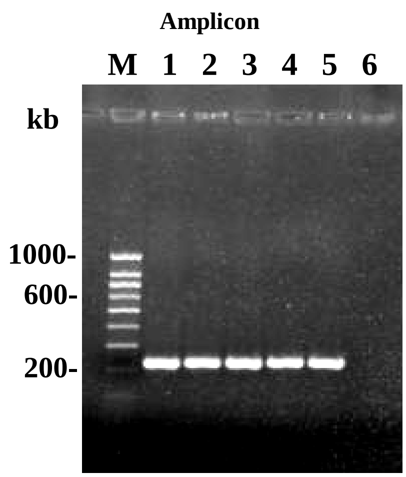


Standard curve generated based on known number of cells of *M. immunogenum*



Real-time PCR amplification graph showing the effect of varying number of *M. immunogenum* cells. A: 10^5 cells, B: 10^4 cells, C: 10^3 cells, D: 10^2 cells, E: 10^1 cells, F: negative control





The dissociation curve (a graph showing the rate of change in fluorescence over time as a function of temperature for each amplicon) is shown for six selected dilutions, A, C, E and F. A clear peak at 88ºC is visible for amplicons; whereas a spurious peak at 81ºC is visible for negative control

PCR end products with expected 228 bp size (lanes 1-5) analyzed on agarose gel 228 bp; Lane 6: Negative control; M: 100 bp DNA size marker (PGC Scientific, USA)

FIGURE 8. Preparation of standard curve for real-time quantitative PCR to measure total mycobacteria using *M. immunogenum* as standard

TABLE 4. Real-time PCR-based quantification of total Mycobacteria (culturable and non-culturable) in MWF

MWF Sample Code	Total Culturable Counts (per ml)		Total Mycobacteria (PCR-based Quantification)	
	All Bacteria*	Mycobacterial Counts**	Ct value	Number of Cells/ml MWF
M09	$6x10^5$	0	28.45	138 = $1.3x10^2$
M10	$6x10^3$	0	27.31	348= $3.4x10^2$
M14	$7x10^5$	0	24.80	2,629= $2.6x10^3$
M16	0	250	18.15	55,9940= $5.5x10^5$
M17	$2x10^4$	40	20.24	103842= $1.0x10^5$
M18	$7x10^3$	0	27.31	348= $3.4x10^2$
M20	$2x10^4$	0	26.13	900= $9.0x10^2$

* = Trypticase Soy Agar was used for total bacterial count
** = Middlebrook 7H10 agar (with OADC enrichment) was used for Mycobacterial count

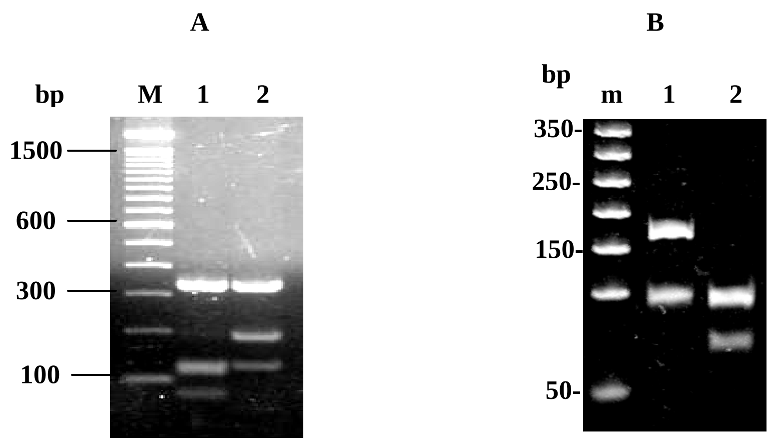


FIGURE 9. PCR-RFLP assays to differentiate between *M. immunogenum* (lane 1) and *M. chelonae* (lane 2). Panel A: AHSPRA (Amplified *hsp* restriction analysis) based on *Nar*I restriction and agarose gel (1.5%) analysis; **Panel B**: ITSPRA (ITS PCR restriction Analysis) based on *Hph*I restriction and polyacrylamide gel (12%) analysis; **M**: 100 bp DNA size marker (Invitrogen); **m**: 50 bp DNA size marker (Promega)

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ACCAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCG M-JY1
ACCAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCG M-JY2
ACCAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGATCCG M-JY3
ACCAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTCGAGGACCCC M-JY4
ACCAACGATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACCCG M-JY5

TACGAGAAGATCGGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACT M-JY1
TACGAGAAGATCGGCGCTGAGCTGGTCAAGGAAGTTGCCAAGAAGACT M-JY2
TACGAGAAGATCGGCGCCGAGCTGGTCAAGGAAGTTGCCAAGAAGACC M-JY3
TACGAGAAGATCGGTGCTGAGCTCGTCAAAGAGGGTCGCCAAGAAGACC M-JY4
TACGAGAAGATCGGCGCCGAGCTGGTCAAAGAGGGTCGCCAAGAAGACC M-JY5

GACGACGTCGCGGGTGACGGCACTACTACCGCCACCGTGCTTGCCCAGG M-JY1
GACGACGTCGCGGGTGACGGCACTACTACCGCCACCGTGCTTGCCCAGG M-JY2
GATGACGTCGCGGGTGACGGCACTACTACCGCCACCGTGCTCGCTCAGG M-JY3
GACGATGTCGCTGGCGACGGCACCACCACCGCCACCGTCCTGGCTCAGG M-JY4
GATGACGTCGCGGGCGACGGCAC-ACCACCGCCACCGTGCTGGCCCAGG M-JY5

CTCTGGTCAAGGAAGGTCCGCGTAACGTCGCTGCCGGCGCCAACCCGCT M-JY1
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CGGCCTGAAGCGCGGTATCGAGAAGGCTGTCGAGGCTGTCACCGCTCGC M-JY5

CTGCTG-ACTCCGCCAAGGAGATCGACACCAAGGAGCAGATCGCGGCCA M-JY1
CTGCTGGACTCCGCCAAGGAGATCGACACCAAGGAGCAGATCGCGGCCA M-JY2
CTGCTGGACTCCGCCAAGGAGATCGACACCAAGGAGCAGATCGCGGCCA M -JY3
CTGCTGAAGTCCGCCAAGGAGGTGGAGACCAAGGAGCAGATCGCTGCCA M-JY4
CTGCTCTCGACCGCCAAAGAGGTCGAGACCAAGGAGCAGATCGCTGCCA M-JY5

CCGCGGGCATCTCCGCGGGTGACCAGTCCATCGGTGATCTGATCGCCGAG M-JY1
CCGCGGGCATCTCCGCGGGTGACCAGTCCATCGGTGATCTGATCGCCGAG M-JY2
CCGCGGGCATCTCCGCGGGTGACCAGTCCATCGGTGATCTGATCGCCGAG M-JY3
CCGCCGGTATCTCCGCCGGTGACCAGTCCGTCGGCGACCTGATCGCCGAG M-JY4
CCGCGGGCATCTCCGCCGGTGACCAGTCGATCGGTGACCTGATCGCCGAG M-JY5

GCCATGGACAAGGTCGGCAACGAGGGTGTCATCACCGTCGAGGAGTCCAA M-JY1
GCCATGGACAAGGTCGGCAACGAGGGTGTCATCACCGTCGAGGAGTCCAA M-JY2
GCCATGGACAAGGTCGGCAACGAGGGTGTCATCACCGTCGAGGAGTCCAA M-JY3
GCCATGGACAAGGTCGGCAACGAGGGTGTCATCACCGTCGAGGAGTCCAA M-JY4
GCTCTGGACAAGGTCGGCAACGAGGGTGTCATCACCGTCGAGGAGTCCAA M-JY5

CACCTTCGGCCTGCAGCTGGAGCTCACCGAGGGTATGCGGTTCGACAAG M-JY1
CACCTTCGGCCTGCAGCTGGAGCTCACCGAGGGTATGCGGTTCGACAAG M-JY2
CACCTTCGGCCTGCAGCTGGAGCTCACCGAGGGTATGCGGTTCGACAAG M-JY3
CACCTTCGGCCTGCAGCTGGAGCTCACCGAGGGTATGCGGTTCGACAAG M-JY4
CACCTTCGGTCTGCAGCTGGAGCTCAACGAGGGTATGCGGTTCGACAAG M-JY5
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FIGURE 10. Sequence Alignment of *hsp* gene amplicon (439 bp) for different mycobacterial isolates (M-JY1, M-JY2, M-JY3, M-JY4 and M-JY5). Nucleotide differences are highlighted by shaded areas.

