ELSEVIER

Molecular and Cellular Probes 18 (2004) 67–73

MOLECULAR AND CELLULAR PROBES

www.elsevier.com/locate/ymcpr

# Real-time PCR assays for genus-specific detection and quantification of culturable and non-culturable mycobacteria and pseudomonads in metalworking fluids

Izhar U.H. Khan, Jagjit S. Yadav*

*Molecular Toxicology Division, Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, OH 45267-0056, USA*

Received 25 June 2003; accepted for publication 12 September 2003

**Abstract**

Genus-specific real-time PCR assays were developed and optimized for the direct culture-independent detection and quantification of Mycobacteria and Pseudomonads in contaminated metalworking fluids (MWF) and the results were compared with conventional culturing using selective media. It included optimization of the direct DNA isolation from the fluid matrix and the amplification conditions using genus-specific primers. Mycobacterium-specific primers based on 65-kDa heat shock protein (*hsp*) gene, and Pseudomonas-specific primers based on 16S rRNA gene were used. A standard curve was developed each for the two model bacterial species *Mycobacterium immunogenum* and *Pseudomonas fluorescens*, representing two important genera frequently isolated from MWF. A minimum quantification limit of 10 cells/ml was achieved although as low as 1 cell/ml yielded a detectable amplicon signal. Of the twenty MWF field samples contaminated with mixed microflora, only two samples yielded putative colonies of Mycobacteria and Pseudomonads by culturing method, while seven samples responded to the genus-specific real-time PCR detection and quantification for each genus. In contrast to the low culturable counts, the real-time PCR based cell counts ranged from $1.3 \times 10^2$ to $5.5 \times 10^5$ cells/ml and $5.2 \times 10^2$ to $7.0 \times 10^5$ cells/ml for Mycobacteria and Pseudomonads, respectively, indicating a significant non-culturable fraction in the fluids, for the two genera. This is the first application of real-time PCR protocol to MWF samples for detection and quantification of total (culturable and non-culturable) Mycobacteria and Pseudomonads without culturing.


*Keywords:* *Mycobacterium*; *Pseudomonas*; Metalworking fluids; Quantitative real-time PCR

## 1. Introduction

Metalworking fluids (MWF) are used in industries for cooling and lubrication during metalworking processes such as drilling, grinding and cutting. Various chemical lubricants, emulsifiers, soaps and other organic materials are used in the formulation of these fluids. Machine workers involved in the metalworking operations are constantly in contact with these fluids; in addition, metalworking operations generate high amounts of mists and aerosols that may persist in the air and the immediate surrounding area. The workers exposed to these fluids due to direct handling of machine parts or due to splashing or spraying, experience various health problems including respiratory illnesses such as hypersensitivity pneumonitis (HP), asthma and bronchitis as well as cancer and skin diseases, e.g. dermatitis [1,2].

Modern water-based MWF support the growth of a wide variety of bacteria and fungi [3]. Conventional culturing methods often yield growth of gram-negative bacteria, particularly, *Pseudomonas* species such as *Pseudomonas fluorescens*, gram-positive bacteria and fungi. Initially, *Pseudomonas* species and their endotoxins were linked to HP in machine workers [1,4]. Subsequent studies have detected growth of acid-fast bacilli of the genus *Mycobacterium* in these fluids and provided evidence for a link between Mycobacteria and HP in machine workers exposed to used MWF [4–6]. MWF-in-use contain large amounts of both culturable (viable) and non-culturable (viable or non-viable) microorganisms and cell fragments which are believed to contribute to the total load of antigens of a causative agent [2,7]. Extensive use of MWF may further increase the non-culturable fraction

* Corresponding author. Tel.: +513-558-4806; fax: +513-558-4397.
*E-mail address:* jagjit.yadav@uc.edu (J.S. Yadav).

0890-8508/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2003.09.004

of its microflora. This emphasizes the need to analyze these fluids for total load (culturable and non-culturable) of microorganisms instead of only culturable flora. This is particularly important for nontuberculous Mycobacteria and Pseudomonads which have been implicated in MWF-linked occupational illnesses [8].

Routine culture-based methods used for the isolation, detection and quantification of these two important genera, *Mycobacterium* and *Pseudomonas*, in contaminated MWF are based on agar plating using available partially selective or enrichment growth media, which putatively yield only the culturable (viable) cells and are time-consuming. Being independent of the viability status of the cell, PCR-based approach could be a reliable and effective alternative to the slow and ambiguous conventional culture-based methods for early identification of these agents without culturing. Hence, the main goal of this study was to develop real-time PCR-based protocols for the purpose. The study involved a two-stage effort: (i) optimization of direct DNA isolation from MWF; (ii) optimization of genus-specific real-time PCR protocols for *Mycobacterium* and *Pseudomonas* to detect and quantify their total number including both culturable as well as non-culturable cells.

