Applied Occupational and Environmental Hygiene
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uaoh20

DNA-Based Methodologies for Rapid Detection, Quantification, and Species- or Strain-Level Identification of Respiratory Pathogens (Mycobacteria and Pseudomonads) in Metalworking Fluids

Jagjit S. Yadav [a], Izhar U. H. Khan [a], Farnaz Fakhari [a] & Mathew B. Soellner [a]
[a] Molecular Toxicology Division, Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, Ohio

Available online: 27 Sep 2010

To cite this article: Jagjit S. Yadav, Izhar U. H. Khan, Farnaz Fakhari & Mathew 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, Applied Occupational and Environmental Hygiene, 18:11, 966-975

To link to this article: http://dx.doi.org/10.1080/10473220390237700

Applied Occupational and Environmental Hygiene, 18: 966–975, 2003

ISSN: 1047-322X print / 1521-0898 online
DOI: 10.1080/10473220390237700

DNA-Based Methodologies for Rapid Detection, Quantification, and Species- or Strain-Level Identification of Respiratory Pathogens (Mycobacteria and Pseudomonads) in Metalworking Fluids

Jagjit S. Yadav, Izhar U. H. Khan, Farnaz Fakhari, and Mathew B. Soellner
Molecular Toxicology Division, Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, Ohio

Mycobacteria and pseudomonads occurring in modern metalworking fluids (MWF) have been implicated in occupational health hazards as causal agents for hypersensitivity pneumonitis (HP) and other respiratory illnesses in machine workers exposed to these fluids and their aerosols. Unlike the conventional cultural and biochemical methods, which are often slow and ambiguous and detect only culturable cells, DNA-based methods offer a time-saving alternative for reliable detection and identification of both culturable and nonculturable bacteria in MWF and for selective quantification of individual genera of pathogens of interest in these fluids. This is the first report on DNA-based direct detection of mycobacteria and pseudomonads in MWF without culturing. Genus-specific PCR approach was successfully applied for screening of field MWF samples originating from different industrial users for detection of mycobacteria or pseudomonads including both culturable and nonculturable cells. PCR in combination with amplicon DNA sequencing led to the identification of *Mycobacterium chelonae*, *Pseudomonas nitroreducens*, and an undefined *Pseudomonas* species from these fluids. Genome fingerprinting by pulsed-field gel electrophoresis (PFGE) on *Mycobacterium* isolates further showed that the isolates represented three strains of *M. chelonae* although the possibility of one of the strains being clonal with *M. immunogenum* cannot be excluded. In parallel efforts, a quantitative competitive PCR method developed based on the *Pseudomonas*-specific PCR was applied to quantify total *P. fluorescens* cells in contaminated metalworking fluid and MWF aerosol without culturing. The DNA-based protocols developed in this study will allow rapid screening of field MWF samples for the presence of both culturable and nonculturable cells and thus facilitate effective fluid management and timely exposure assessment.

Keywords *Mycobacterium*, *Pseudomonas*, Nonculturable, Metalworking Fluid, Hypersensitivity Pneumonitis, PCR, Quantitative PCR, DNA Fingerprinting, PFGE

Modern metalworking fluids (MWF) used in industries for cooling and lubrication during metalworking processes such as turning, drilling, grinding, and cutting are water-based, containing either soluble emulsified oils or synthetic or semi-synthetic type formulations. A recent NIOSH report[1] estimated that about one million U.S. workers are annually exposed to MWF. Recirculation of these fluids might cause microbial colonization as well as lead to mist formation around the machines, creating health problems including respiratory illnesses, cancer, and dermatitis.[2]

The respiratory disorders such as asthma, hypersensitivity pneumonitis, bronchitis, and other respiratory symptoms have been ascribed to chronic exposure to the aerosolized microbial antigens present in these used fluids.[3,4] The microbial antigens may be contributed by both culturable (viable) and nonculturable (viable or non-viable) microorganisms.[2,5] Age and stress factors such as aerosolization may further increase the nonculturable fraction of MWF microflora. This emphasizes the need for analysis of these fluids and their aerosols for total microbial load including both culturable and nonculturable microorganisms.

Since 1991, an estimated 200 hypersensitivity pneumonitis (HP) cases have been associated with the exposure of industrial workers to aerosolized water-based synthetic, semi-synthetic, or soluble oil MWF.[6,7] Conventionally, microflora cultured from water-based MWF has yielded gram-negative bacteria,

particularly *Pseudomonas* species such as *P. fluorescens*, gram-positive bacteria, and fungi.[8] Subsequent studies reported MWF contamination with acid-fast bacilli including *Mycobacterium*.[9] Earlier studies linked Pseudomonads and their endotoxins to HP,[10] whereas recent studies have provided increasing evidence for a link between Mycobacteria and HP in MWF exposures.[6,11] However, these initial studies, which relied on conventional cultural and biochemical methods for identification, led to limited information on species and strains of Mycobacteria occurring in HP-linked MWF. Recently, analysis of cultured isolates of mycobacteria from MWF linked with HP have led to the identification of a novel species *M. immunogenum* reportedly common in these fluids.[12–14]

Conventional culture-based methods used for detection and quantification of the specific genera (*Mycobacterium* and *Pseudomonas*) in contaminated MWF are based on agar plating using selective microbiological media, which makes them time-consuming and often ambiguous, yielding only the viable (culturable) counts. In addition, conventional biochemical and physical methods generally fail to identify cultured isolates to species or strain level, particularly in case of mycobacteria. Therefore, the aim of this study was to develop and apply DNA-based protocols for rapid direct detection and quantification (without culturing) of total Mycobacteria or Pseudomonads in metalworking fluids, and for reliable identification of cultured MWF isolates belonging to the genera *Mycobacterium* and *Pseudomonas*. To our knowledge, this is the first report on development and application of DNA-based methods for direct detection and quantification of mycobacteria and/or pseudomonads in used metalworking fluids without culturing.