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CCTTTCTAAGGAGCACCATTT--CCCAGCCGAAT-----GAGCTTGGGAACA--------TAAAGTGAGTTTCTGTAGT
CCTTTCTAAGGAGCACCATTT--CCCAGCCGAAT-----GAGCTTGGGAACA--------TAAAGTGAGTTTCTGTAGT
CCTTTCTAAGGAGCACCATTT--CCCCGCCGAAT-----GAGCGTGGGAACA--------TAAAGCGGGTTTCTGTAGT
CCTTTCTAAGGAGCACCATTT--CCCCGCCGAAT-----GAGCGTGGGAACA--------TAAAGCGGGTTTCTGTAGT
CCTTTCTAAGGAGCACCATTT--CCCCGCCGAAT-----GAGCGTGGGAACA--------TAAAGCGGGTTTCTGTAGT
CCTTTCTAAGGAGCACCACGAGACCTGGCCGGCCCCGTAGATTGCGGGATCAGCCGATTGTCAGGCGATTCGTTGGA-T

GGTTACTCGCTTG--GTGAATA---------TGTTTTATAAATCCTGTCCACC--CC-------G---TGGGTAGGTAG
GGTTACTCGCTTG--GTGAATA---------TGTTTTATAAATCCTGTCCACC--CC-------G---TGGGTAGGTAG
GGTTTCCTGCTTG--GTGAATA---------TGTTTTATAAATCCTGTCCGCT--CTCGTTATCGAGGTGGATGGGTAG
GGTTTCCTGCTTG--GTGAATA---------TGTTTTATAAATCCTGTCCGCT--CTCGTTATCGAGGTGGATGGGTAG
GGTTTCCTGCTTG--GTGAATA---------TGTTTTATAAATCCTGTCCGCT--CTCGTTATCGAGGTGGATGGGTAG
GGCCTTTCGCCCTGTAGTGGGTGGGGGTCTGGTGCGACAACAAACTTGTAAAACTGCCAGACACACTATTGGGCTTTGAG

TCGGCAAAACGTCGAACTGTCAATAGAATTGAAACGCTGGCACACTG--TTGGGTCCTGAGGCAACACATTGT--GTTG
TCGGCAAAACGTCGAACTGTCAATAGAATTGAAACGCTGGCACACTG--TTGGGTCCTGAGGCAACACATTGT--GTTG
TCGGCAAGACGTCGGACTGTCAAAAGAATTGGAATGCTGGCACACTG--TTGGGTCCTGAGGCAACACATTGT--GTTG
TCGGCAAGACGTCGGACTGTCAAAAGAATTGGAATGCTGGCACACTG--TTGGGTCCTGAGGCAACACATTGT--GTTG
TCGGCAAGACGTCGGACTGTCAAAAGAATTGGAATGCTGGCACACTG--TTGGGTCCTGAGGCAACACATTGT--GTTG
ACAACAGGCCCTGCGA-TGCCCGTGCCTTTGTGGTGGGGGTTGTCGGCCTTCGACCCTTGGGGTTGGTGGTGTTTGTTG

