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Recovery and Quantification of *Mycobacterium Immunogenum* DNA from Metalworking Fluids Using Dual-Labeled Probes

ABSTRACT: Mycobacteria in metalworking fluids (MWF) are associated with hypersensitivity pneumonitis but are difficult to recover using culture. Quantitative PCR is a promising approach to quantify mycobacteria, but three challenges exist: mycobacterial cell lysis, high-yield DNA extraction, and removal of PCR inhibitors. We used *Mycobacterium* spp. primers to amplify polymorphic regions of 16S-rDNA flanked with highly conserved regions. A standard curve was constructed by cloning *M. immunogenum* amplification product. We developed single tube DNA extraction employing mixer mill cell disruption, enzymatic digestions (lysozyme, proteinase K) followed by a mechanical disruption, and column purification. MWF was spiked with *M. immunogenum*, and DNA was successfully extracted. Mycobacterial 16S-RNA genes were quantified by comparing PCR amplification detection (Cycle Threshold) from our samples with that obtained from the standard curve. Recovery and quantification of mycobacterial DNA from spiked samples approached 100 %. A rapid method for quantification of mycobacteria in MWF was demonstrated.

KEYWORDS: metalworking fluid, real-time PCR, mycobacteria, fluorescent probes, hypersensitivity pneumonitis

Introduction

Microbial growth in metalworking fluids (MWF) has become a significant problem for the industry. Bacteria often colonize MWF systems, reducing the system efficiency and inhibiting the performance of MWF in metal boring, hobbing, tapping, grinding, and honing operations [1]. Among the various classes of MWF, those composed of an aqueous oil emulsion with oil in high concentration (soluble oils) or lower concentration (semi-synthetic MWF) are the ones that better support the microbial growth because of their high water activity. The soluble oil MWF are composed of 3–6 % oil emulsified in water and usually contain a variety of additives, including emulsifiers, biocides, antifoaming agents, and corrosion inhibitors [2]. Even if microbicides are added to the fluids, the emulsifiable oils are propitious to microbial growth such as Gram-negative bacteria, e.g., *Pseudomonas spp.* [1–4]. Moreover, we have recently demonstrated that dumping, cleaning, and recharging MWF systems are of little use to rid the MWF systems of microbial problems [5]. Several studies of respiratory disease among workers in contact with soluble MWF have suggested that *Mycobacteria* are associated with Hypersensitivity pneumonitis (HP) outbreaks [6–10]. Mycobacteria are ubiquitous in water environments

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(drinking water, pools, cooling towers) [11,12] and are amplified in MWF systems. The presence of mycobacteria in MWF is not easily detected using culture because of their slow-growing capacity (even if they are classified as rapid growing mycobacteria, they need up to 14 days to form visible colonies) and their variable and alternative culturability state [7]. Since their viability and culturability have little influence on immunogenic properties of bacteria and on the risk for HP development among workers, alternative methods allowing direct and accurate quantification of mycobacteria from MWF environments are to be developed. We have employed the direct count method using epifluorescence microscopy to evaluate bacterial load in MWF systems, but this approach needs to be complemented with a species-specific approach that allows detection and quantification of mycobacteria [2,5]. The major taxa that have been involved in HP outbreaks, *M. chelonae* and *M. immunogenum*, differ in their number of copies of the rRNA operon and their ability to use citrate as carbon source [13]. They also exhibit differences in their 16S rDNA sequences.

Real-time PCR is the method of choice for estimating the concentration of Mycobacteria in an aqueous environment. PCR primers can be designed to specifically amplify conserved sequences amongst mycobacteria at the species or genus level, and dual labeled probes can be used to identify and quantify specific species such as *Mycobacterium immunogenum*. The main problems to overcome are the difficulty of lysing the waxy mycobacteria necessary for high DNA recovery and finding the optimal method for DNA purification in order to perform PCR without inhibitors. The extraction efficiency of microbial DNA has to be evaluated in order to accurately estimate the number of cells in the original samples. Standard lysis protocols that were developed for other microorganisms lead to an underestimation of the Mycobacteria counts and false negatives in cases of low microorganism concentration in MWF, leading to difficult early detection of mycobacterial contamination. Our study aimed to establish a lysis protocol, determine the important purification steps, and quantify mycobacteria using an internal control and dual-labeled probes on a real-time PCR platform.