MATERIALS AND METHODS

Microbial Strains and Culture Conditions

Three reference strains of the genus *Mycobacterium*, *M. chelonae* (ATCC 35752), *M. smegmatis* (ATCC 19420), and *M. bovis* (ATCC 35741), and one reference strain of the genus *Pseudomonas*, *P. fluorescens* (ATCC 13525), from American Type Culture Collection (ATCC), were used for methods development and comparisons. *Mycobacterium* and *Pseudomonas* strains were maintained on Middlebrook 7H10 agar (MBA) with OADC enrichment and *Pseudomonas* Isolation agar (PIA), respectively. The same cultural conditions were used for maintaining isolates of these genera from MWF.

DNA-Based Screening of MWF for Detection of *Mycobacterium* and *Pseudomonas*

MWF samples were screened for the presence of Mycobacteria or Pseudomonads using genus-specific PCR. This warranted an initial optimization of method for DNA recovery from whole MWF samples.

DNA Isolation

Microbial cells present in a given MWF sample were recovered by filtration or centrifugation. Sample (3 ml) was filtered through a 0.22-micron, 13-mm Isopore filter membrane using the manufacturer's vacuum filtration assembly (Millipore, Bedford, MA, USA). Alternatively, the sample (20 ml) was centrifuged consecutively (3×) at 10,000 rpm for 10 min. Cells recovered from the filter or pellet in TE buffer were then lysed by chemical lysis using SDS-CTAB/NaCl lysis[15] or Bactozol (MRC, Cincinnati, OH, USA) and the DNA was separated from the lysate.[16] Quality of total microbial DNA from each sample was examined by agarose gel electrophoresis using 1xTAE buffer and by subjecting it to a general eubacterial PCR using universal primer pair based on the conserved sequences of 16S rRNA gene of *Escherichia coli*.[17]

Genus-Specific PCRs

PCR protocols specific for the genus *Mycobacterium* or for the genus *Pseudomonas* were optimized for application to MWF DNA. A *Mycobacterium*-specific PCR based either on a primer pair derived from its 16S rRNA gene sequence[15] or on a primer pair derived from its heat-shock protein (*hsp*) gene[18] was used. *Pseudomonas*-specific PCR was based on a primer pair derived from its 16S rRNA gene.[19] The PCR reaction was performed using Ex-Taq DNA polymerase and the compatible PCR reagents (Panvera, Madison, WI, USA) in GenAmp PCR System 9700 Machine (Applied Biosystems, Foster City, USA).

Annealing temperature was 50°C for 16S rRNA gene-based PCRs and 60°C for the *hsp*-based PCR. Reaction mixture (50 μl) consisted of varying amounts of genomic DNA template, 1X Ex-Taq buffer with $MgCl_2$, 200 μM of each of the four dNTPs, 1.25 units of Ex-Taq DNA polymerase, and 100 ng each of forward and reverse primers. The PCR conditions included an initial template denaturation at 94°C for 3 min followed by 35 cycles of amplification by repeating denaturation at 94°C for 1 min, annealing at 50°C (or 60°C) for 1 min and extension at 72°C for 1 min. An additional elongation cycle consisting of denaturation at 94°C for 1 min, annealing at appropriate temperature for 1 min and extension at 72°C for 10 min was used before cooling to 4°C.

A 1.5 percent separide gel in 1xTAE containing ethidium bromide (0.5 μg/ml) was used to separate 10 μl of each PCR product using BioRad mini gel electrophoresis apparatus at 70 volts and 70 mA setting for 2 hours. Gels were destained for 10 min before visualizing on UV transilluminator and photographing using Polaroid film.

Isolation of Microorganisms from MWF

A total of 20 samples of metalworking fluids (MWF) in-use originating from different industrial plants at diverse geographic locations were obtained from the Cincinnati-based MWF manufacturing company Milacron. Total microbial load in each sample was estimated by plating on Trypticase Soy Agar (TSA) using spread plate (0.1 ml) method and incubation at 37°C for 48–96 hours. Simultaneously, samples were also plated on Middlebrook 7H10 agar (MBA) and Lowestein-Jensen (LJ) agar to isolate mycobacteria, and on *Pseudomonas* Isolation agar (PIA) to isolate pseudomonads, using incubation

at 37°C and 30°C, respectively, for up to 10 days. Putative *Mycobacterium* and *Pseudomonas* isolates were picked from the two media based on one or more of the morphological criteria such as growth rate, colony morphology, and staining reactions (acid-fast and Gram's) and were pursued further for identification.

PCR and Amplicon DNA Sequencing for Species-Level Identification of Isolates

The cultured isolates were identified at the genus level by 16S rRNA PCR based on protocols described above and the PCR amplicon was purified using Gene Clean kit (BIO 101, USA). The purified amplicon (924 bp for Mycobacteria and 440 bp for *Pseudomonads*) was sequenced at the University DNA Core facility and the sequence data were analyzed using BLAST search against the global database to identify that isolate at the species level. The sequences for individual isolates were compared using MegAlign 1997–2001 (DNASTAR Inc., Madison, WI, USA).

Genomic DNA Fingerprinting for Strain Differentiation

Pulsed-field gel electrophoresis (PFGE) was used to determine genomic DNA fingerprints of each of the mycobacterial isolates. The preparation and digestion of the intact genomic DNA was a modification of the procedure described by Maslow et al.[20] Mycobacterial cells were grown in Trypticase Soy Broth (TSB) for 48–96 hours at 37°C and recovered by centrifugation at 10,000 rpm for 10 min at 4°C.

The pellet was washed and resuspended in 1xTE buffer. Cell suspension was mixed with an equal volume of 1 percent low melting and low gelling Incert agarose (FMC, Philadelphia, PA, USA) at 55°C, carefully dispensed into plug molds, and solidified on an ice block for at least 10 min. After solidification the gel plug was placed in lysis buffer (6 mmol/L Tris, pH 7.6, 1 mol/L NaCl, 10 mmol/L EDTA, pH 7.6, 0.5% Brij-58 [Sigma], 0.2% sodium deoxycholate [Sigma], 0.5% sodium lauryl sarcosine (Sigma]) supplemented with 1 mg/ml lysozyme (EM Science, Gibbstown, NJ, USA) and incubated 48 hours in a water bath at 37°C. Later, proteinase K was added (final concentration 1.0 μg/ml; Roche, Indianapolis, IN, USA) followed by 48 hours incubation at 56°C. The plugs were washed twice in TE buffer at room temperature. Proteinase K was inactivated by incubating twice in presence of phenylmethylsulfonylfluoride (PMSF, Sigma) @ 1.0 mmol/L, for one hour at 56°C.