TCACCCTGCTT-GGTGGTGGGGTGTGGTCTTTGACTTATGGATAGTGGTTGCGAGCATC  M-JY1 (253 bp)
TCACCCTGCTT-GGTGGTGGGGTGTGGTCTTTGACTTATGGATAGTGGTTGCGAGCATC  M-JY2 (253 bp)
TCGCCCTGCTT-GGTGGTGGGGTGTGGACTTTGACTTCTGGATAGTGGTTGCGAGCATC  M-JY3 (263 bp)
TCGCCCTGCTT-GGTGGTGGGGTGTGGACTTTGACTTCTGGATAGTGGTTGCGAGCATC  M-JY4 (263 bp)
TCGCCCTGCTT-GGTGGTGGGGTGTGGACTTTGACTTCTGGATAGTGGTTGCGAGCATC  M. immunogenum ATCC
TTGCCCTGCTTTGGTGGTGGGGTGTGGTGTTTGATTTGTGGATAGTGGTTGCGAGCATC  M-JY5 (296 bp)
```

FIGURE 11. Sequence alignment of 16S rRNA-23S rRNA Internal Transcribed Spacer (ITS) amplicon (~300 bp) for mycobaterial isolates (M-JY1 to M-JY5) as compared to the reference strain. Nucleotide differences are highlighted by shaded areas.

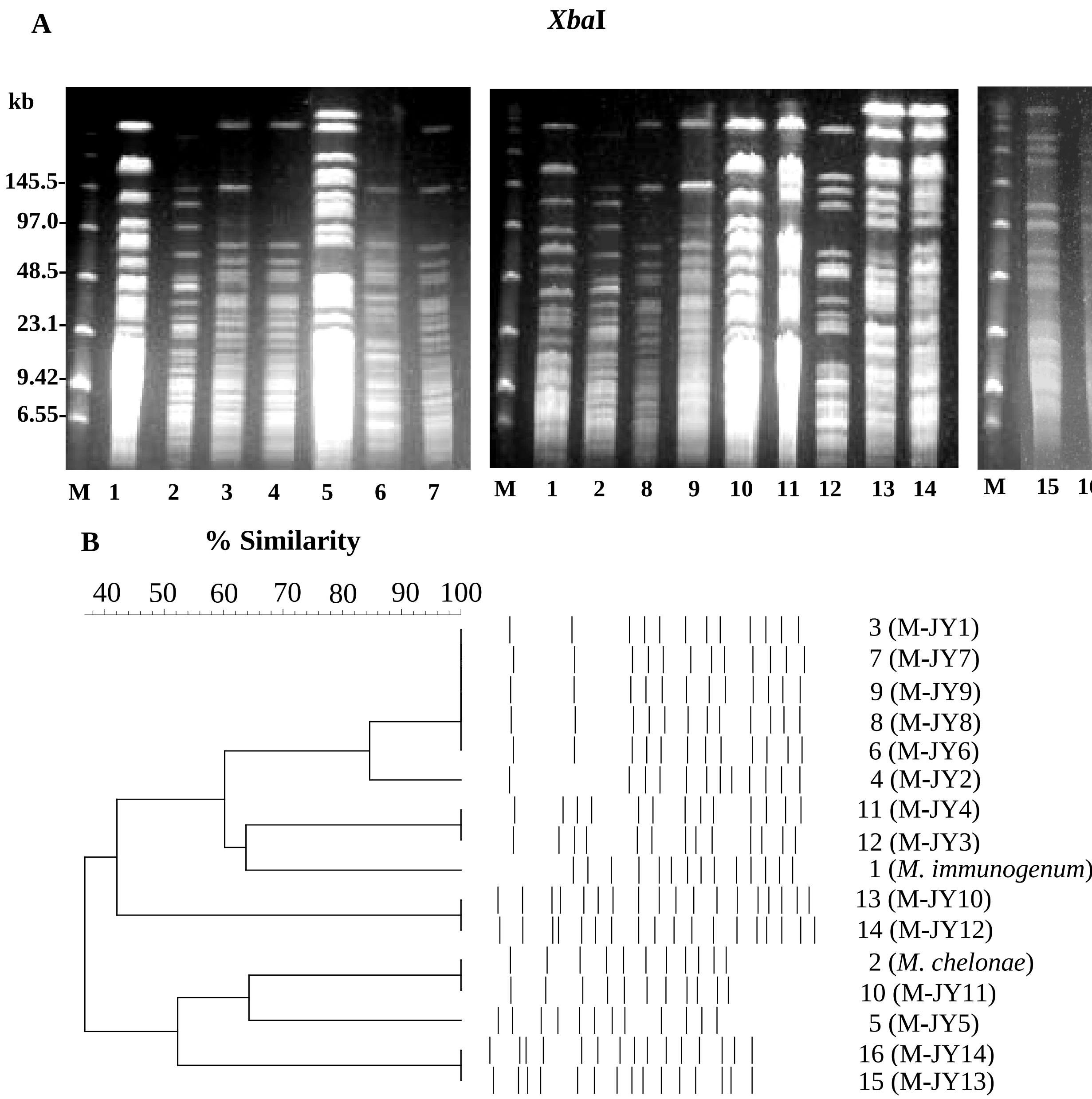


FIGURE 12: **Genomic DNA macrorestriction fragment pattern analysis (MRFPA) by pulsed-field gel electrophoresis (PFGE) of MCC reference strains and mycobacterial isolates from MWF, using *Xba*I restriction enzyme.** (**Panel A**): Lanes 1 and 2: *M. chelonae* and *M. immunogenum*; Lanes 3, 4, 6-10: *M. chelonae* isolates; Lane 5: *M. diernhoferi*; Lanes 11-16: *M. immunogenum* isolates. Lane M: Low range PFG 0.1-200 kb (Sigma, St. Louis, MO) as DNA size marker. (**Panel B**): Dendogram analysis and a schematic diagram showing distinct MRFPA patterns of the genomic DNA from *M. chelonae* and *M. immunogenum* field isolates and reference strains obtained after digestion with *Xba*I restriction enzyme. The lane numbers correspond to the codes of the collected MWF field isolates.

Table 5. Isolation and Genotypic characterization of MWF-associated mycobacterial isolates

PCR-positive samples°	*Isolates*	Mycobacterial isolates identification					