## 2. Materials and methods

### 2.1. Microbial strains and culture conditions

Commonly occurring species of the two genera of medical interest (*Mycobacterium* and *Pseudomonas*) in MWF microflora, *Mycobacterium immunogenum* (ATCC 700506), and *Pseudo monas P. fluorescens* (ATCC 13525), were obtained from the American Type Culture Collection (ATCC) for use in the development of standard curves for estimation of total Mycobacteria and Pseudomonads in MWF. Both the reference strains were grown in Middlebrook 7H9 broth (40 ml) supplemented with Oleic acid-Albumin-Dextrose-Catalase (OADC) enrichment with continuous shaking (150 rpm) at 37 °C for 120 h and 25 °C for 24 h, respectively. Shaking reduced the clumping thereby facilitating the representative sampling as previously described [9].

### 2.2. Metalworking fluids-sample collection and culturing

A total of 20 samples of MWF-in-use originating from different industrial plants at diverse geographic locations were obtained through the Cincinnati-based MWF manufacturing company Milacron. The MWF samples were plated and cultured for total microbial count and selective count for *Mycobacterium* or *Pseudomonas* using general and selective/enrichment media, respectively, as described by Yadav et al [10].

### 2.3. DNA isolation from culture and metalworking fluid matrix

The reference strains, *M. immunogenum* and *P. fluorescens* were grown to 120 Klett reading measured using Klett Photoelectric Colorimeter (Klett, New York, USA). Cell density (cells/ml) in the cultures was measured by spread plating an appropriate dilution (100 μl) on Middlebrook agar with OADC enrichment medium and colonies counted after 120 and 24 h for Mycobacteria and Pseudomonads, respectively. One ml of each culture was centrifuged at 10,000 rpm for 10 min to obtain the cell pellet for DNA isolation. On the other hand, 20 ml of each MWF sample was used for obtaining the cell pellet, by centrifugation consecutively (3 ×) at 10,000 rpm for 10 min using Sorvall RC 5B plus centrifuge machine (Sorvall, USA). Cells washed and recovered from pellet in 1 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) were then lysed and the DNA purified using the Bactozol reagents (MRC, USA). Due to the complexity and rigidity of mycobacterial cell wall and the presence of potential inhibitors in the sample matrix, the original Bactozol DNA isolation protocol was modified as follows to obtain a PCR quality DNA in sufficient yield. The pellet was resuspended in 200 μl of 1 × Bactozyme solution (20 μl enzyme mixed in 180 μl 10 × buffer), by vortexing to obtain a homogenous suspension and incubated at 55 °C for 45 min. The resulting cell suspension was treated with 20 μl Proteinase K (15.6 mg $ml^{-1}$) at 55 °C for 30 min and DNAzol (400 μl) at 55 °C for 15 min. The suspension was extracted with equal quantity of phenol/chloroform/isoamylalcohol (25:24:1). Supernatant was collected and DNA precipitated using 100% ethanol (300 μl). Precipitated DNA pellet was washed with 75% ethanol and solubilized in 50 μl TE buffer. The DNA pellet was repurified using isopropanol precipitation and ethanol (75%) washing and solubilized in 50 μl TE buffer. Quality of the DNA prep was examined by agarose gel electrophoresis using 1 × TAE (0.04 M Tris–acetate, 0.001 M EDTA, pH 7.8) buffer.

### 2.4. Real-time quantitative PCR

*A. Preparation of standard curves*. The standard curve for quantification of Mycobacteria was prepared using genus-specific heat shock protein (*hsp*) gene-based PCR primers optimized in our earlier study to amplify commonly occurring mycobacterial species in MWF [11]. The Mycobacterium-specific oligonucleotide primer pair consisted of primer I with a sequence 5′-CTGGTCAAGGAAGGTCTGCG-3′ and primer II with a sequence 5′-GATGACACCCTCGTTGCCAAC-3′ and targeted the amplification of a 228 bp region of its heat shock protein (*hsp*) gene. Increasing amounts of DNA (1.85, 18.5, 185.0 pg, 1.85 and 18.5 ng) corresponding to varying number of *M. immunogenum* cells ($10^1$, $10^2$, $10^3$, $10^4$ and $10^5$), were amplified by real-time PCR (see below) and

cycle threshold (Ct) values plotted against the cell number. On the other hand, the standard curve for Pseudomonads was prepared based on Pseudomonas-specific primer pair, consisting of primer I with a sequence 5′-GAGTTT-GATCCTGGCTCAG-3′ and primer II with a sequence 5′-CCTTCCTCCCAACTT-3′, targeting a 440 bp region of the 16S rRNA gene [10]. Varying amounts of DNA (0.775, 7.75, 77.5, 775 pg, 7.75 and 77.5 ng) corresponding to varying number of *P. fluorescens* cells ($10^1$, $10^2$, $10^3$, $10^4$, $10^5$ and $10^6$) were used.