Plugs were washed extensively with 1xTE buffer. DNA digestion in the plugs was carried out overnight at 37°C using the restriction enzyme *Xba*I or *Dra*I (40U each). The large restriction fragments were separated using the pulsed-field gel electrophoresis system CHEF-DRIII (Bio-Rad, Hercules, CA, USA) with 0.5xTBE as running buffer. Pulse time was ramped from 3 to 12 s following *Xba*I digestion for 24 h and 5 to 35 s following *Dra*I digestion for 20 h at 6 V. The gel was stained with 10-mg/ml ethidium bromide (Sigma) and photographed (EDAS 290 Kodak, Rochester, NY, USA).

Quantitative Competitive PCR to Measure Pseudomonads in MWF

A quantitative competitive PCR based on *Pseudomonas*-specific PCR was optimized and applied to estimate total *Pseudomonas* cells present in simulated MWF contaminated with mixed flora and its simulant-generated aerosol sample obtained from the department's Aerosol Research Laboratory and in an in-use MWF obtained from field. The aerosol generation protocol along with the simulant experimental setup has been described in details in a previous publication.[21] It involved a genus-specific amplification described above except that a competitor DNA with the same primer binding sites as the target was included. The competitor DNA was constructed by deletion of a 48 bp segment in the target amplicon generated from *P. fluorescens* using an overlap-extension PCR strategy.[22]

For template DNA isolation, a method involving phenol-chloroform extraction was used with modifications.[16] One ml cell suspension was filtered through a 0.22-micron, 13-mm Isopore filter membrane (Millipore, Bedford, MA, USA) using Millipore vacuum filtration apparatus. The cells were recovered in 1 ml of TE buffer and subjected to lysozyme-mediated lysis followed by purification of the crude lysate by phenol-chloroform extraction and polysaccharide removal by CTAB (cetyltrimethyl ammonium bromide, Sigma). DNA concentrations and purity (A_{260}/A_{280}) were measured spectrophotometrically.

A standard curve was prepared based on serial amplifications by genus-specific PCR using a selected amount (78 famto gram) of competitor DNA and increasing amount of standard DNA (*P. fluorescens* genomic DNA) extracted from 10^1, 10^2, 10^3, 10^4, and 10^5 cells spiked in 1 ml of MWF. Amplicons separated on the gel electrophoresis as described above were quantified by Nucleotech's Fluorescent Gel Analyzer and amplicon signal intensity ratio (competitor: target) was determined and plotted against cell number. The experimental DNA (1 μl of the total 10 μl) isolated from MWF liquid sample or MWF aerosol sample (1 ml each) was co-amplified with the selected amount of competitor (78 fg) and the signal ratio was used to determine the corresponding cell number from the standard curve. Number of cells/ml was then calculated.

RESULTS

PCR-Based Method for Selective Detection of Mycobacteria or Pseudomonads in MWF Without Culturing

As a first step toward the development of the DNA-based screening method for genus-specific detection of Mycobacteria or Pseudomonads in MWF samples without culturing, total DNA isolation from these samples was optimized. Relative recovery 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

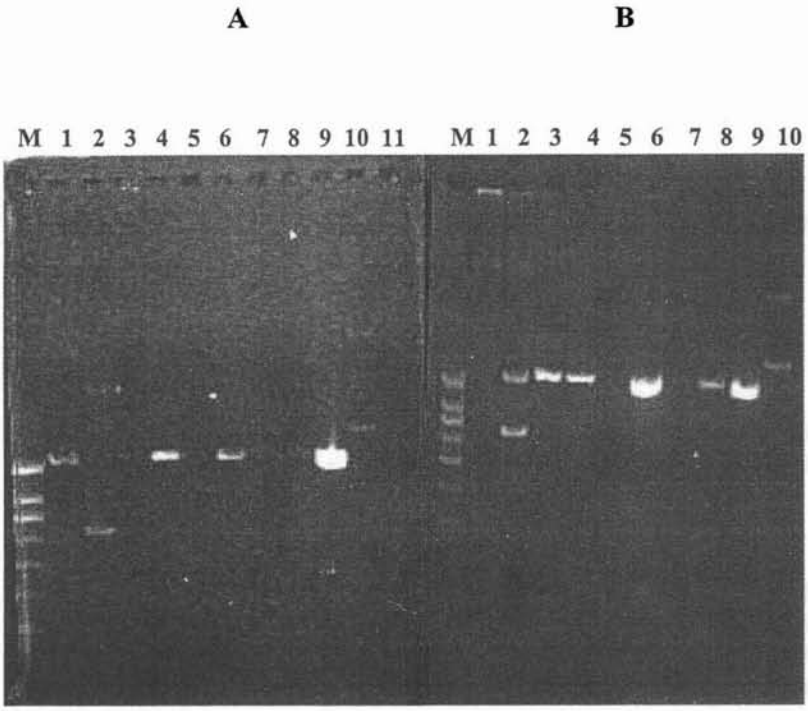

FIGURE 1

PCR evaluation of the total microbial DNA isolated from different MWF samples with homogeneous watery consistency, using filtration (panel A) or centrifugation (panel B). The PCR amplification was based on universal eubacterial primers that yielded an expected 1039 bp amplicon, as described in Materials and Methods. 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).

centrifugation pre-step in the procedure yielded more DNA in comparison to filtration, indicating the former to be the method of choice for cell recovery (see Figure 1). The two methods used for cell lysis yielded comparable amounts of DNA (data not shown). The quality of the extracted DNA for amplification was also evaluated based on the diagnostic PCR. Eleven of the 16 DNA samples extracted showed positive PCR reactions indicating their amplifiable quantity and quality (see Table I).

The two genus-specific PCR protocols, *Mycobacterium*-specific (16S rRNA gene-based and *hsp*-gene based) and *Pseudomonas*-specific (16S rRNA gene-based), were optimized to selectively amplify control DNA from the reference strains of the two genera without any cross reactions. The genus-specific PCR protocols were then applied to screen the extracted DNA from individual MWF samples for the presence of *Mycobacterium* or *Pseudomonas* or both.