Material and Methods

Mycobacteria Growth

ATCC and environmental Mycobacteria from MWF and peat moss were grown on Middlebrook 7H10 agar with OADC and in Middlebrook 7H9 ADC broth. The media were incubated at 30°C with 5 % CO_2. Isolates' identity was regularly controlled by acid-fast staining and microscopic observations.

Identity of Environmental Strains

The DNA from peat moss stains was amplified with the PCR primer and conditions from Kox [14]. Sequencing was done on the PCR products using a 3730/XLDNA Analyzer (Applied Biosystems, Foster City, CA), and the sequences were compared to those present in Genbank.

Mycobacterium Immunogenum Stock Suspension

A stock suspension of *M. immunogenum* was prepared in PBS + 0.05 % Tween 20. The total number of cells was determined using fluorescence microscopy and DAPI staining (Molecular

Probes, Eugene, OR) [5]. The final suspension was adjusted to 10^8 cells/ml in a level 2 laminar flow hood.

MWF Spike and Total Counts

A neat, never used 5 % MWF solution was prepared using concentrated MWF and distilled water. Serial dilutions of *M. immunogenum* in MWF (10^7–10^1) were performed by inoculating stock suspension (10^8/ml) into the 5 % MWF solution. Concentrations were controlled by fluorescence microscopy and DAPI staining.

DNA Extraction

Spiked MWF samples (1.5 ml) were centrifuged at 21 000xG for 10 min in 2.0 ml microtubes (Eppendorf, Mississauga, ONT). The pellet was washed with 500 µl of PBS and then re-centrifuged at 21 000xG for 5 min. The cell pellet was re-suspended in 50µl of TNE buffer (12 mM Tris-HCl, 2 M NaCl, 0.2 M EDTA, pH 7.8), digested with 5 µl of lysozyme (740 mg/ml) at 37°C for 1 h, and pulse-vortexed every 10 min. 180 µl of ATL digestion buffer provided in the Qiagen QIAamp®[2] DNA Mini Kit (content unknown) and 20 µl of Qiagen proteinase K was added to the lysozyme samples. They were then digested for 2 h at 56°C, pulse-vortexing every 10 min. After the digestion, 200 µl of AT lysis buffer provided in the Qiagen QIAamp®[2] DNA Mini Kit (unknown composition) was added, and the samples incubated at 70°C for 10 min. Mechanical disruption was performed using a MM301 Mixer Mill with 3 mm tungsten beads (Retsch, Germany). The disruption program consisted of two cycles for 2 min at 30 agitation/s. Samples were then centrifuged at 3000xG for 5 min to remove foam before adding 200 µl of ice-cold 99 % ethanol. The samples were applied to silica based columns[2] for the DNA cleaning procedure using the manufacturer's protocol. DNA was eluted twice using 50 µl of Qiagen elution buffer for each sample.

Realtime PCR

Primers from Kox were modified (see Table 1) to increase specificity to *M. immunogenum*. The two degenerated bases of the pMyc14 primer were replaced by guanines and the second base in 3' of the reverse pMyc7 primer was replaced by thymine. A 23 bp dual-labeled probe (FAM-5' CCG CAT GCT TCA TGG TGT GTG GT 3'-BHQ1) has been designed using the sequences from MWF *M. immunogenum* strains and sequences available on NCBI Genbank.

The PCR mix was composed of 1X QuantiTect Probe PCR buffer® (Tris-HCl, KCl, $(NH_4)_2SO_4$, 4mM $MgCl_2$, Hotstar® Taq DNA Polymerase, dATP, dCTP, dGTP, dTTP/UTP, and ultra pure PCR-grade water, pH 8.7), 0.4 µM of each primer and 0.1 µM of probes. Twenty micro-liters of this mix were used, and 5 µl of each samples were added to the PCR mixture for amplification. The amplification was done on a Smartcycler2 platform (Cepheid, Sunnyvale, CA), and the PCR program consisted of 15 min hot start at 94°C, followed by 35 cycles of 3 s at 94°C and 1 min at 61.5°C. Fluorescence was acquired in Channel 1 (Excitation: 450–495 nm, Emission: 510–527 nm) for each cycle. FAM fluorophore has a maximum absorbance at 495 nm and maximum emission at 520 nm.

[2] Qiagen, Mississauga, ONT.