Real-time PCR reaction for both the genera was performed using Hot Start *Taq* DNA polymerase and the compatible Sybr Green PCR master mix (Stratagene, CA, USA) using 7900 HT ABI Prism (Applied BioSystems, Foster City, USA). Reaction mixture (20 μl) consisted of varying amount of genomic DNA template, 10 μl of 2 × master mix (containing reaction buffer, $MgCl_2$, dNTPs, Sybr Green and Hot Start *Taq* DNA polymerase), and 40 ng each of the forward and reverse primers. A passive reference dye supplied with the kit was added at 300 nM to compensate for non-PCR-related variations in fluorescence. PCR amplification program for *Mycobacterium* involved initial melting cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s (denaturation), 58 °C for 15 s (annealing) and 72 °C for 30 s (extension). For *Pseudomonas*, the PCR conditions included an initial template denaturation at 95 °C for 10 min followed by 40 cycles of amplification by repeating denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. An additional extension at 72 °C for 3 min was used before cooling to 4 °C. In order to check the quality of amplification in both reactions, a melting profile was generated for each amplicon over a temperature range of 60–95 °C.

*B. Detection and quantification of Mycobacteria and Pseudomonads in MWF*. Using the above optimized real-time PCR amplification conditions, known aliquots of the isolated DNA from MWF samples were used for the detection of *Mycobacterium* and *Pseudomonas* as well as quantification of total number of their cells (culturable and non-culturable) per ml based on the developed standard curves. The quality of amplification reactions was confirmed by analyzing the melting peaks of amplicons and comparing them to the standard melting peaks obtained for *M. immunogenum* and *P. fluorescens* amplicons, respectively. Additionally, the amplification quality was confirmed by electrophoresing (70 V, 1 h) the PCR product (10 μl) using 1% Trevigel gel matrix (Trevigen, USA) in 1 × TAE buffer containing ethidium bromide (0.5 $\mu g/ml^{-1}$) and 100 bp DNA size marker (PGC Scientifics, USA) using BioRad mini gel electrophoresis apparatus. Gels were visualized on UV transilluminator and photographed using Kodak Edas 290 gel documentation system (Kodak, USA).

## 3. Results

### 3.1. Culturable Mycobacteria and Pseudomonads in MWF

All the 20 MWF samples showed high load of mixed bacterial flora ($3 \times 10^3$–$4 \times 10^6$ CFU/ml) when cultured on the general bacterial growth medium Trypticase Soy Agar. However, when cultured on selective media, only two of the 20 samples yielded putative colonies each of the genus *Mycobacterium* on Middlebrook agar (40–250 CFU/ml for samples S-16 and S-17) and of *Pseudomonas* on

Table 1
Real-time PCR-based quantification of total Mycobacteria and Pseudomonads (culturable and non-culturable) and selective culturing-based quantification of culturable Mycobacteria and Pseudomonads in MWF containing mixed microflora

| MWF sample code | Culturable counts/ml | | | Total Mycobacteria (PCR-based quantification) | | Total Pseudomonads (PCR-based quantification) | |
|---|---|---|---|---|---|---|---|
| | All bacteria[a] | Mycobacterial counts[b] | Pseudomonads counts[c] | Ct value | Number of cells/ml MWF | Ct value | Number of cells/ml MWF |
| S-05 | $3 \times 10^4$ | 0 | 0 | – | 0 | 17.48 | $6.7 \times 10^4$ |
| S-09 | $6 \times 10^5$ | 0 | 0 | 28.45 | $1.3 \times 10^2$ | 15.64 | $2.7 \times 10^5$ |
| S-10 | $6 \times 10^3$ | 0 | 0 | 27.31 | $3.4 \times 10^2$ | 21.60 | $2.8 \times 10^3$ |
| S-12 | $4 \times 10^6$ | 0 | $2 \times 10^3$ | – | 0 | 14.33 | $7.0 \times 10^5$ |
| S-13 | $3 \times 10^3$ | 0 | 600 | – | 0 | 14.39 | $7.0 \times 10^5$ |
| S-14 | $7 \times 10^5$ | 0 | 0 | 24.80 | $2.6 \times 10^3$ | – | 0 |
| S-16 | 0 | 250 | 0 | 18.15 | $5.5 \times 10^5$ | – | 0 |
| S-17 | $2 \times 10^4$ | 40 | 0 | 20.24 | $1.0 \times 10^5$ | 22.90 | $1.1 \times 10^3$ |
| S-18 | $7 \times 10^3$ | 0 | 0 | 27.31 | $3.4 \times 10^2$ | 23.85 | $5.2 \times 10^2$ |
| S-20 | $2 \times 10^4$ | 0 | 0 | 26.13 | $9.0 \times 10^2$ | – | 0 |