Mycobacterium genus-specific PCR based on the 16S rRNA gene allowed an amplification of the expected 924 bp fragment from 11 of the 20 samples (see Figure 2A). However, only 5 samples exhibited positive amplification reaction with the *hsp*-gene based protocol by yielding the expected 439 bp amplicon (see Figure 2B). On the other hand, 9 of the 20 samples gave positive signals with the *Pseudomonas* genus-specific PCR yielding a 440 bp amplicon (see Figure 2C). Analyses based on universal- and genus-specific PCR are summarized in Table I.

Isolation of *Mycobacterium* and *Pseudomonas* Species from MWF

All MWF samples were plated on MBA for Mycobacterial isolation and PIA for Pseudomonads isolation. Fourteen samples yielded colonies on MBA, whereas five samples yielded colonies on PIA (see Table II). Not all samples found positive based on direct PCR screening yielded colonies on the agar media (see Table II). Total bacterial count (culturable) as well as colony isolates on different growth media are summarized in Table I.

DNA-Based Identification of the Isolates

Genus-level identification was performed using the appropriate genus-specific PCR on the isolates (see Table II). Of the 14 colony isolates from MBA, only 3 were confirmed as *Mycobacterium* by *Mycobacterium*-specific PCRs (based on 16S rRNA gene and heat-shock protein [*hsp*] gene). Genus identity of only 2 of the 7 PIA isolates was confirmed as *Pseudomonas* using the *Pseudomonas*-specific PCR. Reference strains of both genera served as positive controls for comparison in these amplifications. Amplicon sequencing was performed for species-level identification.

TABLE I

Microbiological analysis[A] and PCR-based screening of field MWF samples for total bacterial contamination[B] and detection of mycobacteria[C] and pseudomonads[D]

Sample code	Sample type	pH	Culturable bacterial counts (cfu/ml)[A]	Eubacterial universal PCR[B]	Genus-specific PCR	
					Mycobacterium-specific[C]	*Pseudomonas*-specific[D]
MWF 1	Synthetic	8.5	0	ND	−	−
MWF 2	Synthetic	7.5	1×10^6	ND	−	+
MWF 3	Synthetic	7.2	2×10^6	ND	+	+
MWF 4	Synthetic	7.2	0	ND	+	−
M-001	Drawing	8.3	3×10^4	−	−	+
M-002	Semi-synthetic	8.7	5×10^5	−	−	−
M-003	Semi-synthetic	8.1	4×10^6	−	−	−
M-004	Synthetic	6.8	6×10^6	+	−	−
M-005	Synthetic	8.2	6×10^5	+	+	+
M-006	Synthetic	6.6	6×10^3	+	+	+
M-007	Soluble oil	8.1	5×10^4	−	−	−
M-008	Semi-synthetic	6.2	4×10^6	+	+	+
M-009	Semi-synthetic	7.6	3×10^3	+	+	+
M-010	Semi-synthetic	7.3	7×10^5	+	+	+
M-011	Semi-synthetic	8.4	0	−	−	−
M-012	Semi-synthetic	8.4	0	+	+	−
M-013	Semi-synthetic	8.0	2×10^4	+	+	+
M-014	Double mix	8.3	7×10^3	+	+	−
M-015	Synthetic	8.9	0	+	−	−
M-016	Synthetic	8.7	2×10^4	+	+	−

ND = Not done.
[A]Trypticase Soy Agar was used.
[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 924 bp fragment of the 16S rRNA gene.
[D]*Pseudomonas*-specific PCR was based on amplification of 440 bp fragment of the 16S rRNA gene.

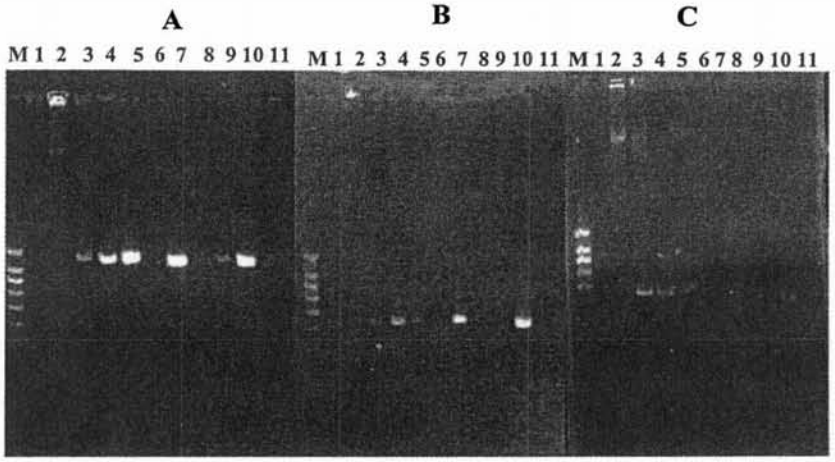

FIGURE 2

Genus-specific PCR amplification of total DNA from selected MWF samples: (A) *Mycobacterium*-specific PCR based on 16S rRNA gene with an expected amplicon of 924 bp; (B) *Mycobacterium*-specific PCR based on heat-shock protein (*hsp*) gene with an expected amplicon of 439 bp; (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 II
Cultural isolation[A] of putative mycobacteria and pseudomonads from MWF and confirmation of the colony isolates by genus-specific PCRs[B]

	Mycobacteria		Pseudomonads	
Sample code	Middle-Brook 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	−	−	−	−

[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.

Based on 924 bp amplicon DNA sequence analysis using BLAST homology search, the three *Mycobacterium* isolates (M-JY1, M-JY2, and M-JY3) were putatively identified as *M. chelonae*. An additional acid-fast bacillus isolate originating from reverse osmosis water from an industrial plant with MWF operation, obtained from Milacron along with the MWF samples, was identified as *M. diernhoferi*. One of the two *Pseudomonas* isolates showed closest BLAST homology to *Pseudomonas nitroreducens*, whereas the other isolate matched an undefined *Pseudomonas* species. Sequences for the 16S rRNA gene amplicon (924 bp) of all *Mycobacterium* isolates are compared in Figure 3.