		Species-level•		Sequence-based genotype■		PFGE genotype□	
		*hsp*65 gene	16S-23S ITS region	*hsp*65 gene	16S-23S ITS region	*Xba*I	*Spe*I
S-16	M-JY1	*M. chelonae*	*M. chelonae*	C-1	C-1	C-1	C-1
S-17	M-JY2	*M. chelonae*	*M. chelonae*	C-1	C-1	C-2	C-1
S-18	M-JY3	*M. immunogenum*	*M. immunogenum*	I-1	I-1	I-1	I-2
S-21	M-JY4	*M. immunogenum*	*M. immunogenum*	I-1	I-1	I-1	I-1
S-22	M-JY5	*M. diernhoferi*	*M. diernhoferi*	D-1	D-1	D-1	D-1
S-23	M-JY6	*M. chelonae*	*M. chelonae*	C-1	C-2	C-1	C-2
S-24	M-JY7	*M. chelonae*	*M. chelonae*	C-1	C-1	C-1	C-1
S-37	M-JY8	*M. chelonae*	*M. chelonae*	C-1	C-1	C-1	C-1
S-38	M-JY9	*M. chelonae*	*M. chelonae*	C-1	C-1	C-1	C-1
S-40	M-JY10	*M. immunogenum*	*M. immunogenum*	I-1	I-1	I-2	I-1
S-46	M-JY11	*M. chelonae*	*M. chelonae*	C-2	C-3	C-3	C-3
S-51	M-JY12	*M. immunogenum*	*M. immunogenum*	I-1	I-1	I-2	I-1
S-94	M-JY13	*M. immunogenum*	*M. immunogenum*	I-1	I-1	I-3	I-1
S-95	M-JY14	*M. immunogenum*	*M. immunogenum*	I-1	I-1	I-3	I-1

° Samples that showed positive signal in PCR screening by *Mycobacterium*-specific PCRs and yielded isolates

• PCR restriction analysis of either *hsp*65 gene segment or ITS region

■ Nucleotide sequencing of either *hsp*65 gene segment or ITS region

□ Pulsed-field gel electrophoresis (PFGE) on the *Xba*I or *Spe*I digested genomic DNA

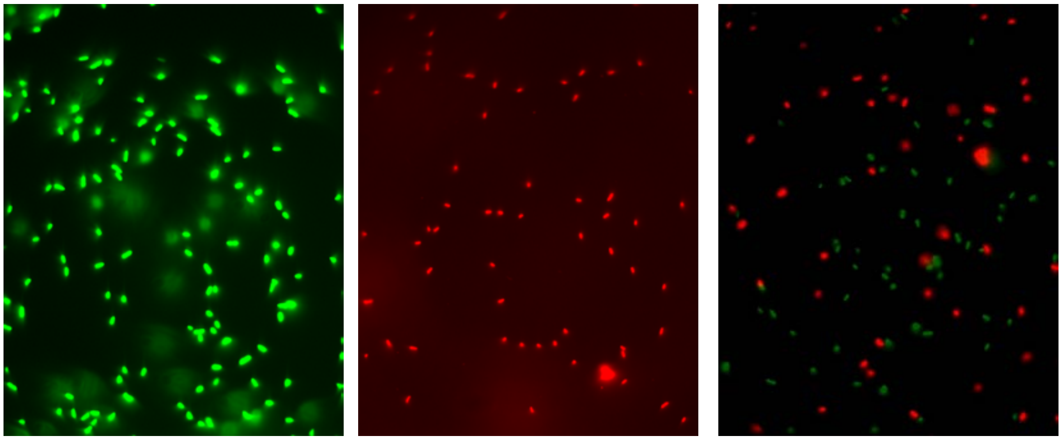
C = *M. chelonae*

I = *M. immunogenum*

D = *M. diernhoferi* (water isolate from MWF diluent water)

A. *P. fluorescens*

Viable **Non-viable** **Viable and non-viable**



B. *M. immunogenum*

Viable **Non-viable** **Viable and non-viable**

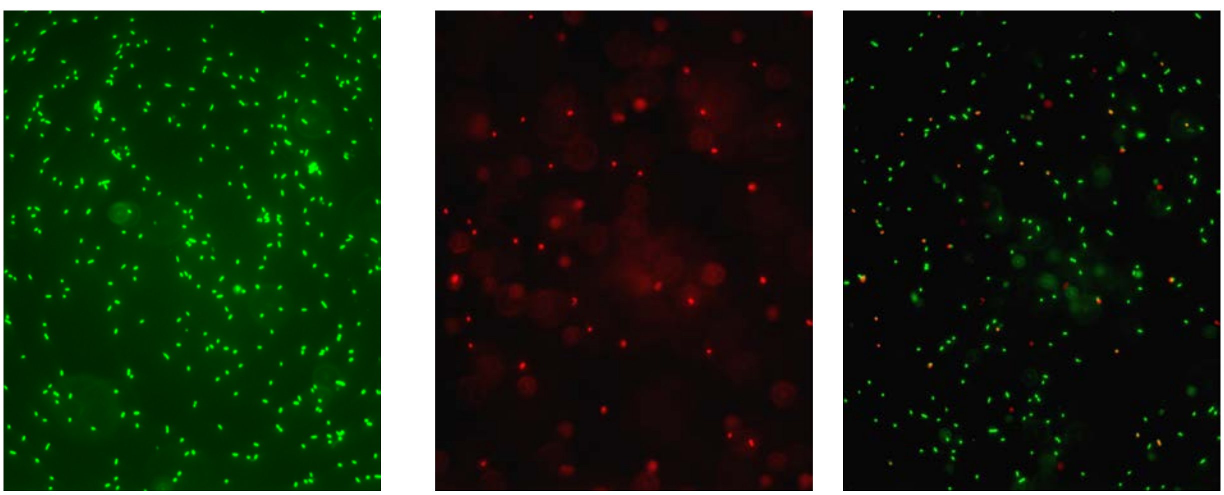


FIGURE 13. Differentiation of viable and non-viable cells of *P. fluorescens* (Panel A) and *M. immunogenum* (Panel B) by fluorescence microscopy