[a] Trypticase Soy Agar (TSA) was used for total culturable bacterial count.
[b] Middlebrook 7H10 agar (MBA) supplemented with OADC enrichment was used for culturable Mycobacterial count.
[c] Pseudomonas Isolation Agar (PIA) was used for culturable Pseudomonads count.

Pseudomonas Isolation Agar ($6 \times 10^2 - 2 \times 10^3$ CFU/ml for samples S-12 and S-13) as summarized in Table 1.

### 3.2. Optimization of real-time PCR conditions and development of standard curves

Real-time PCR-based protocol was optimized for the reference strains *M. immunogenum* and *P. fluorescens* using the 7900 HT ABI Prism system. The amplification primers used were specific for the two genera as described previously [10,11]. The standard curves for quantitation were generated based on *M. immunogenum* cell number ranging from $10^1$ to $10^5$, and *P. fluorescens* cell number ranging from $10^1$ to $10^6$. For both genera, the quantification limit was as low as 10 cells/ml (Figs. 1A and 2), although a minimum of 1 cell/ml was detectable based on amplification growth curve signal and a minor melting peak (data not shown). The standard curves for *M. immunogenum* and *P. fluorescens* showed correlation coefficients ($R^2$) of 0.998347 and 0.990507, respectively (Figs. 1A and 2). Typical growth curve for the amplicon was detected for *M. immunogenum* based upon the fluorescent signal measured by the ABI Prism system following the annealing step at 58 °C (Fig. 1B). Similar amplification growth curve was obtained for *P. fluorescens* (data not shown). A dissociation curve analysis of PCR products showed that the melting temperature of the *M. immunogenum* amplicon was 88 °C (Fig. 1C) while the negative control without DNA template did not show such melting peak and instead showed a nonspecific background product with a minor melting peak at 81 °C (Fig. 1B and C). Likewise, in case of *P. fluorescens*, a melting peak for each amplicon was observed at 83.5 °C (data not shown). Agarose gel electrophoresis of the PCR products (228 bp) for *M. immunogenum* confirmed the desired quality of amplification showing lack of formation of any primer dimers (Fig. 1D). Similar amplification quality was observed for *P. fluorescens* (data not shown).