Strain Differentiation of Mycobacterial Isolates by DNA Fingerprinting

Large restriction fragment analysis of the mycobacterial isolates (M-JY1, M-JY2, and M-JY3) was determined by PFGE using *Xba*I and *Dra*I restriction digestions. Isolate M-JY2 did not respond to PFGE analysis. The strains M-JY1 and M-JY3 that previously showed identical amplicon sequence (see Figure 3) had non-identical PFGE patterns with *Xba*I (see Figure 4). Considering DNA sequence analysis and PFGE patterns, the three mycobacterial isolates (M-JY1, M-JY2, and M-JY3) appeared to represent different strains.

Competitive PCR-Based Quantification of *Pseudomonas* in MWF Without Culturing

Pseudomonas cells were quantified in MWF liquid and aerosol samples containing mixed microbial population using the optimized quantitative competitive PCR based on *Pseudomonas*-specific protocol. A standard curve prepared by spiking *P. fluorescens* in MWF, as described in Materials and Methods, was used (see Figure 5). The results on spiked metalworking fluid and the derived aerosol samples and on in-use MWF from the field are presented in Table III. The number

TABLE III
Quantification of *Pseudomonas* in MWF and MWF aerosols using the developed quantitative competitive PCR

	Percent area[A]				
Sample	T	C	Ratio (T/C)	Log ratio (Y value)	Cells/ml[B]
MWF[C] (before aerosolization)	33.79	66.21	0.510	−0.292	3.648×10^3
MWF (after aerosolization)	31.23	68.77	0.454	−0.343	3.206×10^3
MWF aerosol	23.21	76.79	0.302	−0.520	2.046×10^3
MWF field sample[D]	42.01	57.99	0.7244	−0.140	4.02×10^8

[A]Percent area: T = Target signal; C = Competitor signal.
[B]Derived from the standard curve.
[C]MWF simulated with bacterial mixture containing *P. fluorescens*.
[D]MWF 3 sample from Table I.

```
GGGAACTGGGTCTAATACCGGATAGGACCGCATGCTTCATGGTGTGT  M-JY1; M-JY3
GGGAACTGGGTCTAATACCGGATAGGACCACACACTTCATGGTGAGT  M-JY2

GGTGCAAAGCTTTTGCGGTGTGGGATGAGCCCGCGGCCTATCAGCT  M-JY1; M-JY3
GGTGCAAAGCTTTTGCGGTGTGGGATGAGCCCGCGGCCTATCAGCT  M-JY2

TGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCC  M-JY1; M-JY3
TGTTGGTGGGGTAATGGCCCACCAAGGCGACGACGGGTAGCCGGCC  M-JY2

TGAGAGGGTGACCGGCCACACTGGGACTGAGATACGGCCCAGACTC  M-JY1; M-JY3
TGAGAGGGTGACCGGCCACACTGGGACTGAGATACGGCCCAGACTC  M-JY2

CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT  M-JY1; M-JY3
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT  M-JY2

GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC  M-JY1; M-JY3
GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC  M-JY2

CTCTTTCAGTAGGGACGAAGCGAAAGTGACGGTACCTACAGAAGAA  M-JY1; M-JY3
CTCTTTCAGTAGGGACGAAGCGAAAGTGACGGTACCTACAGAAGAA  M-JY2

GGACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCC  M-JY1; M-JY3
GGACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCC  M-JY2

GAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTG  M-JY1; M-JY3
GAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTG  M-JY2

TCGCGTTGTTCGTGAAAACTCACAGCTTAACTGTGGGCGTGCGGGCG  M-JY1; M-JY3
TCGCGTTGTTCGTGAAAACTCACAGCTTAACTGTGGGCGTGCGGGCG  M-JY2

ATACGGGCAGACTAGAGTACTGCAGGGGAGACTGGAATTCCTGGTGT  M-JY1; M-JY3
ATACGGGCAGACTAGAGTACTGCAGGGGAGACTGGAATTCCTGGTGT  M-JY2
```

FIGURE 3

Sequence comparison of 16S rRNA gene amplicon (924 bp) for different mycobacterial isolates (M-JY1, M-JY2, M-JY3). The shaded areas indicate the differences in nucleotide sequence.