TABLE 6: Development of a Biocidal assay to estimate total biocide load in field samples of metalworking fluids (MWF) using different test bacterial species

Sample Code	*E. coli* (mm)		*Bacillus* (mm)		*P. fluorescens* (mm)		*M. smegmatis* (mm)	
	Well	Disc	Well	Disc	Well	Disc	Well	Disc
MWF 1	-	-	-	-	-	-	-	-
MWF 2	-	-	-	-	10	14		-
MWF 3	-	-	-	-	-	14	-	-
MWF 4	-	-	-	-	-	-	-	-
M-001	10	-	-	-	16	16	25	30
M-002	-	-	-	-	-	-	-	12
M-003	-	-	-	-	-	-	-	-
M-004	-	-	-	-	-	-	-	10
M-005	-	-	-	-	-	-	-	-
M-006	-	-	-	-	12	20	-	12
M-007	-	-	-	-	13	16	-	30
M-008	-	-	-	-	-	-	-	-
M-009	-	-	-	-	0	16	-	-
M-010	-	-	-	-	10	14	-	-
M-011	-	-	10	14	11	20	10	12
M-012	-	-	11	14	11	18	-	14
M-013	-	-	-	-	10	16	30	34
M-014	-	-	11	-	15	16	35	30
M-015	-	-	-	-	-	20	-	14
M-016	-	12	-	-	-	-	-	-
4360	ND*	ND	ND	ND	ND	ND	ND	ND
4362	ND	ND	ND	ND	ND	ND	ND	ND
SS1	15	-	16	20	20	-	10	-
SS2	-	-	20	-	10	-	-	-
A305881	-	-	-	-	-	-	NG**	NG
B304896	-	-	-	-	-	-	NG	NG
B304898	-	-	-	-	-	-	NG	NG
B305805	-	-	-	-	-	-	NG	NG
B305806	-	-	-	-	-	-	NG	NG
B305814	-	-	-	-	-	-	NG	NG
B306193	-	-	-	-	-	-	16	18
A306219	-	-	-	-	-	-	-	10
A306237	-	-	-	-	-	-	-	12
A306238	-	-	-	-	-	-	-	-
A306241	-	-	-	-	-	-	-	10
A306242	-	-	-	-	-	-	-	12

* = Not done
** = No growth

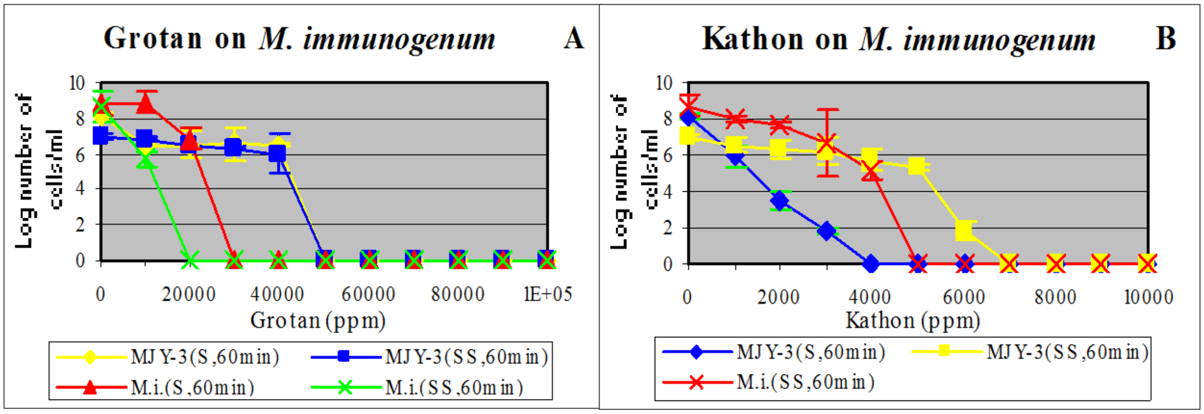


FIGURE 14: Dose-response analysis for biocide efficacy of two selected commercial biocides on *M. immunogenum* strains in synthetic and semi-synthetic fluids. Abbreviations: Mi (*M. immunogenum* ATCC strain 700506); MJY3 (*M. immunogenum* isolate M-JY3); S (synthetic MWF); SS (semi-synthetic MWF).

9. REPRINTS OF PUBLISHED PAPERS (5R01 OH007364)

1. Yadav, J. S., I. U. H. Khan, F. Fakhari, and M. B. Soellner. 2003. 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.* **18:**966-975.