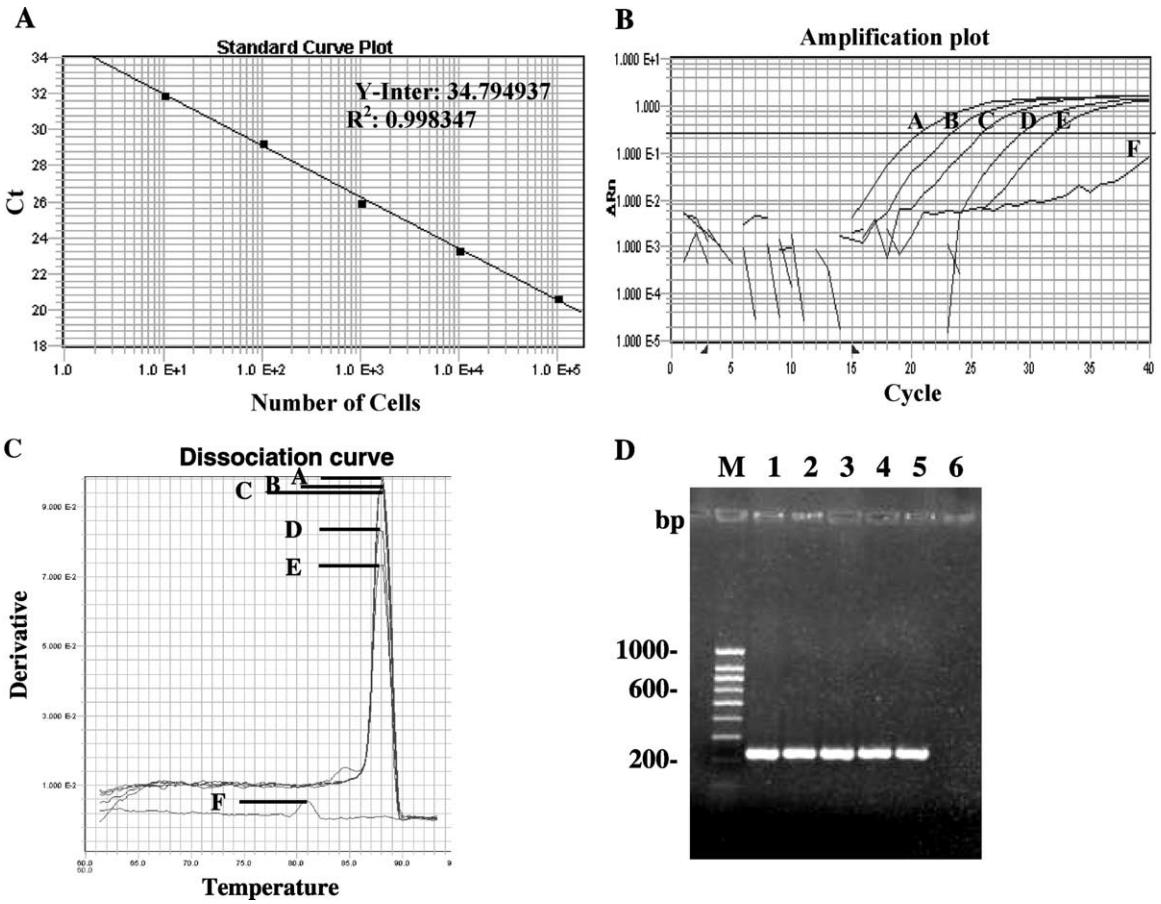


Fig. 1. Preparation of standard curve for *M. immunogenum* using genus-specific quantitative real-time PCR. Panel A: Standard curve generated based on amplification of DNA from increasing number of cells using Mycobacterium-specific *hsp* gene primers targeting a 228 bp region. Panel B: Real-time PCR amplification growth curves corresponding to varying number of cells (A–E: $10^5$, $10^4$, $10^3$, $10^2$, and $10^1$ cells, F: negative control). Panel C: The dissociation curve or melting curve (a graph showing the rate of change in fluorescence over time as a function of melting temperature for each amplicon) for the five amplicons (A through E) showing a clear peak at 88 °C unlike the negative control, confirming the specific nature of amplicons. Panel D: The PCR end products with expected 228 bp size (lanes 1–5) analyzed on agarose gel; Lane 6: Negative control; M: 100 bp DNA size marker (PGC Scientific, USA).

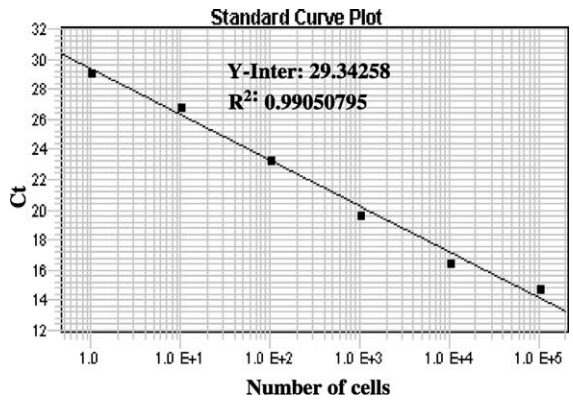


Fig. 2. Standard curve for *P. fluorescens* (ATCC 13525) using genus-specific quantitative real-time PCR. Increasing amounts of DNA corresponding to varying number of cells ($10^1$–$10^6$) were amplified with real-time PCR using Pseudomonas genus-specific 16S rRNA gene primers targeting a 440 bp sequence.

### 3.3. Real-time PCR detection and quantification of total (cultrable and non-culturable) Mycobacteria and Pseudomonads in MWF

Microbial DNA isolated directly from MWF samples without culturing were analyzed using the optimized real-time PCR protocol for the presence of the genera *Mycobacterium* and *Pseudomonas*. The developed genus-specific protocols enabled detection and quantification of total (culturable and non-culturable) Mycobacteria or Pseudomonads in MWF. Of the 20 samples, seven samples (two different sets) yielded amplification signals for *Mycobacterium* or *Pseudomonas*, while only two samples (two different sets) yielded isolates for each of the two genera on selective media (Table 1). Total number of cells for each genus was quantified using the respective standard curve. Total Mycobacteria in the MWF samples ranged from $1.3 \times 10^2$ to $5.5 \times 10^5$ cells/ml, whereas Pseudomonads ranged from $5.2 \times 10^2$ to $7.0 \times 10^5$ cells/ml (Table 1). All the PCR positive samples showed amplicon melting peaks comparable to the standards, i.e. 88 and 83.5 °C for *Mycobacterium* and *Pseudomonas*, respectively (Fig. 3). Agarose gel electrophoresis confirmed the quality of amplification of the expected size amplicons for all MWF samples (data not shown).