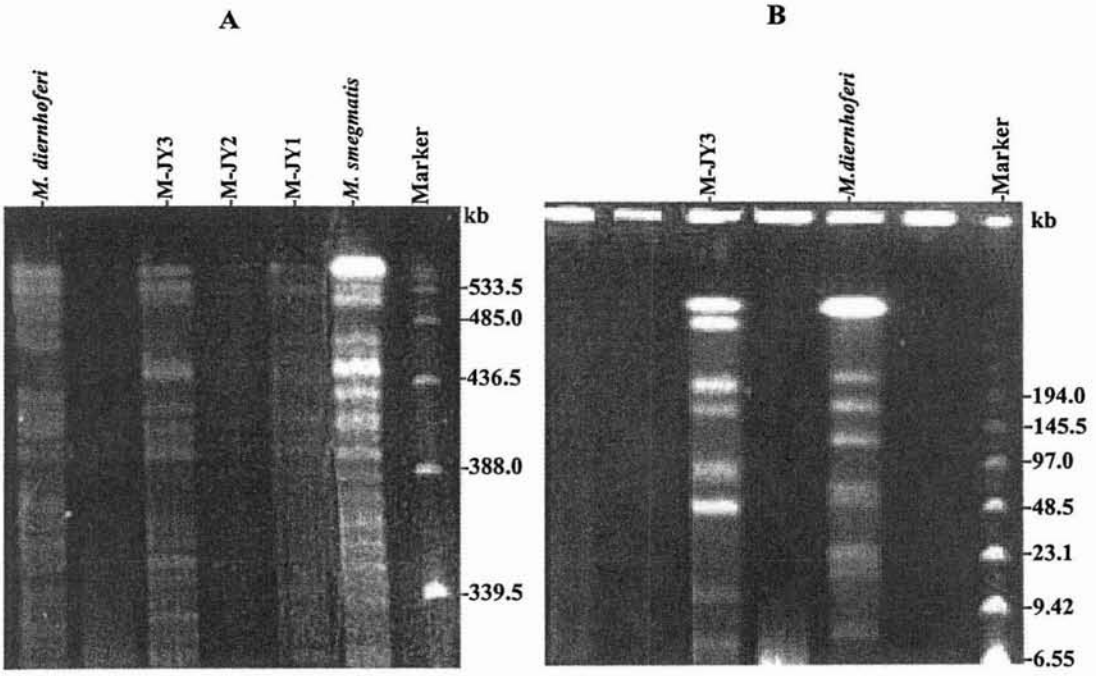

FIGURE 4

Pulsed-field gel electrophoretic (PFGE) patterns of genomic DNA of the mycobacterial isolates using restriction enzyme *Xba*I (panel A) and *Dra*I (panel B). *M. smegmatis* served as a method control. M = Lambda ladder PFG 50-1000 kb (panel A) and low range PFG 0.1–200 kb (panel B) as size markers. *M. diernhoferi*, an isolate from reverse osmosis water from an industrial plant with MWF operations, was used for comparison.

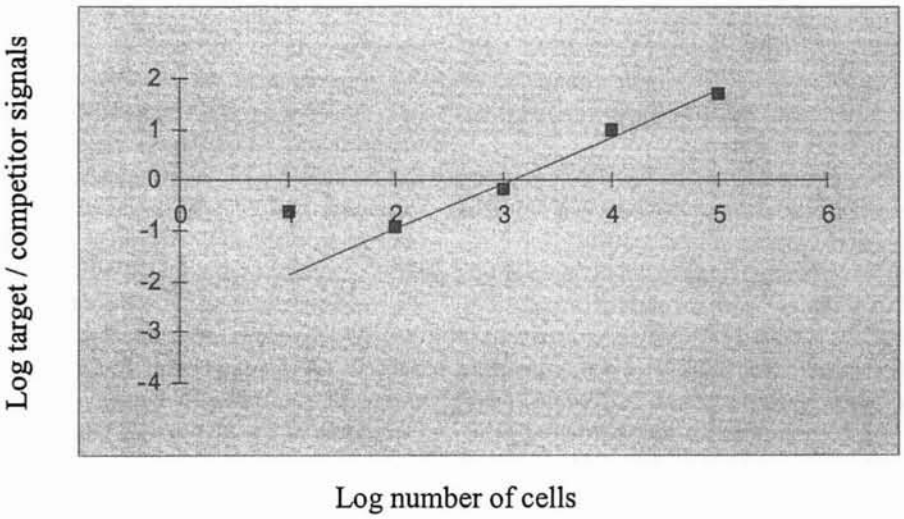

FIGURE 5

Standard curve for quantitative competitive PCR to measure *Pseudomonas*. DNA extracted from serially diluted cell suspensions containing 10^5, 10^4, 10^3, 10^2, and 10^1 cells/ml was co-amplified with a selected amount of competitor DNA (78 fg) and the target and competitor signals 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. $y = ax + b(a = 0.908; b = -2.78; r = 0.995)$.

represented both culturable as well as nonculturable cells of *Pseudomonas*. This shows the feasibility of the optimized quantitative PCR method for use on both liquid and air samples of MWF. A minimum of 100 cells/ml fluid could be quantified effectively, although as low as 10 cells/ml could be detected as shown in Figure 5. Furthermore, this method proved useful for genus-specific quantitation of *Pseudomonas* in MWF samples containing a mixed bacterial population.

DISCUSSION

Microbial contaminants in modern water-based metalworking fluids, particularly mycobacteria and pseudomonads, have been implicated in respiratory disorders such as hypersensitivity pneumonitis in the exposed machine workers.[1–3] Pseudomonads have been conventionally reported as a part of the culturable gram-negative microflora of water-based fluids,[10] whereas mycobacteria have emerged lately in the reported microflora of these fluids linked with respiratory disorders.[6,9,11–14]

One possible reason for lack of reports on mycobacteria in initial studies could be that these organisms evaded detection in routine culturing efforts applied because of their requirement for more selective culturing media and temperature and relatively long incubation time. Another reason for emergence of mycobacteria in modern MWF in-use could be the use of formaldehyde-releasing biocides, which may give selective advantage to resistant Mycobacteria by inhibiting the sensitive predominant bacterial flora.

Regardless of their history of incidence in MWF, both mycobacteria and pseudomonads occur in modern MWF formulations[8] and both are the suspected antigens for respiratory illnesses such as HP.[10,11] This warrants detection of both of these genera in MWF and their aerosols and estimation of their total load (culturable and nonculturable) in these matrices.[5]

Current practice for detection and quantification of these genera in MWF is mostly based on culturing and/or cell wall staining, which often yield ambiguous results. Selective culturing of one genus in a mixed flora such as in MWF is a difficult task. There are no absolute selective media available for either Mycobacteria or Pseudomonads. For instance, Middlebrook agar and *Pseudomonas* Isolation Agar meant for culturing *Mycobacteria* and *Pseudomonas* respectively often yield growth of other bacteria in lab culturing of these fluids (see Results).

Another commonly used selective medium for mycobacteria, Lowestein-Jensen medium, does not support the growth of all mycobacteria. Besides, the culturing method only yields culturable counts and does not account for nonculturable (viable or non-viable) cells, which have been considered as important contributors to microbial antigens and endotoxins in MWF.[2,10] For instance, Mattsby-Baltzer and co-workers[2] have demonstrated that viable counts of the aerosol grossly underestimated the microbial mass and endotoxins inhaled by the metal workers since the viable counts in the MWF represented less than 10 percent of the bacterial mass. Nonculturable population is prevalent in harsh environments such as MWF because of complex chemical composition and heavy use of biocides.

DNA-based strategy for detection and quantification of individual genera of microorganisms in MWF holds promise for the reasons that it does not rely on time-consuming and nonselective culturing and that it accounts for both viable and nonviable cells. Such culture-independent methods have not been reported for application to metalworking fluids. In the optimization of these methods for MWF, one of the critical steps is the

recovery of DNA of amplifiable quality and quantity from these complex matrices. Quality of DNA isolated from MWF samples is crucial in yielding successful PCR amplifications. Certain inhibitory substances carried over from the metalworking fluids could contribute to such PCR inhibitions.