2. Khan, I. U. H., and J. S. Yadav. 2004a. Development of a single-tube, cell lysis-based, genus-specific PCR method for rapid identification of mycobacteria: optimization of cell lysis, PCR primers and conditions, and restriction pattern analysis. *J. Clin. Microbiol.* **42:**453-457.
3. Khan, I. U. H., and J. S. Yadav. 2004b. Real-time PCR assays for genus-specific detection and quantification of culturable and non-culturable mycobacteria and pseudomonads in metalworking fluids. *Mol. Cell. Probes*. **18**: 67-73.
4. Selvaraju, S. B., I. U. H. Khan, and J. S. Yadav. 2005. A new method for species identification and differentiation of *Mycobacterium chelonae* complex based on amplified *hsp*65 restriction analysis (AHSPRA). *Mol. Cell. Probes*. **19**:93-99.
5. Khan, I. U. H., S. B. Selvaraju, and J. S. Yadav. 2005. Method for rapid identification and species differentiation for *Mycobacterium chelonae* complex based on 16S-23S Ribosomal DNA internal transcribed spacer (ITS) PCR-restriction analysis (ITSPRA). *J. Clin. Microbiol*. **43**(9): 4466-4472 .
6. Khan, I. U. H., S. B. Selvaraju and J. S. Yadav. 2005. Occurrence and characterization of multiple novel genotypes of *Mycobacterium immunogenum* and *Mycobacterium chelonae* in metalworking fluids. *FEMS Microbiol. Ecol*. **54**:329-338.
7. Selvaraju, S. B., I. U. H. Khan, and J. S. Yadav. 2005. Biocidal activity of formaldehyde and nonformaldehyde biocides toward *Mycobacterium immunogenum* and *Pseudomonas fluorescens* in pure and mixed suspensions in simulated synthetic metalworking fluid and saline. *Appl. Environ. Microbiol*. **71**(1):542-546.
8. 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) (in press).