## 4. Discussion

Mycobacteria and Pseudomonads colonize in modern MWF formulations and are the suspected antigens for respiratory illnesses, particularly HP [1,3]. 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 [7]. Conventional practice for detection and quantification of these genera in MWF is mostly based on culturing and staining, which often yield ambiguous results. Selective culturing of these genera from

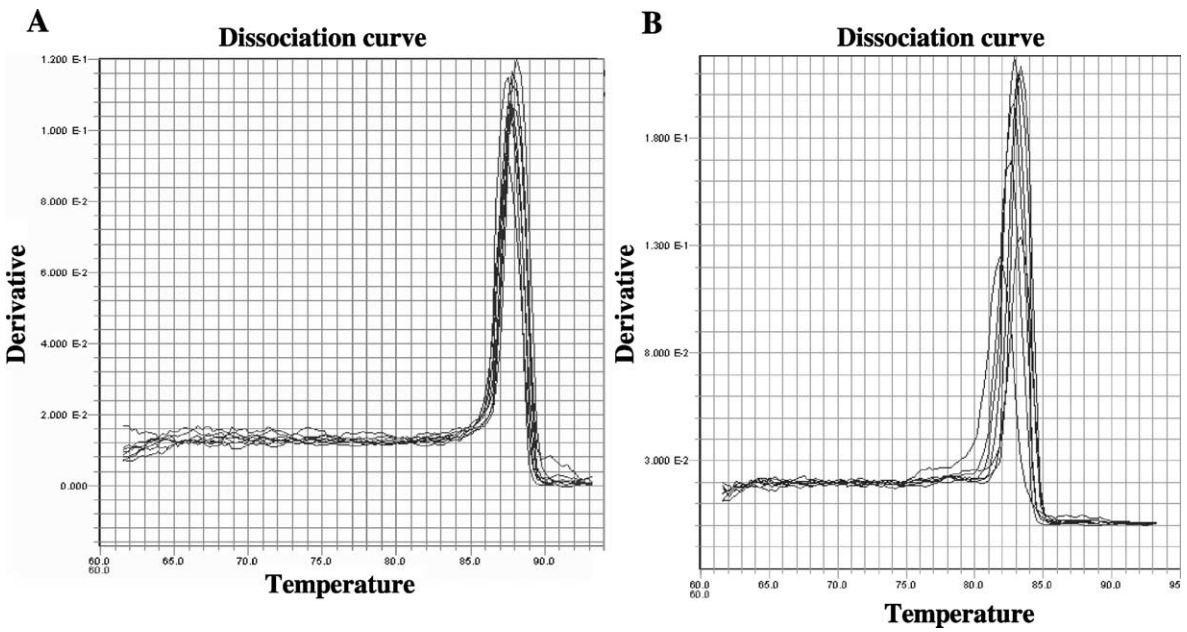


Fig. 3. Melting curves for genus-specific amplicons generated during real-time PCR analysis of the MWF samples. Panel A: The melting curves for mycobacterial amplicons showing the same melting temperature (88 °C) for all amplicons from seven MWF samples positive for *Mycobacteria* corresponding to the melting peak of *M. immunogenum* amplicon used as standard. Panel B: The melting curves for Pseudomonas-specific amplicons showing typical melting temperature (83.5 °C) for all seven MWF samples positive for *Pseudomonas* (Table 1), corresponding to the melting peak of *P. fluorescens* amplicon used as standard.

mixed flora such as in MWF, is a difficult task. Moreover, the culturing method only yields culturable counts and does not account for non-culturable (viable or non-viable) fraction which has been considered as important contributor to microbial antigen load and endotoxin levels in MWF [2,4]. This study reports the optimization of a real-time PCR-based protocol for direct detection and quantification of total cells including culturable and non-culturable (viable/nonviable) cells, of *Mycobacterium* or *Pseudomonas* in a mixed microbial population in field MWF samples. Real-time PCR assays for selected species of the genera *Mycobacterium* (*Mycobacterium tuberculosis* and *Mycobacterium avium* subsp. *paratuberculosis*) and *Pseudomonas* meant for clinical and other environmental applications have been recently evaluated [12–16]. The developed real-time protocol will offer numerous advantages over the end point-based PCR quantification reported by us earlier [10] because of its rapidity and the ability to analyze in actual time, most desirable for high throughput applications. Also it does not require post PCR handling by running agarose gel, which reduces the chances of cross contamination in multiple PCR assays in a given setting [17–21]. In addition, the developed protocol uses fluorescent dye Sybr Green as against the cost-intensive fluorogenic probes [18].