In this study, we have developed a method for DNA recovery from field samples of MWF and the results show that the DNA preparations are in general suitable both in quantity and quality for PCR reactions. Nevertheless, further optimization efforts may be useful for difficult sample matrices and for samples with low cell number such as for sample 11 (for *Mycobacterium*) and sample 4 (for *Pseudomonads*) (see Table I). Colony isolation from these two samples but detection of no PCR signal could be because of very low cell number for that particular genus or because of inefficient DNA recovery. Compared to filtration, centrifugation pre-step for recovery of cells from MWF seemed a more efficient and feasible method that could be easily adapted in a diagnostic laboratory. Since the quantity of total DNA from field samples is usually low, use of a universal eubacterial PCR as a tool to judge the DNA quality and quantity for subsequent PCR applications proved useful.

Genus-specific PCRs adapted for application to MWF proved useful for selective detection of a given genus (*Mycobacterium* or *Pseudomonas*) in a mixed microbial population in field MWF samples. While applying the *Mycobacetrium*-specific PCR to the MWF samples, the 16S rRNA gene-based protocol yielded more positives than the *hsp* gene-based protocol. This may be either because of relatively relaxed selectively of 16S rRNA gene-based primers as compared to the *hsp*-based primers or because of absence of the target *hsp* gene or its specific primer sequence in some Mycobacteria in the negative samples. The former seems plausible, as only one of the two primers was specific for Mycobacteria in the 16S rRNA-based method. Our current efforts toward designing more specific primer pair from the recently released genomic information of individual Mycobacteria are underway to increase selectivity of these approaches for diverse environmental Mycobacteria with expected genotypic diversity.

The PCR-based method for screening field MWF samples for individual genera is relatively rapid as it takes an estimated 6–8 hours compared to the conventional culturing based approach, which may take an estimated 3–10 days. This is particularly useful for mycobacteria, including rapidly growing mycobacteria (RGM), which normally take up to a week to grow on plates. The time frame for the PCR approach can be further reduced to 2–3 hours if a real-time platform is used instead of end-product detection. Our results (see Tables I and II) show that the developed PCR detection approach can identify even nonculturable Mycobacteria or Pseudomonads in the field samples. The optimized quantitative competitive format of the PCR can estimate total bacterial load including culturable and nonculturable (viable or non-viable) cells in MWF containing mixed microbial flora (see Table III).

Mycobacterium from metalworking fluids has been conventionally identified by biochemical and physicochemical approaches including HPLC. Distinguishing the closely related mycobacteria at the species level or strain level such as *M. immunogenum*, *M. chelonae* and *M. abscessus* is particularly challenging. DNA fingerprinting of mycobacterial isolates has become an important technique for studying epidemiological relationship of environmental mycobacteria.[23] Lately, *M. immunogenum*[12,13] has been claimed to be the prominent if not the sole contaminant in different metalworking fluids based on DNA screening of multiple MWF isolates drawn from geographically diverse locations.[14] In contrast to this, we found that our three mycobacterial isolates originating from MWF were identifiable as *M. chelonae* based on DNA sequencing of their 924 bp amplicon from 16S rRNA gene.

In view of the fact that *M. chelonae* and *M. immunogenum* are closely related, we performed PFGE on our isolates (using *Xba*I and *Dra*I restriction digestion) to further identify and distinguish them from each other. Two (M-JY1 and M-JY3) of the three putative *M. chelonae* isolates responded to *Xba*I-based analysis, whereas only M-JY3 responded to *Dra*I-based analysis. The two isolates showed non-identical PFGE patterns with *Xba*I. The M-JY3 pattern with *Xba*I appeared to match *M. immunogenum Xba*I pattern[12] but its *Dra*I pattern was distinctly different from the *Dra*I pattern of *M. immunogenum*,[14] indicating that M-JY3 may be related or clonal with *M. immunogenum*. In contrast, the M-JY1 pattern with *Xba*I was distinctly different from *M. immunogenum* indicating that this represents a different species, most likely *M. chelonae* as indicated by DNA sequence information. In this regard, it is worth considering that growth rates of the three isolates on MBA were different; M-JY1 showed a relatively rapid growth within 48–72 hours compared to M-JY2 (3–4 days) and M-JY3 (7–10 days). Growth rate of M-JY3 is consistent with *M. immunogenum*[13] but M-JY1 showed a distinctly different growth rate than *M. immunogenum* and thus appears to be a rapidly growing *M. chelonae*.

CONCLUSIONS AND RECOMMENDATIONS

Availability of the developed PCR-based approach for genus-specific detection and quantification of Mycobacteria or Pseudomonads in MWF without culturing will allow rapid screening of field MWF samples for the presence of both culturable and nonculturable cells and thus facilitate efficient fluid management and timely exposure assessment. The study also reveals the presence of diverse species and strains of *Mycobacterium* in MWF in-use using DNA-based analysis for their identification to species and strain level. More extensive direct screening of field MWF samples using the developed DNA-based protocols is likely to yield novel strains or species of *Mycobacterium* from metalworking fluids used in diverse machining operations across the industries. Availability of these strains adapted to metalworking fluids will facilitate development of novel biocides and mechanistic studies on health effects of MWF exposures.

ACKNOWLEDGMENTS

We thank Milacron management for providing the used metalworking fluid samples and the Department's Aerosol Research

Laboratory for providing a simulated metalworking fluid and its aerosol sample. This study was supported in part by a NIOSH (CDC) R01 grant (1R01OH007364-01A1) and by NIOSH Pilot Research Project Training Program Grant #T42/CCT510420 through the University of Cincinnati Education and Research Center (UC/ERC).