Another significant outcome of this study is the first development of a method for DNA recovery and purification from field samples of MWF without culturing for downstream applications in real-time PCR detection and quantification. The DNA yields showed that the centrifugation versus filtration is an efficient and feasible pre-step for recovery of cells from MWF (data not shown), and is easily adaptable in an analytical or diagnostic laboratory. Various types of MWF contain diverse co-occurring contaminants including organic and inorganic debris and metal particles which may act as inhibiting factors in cell recovery and PCR reaction [10]. The developed DNA extraction procedure yielded a DNA with a quality and quantity suitable for downstream quantitative PCR application, unlike other DNA isolation protocols tested in this study (data not shown).

Mycobacterium-specific heat shock protein (*hsp*) gene, which shows hypervariability among species, has been used as an effective target for detection and species differentiation in Mycobacteria by PCR in combination with nucleotide sequencing or restriction enzyme analysis [8,22,23]. However, the reported primers failed to amplify all Mycobacterial species particularly those occurring in MWF such as *M. immunogenum* [10]. Hence, in this study a primer pair designed based on *hsp* gene sequences conserved among species and flanking a hypervariable region was used for development of standard curve as well as detection and quantification of MWF mycobacteria [11].

The developed genus-specific real-time protocol for *Mycobacterium* or *Pseudomonas* showed a minimum quantification limit of 10 cells/ml corresponding to the DNA concentration of 1.8 and 0.7 pg, respectively, although a minimum detection limit achievable with an amplification growth curve and minor melting peak was 1 cell/ml (data not shown). The culturable counts from the positive two MWF samples ranged from $4 \times 10^1$ to $2 \times 10^3$ CFU/ml for Mycobacteria and 600 to $2 \times 10^3$ CFU/ml for Pseudomonads. The corresponding total counts based on the developed quantitative PCR ranged from $1.3 \times 10^2$ to $5.5 \times 10^5$ CFU/ml and $5.2 \times 10^2$ to $7.0 \times 10^5$ CFU/ml, respectively. Vast difference in the detection and quantification outcome by the quantitative PCR versus the selective culturing method may be ascribed to the presence of large number of non-culturable cells (viable but non-culturable and non-viable cells) of the test genera in these fluids.

The results show that the real-time PCR-based assay for screening field MWF samples for the individual test genera is fairly rapid as it takes an estimated 2–3 h time depending on the genus, including the DNA extraction step (60–90 min) and the real-time PCR protocol (90 min–2 h) as compared to the conventional end-point-based PCR and culturing based approach which may take an estimated 6–8 h and 3–10 days, respectively. This is particularly useful for mycobacteria including even rapidly growing mycobacteria (RGM), which normally take up to a week to grow on plates. The optimized procedure uses a 384 well microtiter plate format, which can allow testing of a large number of samples as well as multiple targets with common thermal cycling conditions on the same analysis plate.

## 5. Conclusions

The study has led to the first development and application of real-time quantitative PCR assay for used MWF based on DNA extracted directly from the fluid matrix without culturing. Application of the developed genus-specific real-time PCR assays to the field MWF samples revealed the presence of a significant non-culturable fraction of both Mycobacteria and Pseudomonads in these fluids. The developed assays could, therefore, provide tools for early and reliable detection of microbial contaminants (culturable and non-culturable) in field samples, a critical factor in intervention for reducing and eliminating the risk of Mycobacteria and Pseudomonads in occupational health.

## Acknowledgements

The study was supported by the NIOSH-CDC grant 1R01OH007364 (JSY). We thank Milacron management for providing the used metalworking fluid samples for isolation of Mycobacteria, and Venkataramanan Subramanian and Kun Zhang for initial assistance in the use of real-time PCR equipment.

## References


[1] Rossmoore HW. Microbial degradation of water-based metalworking fluids. In: M. Moo-Young, C. L. Cooney, A. E. Humphrey, editors. Comprehensive Biotechnology, vol. 3.; 1986. p. 249–69. Pergamon, New York.

[2] Mattsby-Baltzer I, Sandin M, Ahalström B, Allenmark S, Edebo M, Falsen E, Pedersen K, Rodin N. Microbial growth and accumulation in industrial metal-working fluids. Appl Environ Microbiol 1989;55: 2681–9.

[3] Hill EC, Al-Zubaidy T. Some health aspects of infections in oil and emulsions. Tribiol Int 1979;8:161–4.

[4] Bernstein DI, Lummus ZL, Santilli G, Siskosky J, Berstein IL. Machine operator's lung. A hypersensitivity pneumonitis disorder associated with exposure to metalworking fluid aerosols. Chest 1995; 108:636–41.

[5] Muilenberg ML, Berge HA, Sweet T. Hypersensitivity pneumonitis and exposure to acid-fast bacilli in coolant aerosols. J Allergy Clin Immunol 1993;91:311.

[6] Kreiss K, Cox-Ganser J. Metalworking fluid-associated hypersensitivity pneumonitis: a workshop summary. Am J Ind Med 1997;32: 423–32.

[7] Lonon MK, Abanto M, Findlay RH. A pilot study for monitoring changes in the microbiological component of metalworking fluids as a function of time and use in the system. Am Ind Hyg Assoc J 1999;60: 480–5.

[8] Wallace Jr RJ, Zhang Y, Wilson RW, Mann L, Roosmoore L. Presence of a single genotype of the newly described species *Mycobacterium immunogenum* in industrial metalworking fluids associated with hypersensitivity pneumonitis. Appl Environ Microbiol 2002;68:5580–4.

[9] Hughes VM, Stevenson K, Sharp JM. Improved preparation of high molecular weight DNA for pulsed-field gel electrophoresis from mycobacteria. J Microbiol Meth 2001;44:209–15.

[10] Yadav JS, Khan IUH, Fakhari F, Soellner MB. DNA-based methodologies for rapid detection, quantification, and species- or strain-level identification of respiratory pathogens (Mycobacteria and Pseudomonads) in metalworking fluids. Appl Occup Environ Hyg 2003;18;966–75.

[11] Khan IUH, Yadav JS. Development of a single-tube cell lysis-based genus-specific PCR method for rapid detection of Mycobacteria: optimization of cell lysis, PCR primers and conditions, and restriction pattern analysis. J Clin Microbiol 2003; in press.

[12] Jaffe RI, Lane JD, Bates CW. Real-time identification of *Pseudomonas aeruginosa* direct from clinical samples using a rapid extraction method and polymerase chain reaction (PCR). J Clin Lab Anal 2001; 15:131–7.

[13] Kraus G, Cleary T, Miller N, Seivright R, Young AK, Spruill G, Hnatyszyn J. Rapid and specific detection of the *Mycobacterium tuberculosis* complex using fluorogenic probes and real-time PCR. Mol Cell Probes 2001;15:375–83.

[14] O'Mahony J, Hill C. A real time PCR assay for the detection and quantitation of *Mycobacterium avium* subsp. *paratuberculosis* using SYBR Green and the Light Cycler. J Microbiol Meth 2002;51: 283–93.

[15] Fang Y, Wu WH, Pepper JL, Marras SA, Nelson EA, Epperson WB. Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. J Clin Microbiol 2002;40:287–91.

[16] Torres MJ, Criado A, Ruiz M, Llanos AC, Palomares JC, Aznar J. Improved real-time PCR for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates. Diagn Microbiol Infect Dis 2003;45:207–12.

[17] Bellin T, Pulz M, Matussek A, Hempen HG, Gunzer F. Rapid detection of enterohemorrhagic *Escherichia coli* by real-time PCR with fluorescent hybridization probes. J Clin Microbiol 2001;39: 370–4.

[18] Taylor MJ, Hughes MS, Skuce RA, Neill SD. Detection of *Mycobacterium bovis* in bovine clinical specimens using real-time fluorescence and fluorescence resonance energy transfer probe rapid-cycle PCR. J Clin Microbiol 2001;39:1272–8.

[19] Wellinghausen N, Frost C, Marre R. Detection of legionellae in hospital water samples by quantitative real-time LightCycler PCR. Appl Environ Microbiol 2001;67:3985–93.

[20] Sails AD, Fox AJ, Bolton FJ, Wareing DR, Greenway DL. A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture. Appl Environ Microbiol 2003;69: 1383–90.

[21] Gruntzig V, Nold SC, Zhou J, Tiedje M. *Pseudomonas stutzeri* nitrite reductase gene abundance in environmental samples measured by real-time PCR. Appl Environ Microbiol 2001;67:760–8.

[22] Yakrus MA, Hernandez SM, Floyd MM, Sikes D, Butler WR, Metchock B. Comparison of methods for identification of *Mycobacterium abscessus* and *M. chelonae* isolates. J Clin Microbiol 2001;39: 4103–10.

[23] Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T. Rapid identification of *Mycobacteria* to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 1993;31:175–8.