REFERENCES

1. Centers for Disease Control and Prevention: Criteria for a Recommended Standard: Occupational Exposure to Metalworking Fluids, NIOSH Pub. No. 98-102. NIOSH, Cincinnati, OH (1998).
2. Mattsby-Baltzer, I.; Sandin, M.; Ahalström, B.; et al.: Microbial Growth and Accumulation in Industrial Metal-Working Fluids. Appl Environ Microbiol 55(10):2681–2689 (1989).
3. Lummus, Z.L.; Lockey, J.E.; Bernstein, I.L.; et al.: Microbial Flora of Metalworking Fluids Associated with Occupational Respiratory Disorders. J Allergy Clin Immunol 1:166 (1998).
4. Freeman, A.; Lockey, J.; Hawley, P.; et al.: Hypersensitivity Pneumonitis in a Machinist. Am J Indus Med 34(4):387–392 (1998).
5. Lonon, M.K.; Abanto, M.; Findlay, R.H.: A Pilot Study for Monitoring Changes in the Microbiological Component of Metalworking Fluids as a Function of Time and Use in the System. Am Indus Hyg Assoc J 60(4):480–485 (1999).
6. Kreiss, K.; Cox-Ganser, J.: Metalworking Fluid-Associated Hypersensitivity Pneumonitis: A Workshop Summary. Am J Indus Med 32(4):423–432 (1997).
7. ACGIH® Symposium: Health Effects of Mineral Oil Mist and Metalworking Fluids. ACGIH, Cincinnati, OH (2002).
8. Rossmoore, H.S.: Microbiology of Metalworking Fluids: Deterioration, Disease and Disposal. J Soc Tribiol Lub Eng 2:113–118 (1995).
9. Muilenberg, M.L.; Berge, H.A.; Sweet, T.: Hypersensitivity Pneumonitis and Exposure to Acid-Fast Bacilli in Coolant Aerosols. J Aller Clin Immunol 91:311 (1993).
10. Bernstein, D.I.; Lummus, Z.L.; Santilli, G.; et al.: Machine Operator's Lung. A Hypersensitivity Pneumonitis Disorder Associated with Exposure to Metalworking Fluid Aerosols. Chest 108(3):636–641 (1995).
11. Shelton, G.B.; Flanders, W.D.; Morris, K.G.: *Mycobacterium* sp. as a Possible Cause of Hypersensitivity Pneumonitis in Machine Workers. Emerging Infect Dis 5(2):270–273 (1999).
12. Moore, S.J.; Christensen, M.; Wilson, W.R.; et al.: Mycobacterial Contamination of Metalworking Fluids: Involvement of Possible New Taxon of Rapidly Growing *Mycobacterium*. Am Indus Hyg Assoc J 61:205–213 (2000).
13. Wilson, R.W.; Steingrube, V.A.; Bottger, E.C.; et al.: *Mycobacterium immunogenum* sp. nov., a Novel Species Related to *Mycobacterium abscessus* and Associated with Clinical Disease, Pseudo-outbreaks and Contaminated Metalworking Fluids: An International Cooperative Study on Mycobacterial Taxonomy. Int J Syst Evol Microbiol 51(5):1751–1764 (2001).
14. Wallace, R.J. Jr.; Zhang, Y.; Wilson, R.W.; et al.: Presence of a Single Genotype of the Newly Described Species *Mycobacterium immunogenum* in Industrial Metalworking Fluids Associated with Hypersensitivity Pneumonitis. Appl Environ Microbiol 68(11):5580–5584 (2002).
15. Talaat, A.M.; Reimschuessel, R.; Trucksis, M.: Identification of Mycobacteria Infecting Fish to the Species Level Using Polymerase Chain Reaction and Restriction Enzyme Analysis. Vet Microbiol 58:229–237 (1997).
16. Ausubel, F.M.; Brent, R.; Kingston, R.E.; et al.: Preparation of Genomic DNA from Bacteria. In: Current Protocols in Molecular Biology, vol. 1. John Wiley & Sons, Inc. (1988–2000).
17. Springer, B.; Bottger, E.C.; Kirschner, P.; et al.: Phylogeny of the *Mycobacterium chelonae*-Like Organism Based on Partial Sequencing of the 16S rRNA Gene and Proposal of *Mycobacterium mucogenicum* sp. nov. Int J Syst Bacteriol 45(2):262–267 (1995).
18. Telenti, A.; Marchesi, F.; Balz, M.; et al.: Rapid Identification of Mycobacteria to the Species Level by Polymerase Chain Reaction and Restriction Enzyme Analysis. J Clin Microbiol 31:175–178 (1993).
19. Johnsen, K.; Enger, O.; Jacobsen, C.S.; et al.: Quantitative Selective PCR of 16S Ribosomal DNA Correlates Well with Selective Agar Plating in Describing Population Dynamics of Indigenous *Pseudomonas* spp. in Soil Hot Spots. Appl Environ Microbiol 65:1786–1789 (1999).
20. Maslow, J.N.; Slutsky, M.A.; Arbeit, R.D.: Application of Pulsed-Field Gel Electrophoresis to Molecular Epidemiology. In: Diagnostic Molecular Microbiology. Principles and Applications, D.H. Persing; T.F. Smith; F.C. Tenover; T.J. White, eds., pp. 563–572. American Society of Microbiology, Washington, DC (1993).
21. Mainelis, G.; Gorny, R.L.; Reponen, T.; et al.: Effect of Electrical Charges and Fields on Injury and Viability of Airborne Bacteria. Biotechnol Bioeng 79(2):229–241 (2002).
22. Hallier-Soulier, S.; Ducrocq, V.; Mazure, N.; et al.: Detection and Quantification of Degradative Genes in Soils Contaminated with Toluene. FEMS Microbiol Ecology 20:121–133 (1996).
23. Fraser, V.; Wallace, R.J. Jr.: Nontuberculous Mycobacteria. In: Hospital Epidemiology and Infection Control, C. Glen Mayhall, ed. Williams and Wilkins, Baltimore (1996).

APPENDIX A

Abbreviations Used in This Study

Abbreviations	Full form
ATCC	American Type Culture Collection
CTAB	Cetyltrimethyl Ammonium Bromide
HP	Hypersensitivity Pneumonitis
LJ	Lowestein-Jensen Medium
MBA	Middlebrook Agar
MWF	Metalworking Fluids
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PIA	*Pseudomonas* Isolation Agar
PMSF	Phenylmethylsulfonyl Fluoride
RGM	Rapidly Growing Mycobacteria
SDS	Sodium Dodecyl Sulfate
TAE	Tris-Acetate-EDTA Buffer
TBE	Tris-Boric Acid-EDTA Buffer
TE	Tris-EDTA Buffer
TSA	Trypticase Soy Agar