TABLE 1—*Sequences of the oligonucleotides used in quantitative PCR to amplify* Mycobacterium immunogenum.

Oligonucleotide Names	Uses	Sequences
pMycImmF	Forward primer	5'-GGG GTA CTC GAG TGG CGA AC-3'
pMycImmR	Reverse Primer	5'-GGC CGG CTA CCC GTT GTC 3'
PMycImmP	Dual labeled probe	FAM-5'-CCG CAT GCT TCA TGG TGT GTG GT-3' BHQ1

Specificity and Sensitivity of the Primers Probe Set

One hundred nanograms of DNA from different strains, previously amplified with original Kox primers were used to determine the specificity of the PCR assay (see Table 2). The DNA was quantified using a Genequant pro spectrophotometer (Biochrom, Cambridge, UK). Sensitivity was estimated by building standard curves using both genomic DNA from *M. immunogenum* and serial dilutions of a plasmid containing the amplified sequence. In both cases, we built standard curves, using from 10^1–10^7 copy/reaction of the target gene.

TABLE 2—*Environmental and ATCC strains used to determine the specificity of the quantitative PCR assay.*

Strains	Amplification with Primers	Fluorescent Signal with Primers and Probes
M. immunogenum	Yes	Yes
M. chelonae	Yes	No
M. fortuitum	Yes	No
M. palustre	Yes	No
M. bohemicum	Yes	No
M. malmoense	Yes	No
M. intracellular	Yes	No
M. interjectum	Yes	No
M. terrae	Yes	No
M. avium	Yes	No

Evaluation of the Recovery Rate from Spiked MWF: Study Design

Each spiked sample at various concentrations was extracted in triplicate, using each extraction protocol. For each, replicate PCR was performed three times, using the previously described realtime PCR conditions. Cycle thresholds obtained were compared to the plasmidic standard curves, and the number of copies of the 16S rDNA was evaluated for each condition. In order to evaluate the extraction and detection efficiency, the number of copies was divided by two since each *M. immunogenum* cell has two copies of the 16S rRNA gene, and the number was compared to the number of cells spiked in the initial fluid.

Statistical Analyses

Comparisons between extraction procedures for DNA detection were performed using a one way ANOVA. Continuous values were log-transformed to assess the normality and variance

assumptions. Values expressed in percentages were analyzed using an arcsinus transformation on the square root. The normality assumptions were verified with the Shapiro-Wilk test; the Brown and Forsythe's variation of Levene's test statistics were used to verify the homogeneity of variances. The results were considered significant with p-values < 0.05. The data were analyzed using the statistical package program SAS v8.2 (SAS Institute Inc., Cary, NC).

Results

Specificity of the Probe/Primer Set

The strains (Table 2) were tested by PCR for the assay specificity. Figure 1 (61.5°C) demonstrates that the assay is specific to *M. immunogenum* with a cycle threshold of 21.66, compared to 33.61 for *Mycobacterium avium*, and nothing for the other strains.

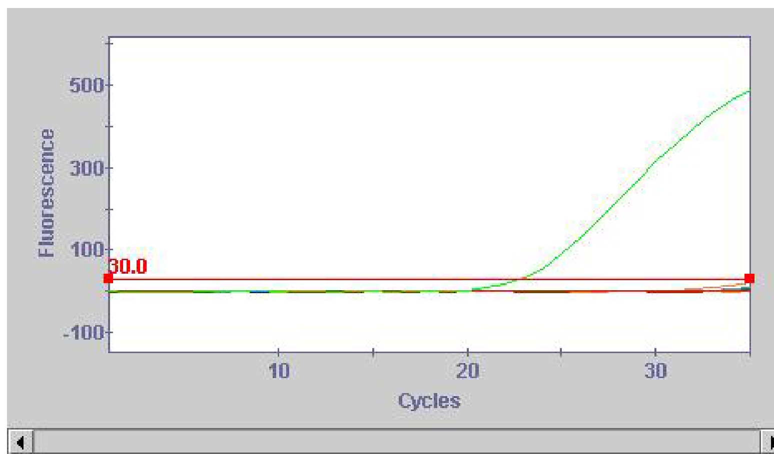

FIG. 1—*Specificity of the primer/dual labeled probe on different* Mycobacterium spp. *species at 61.5°C (*Mycobacterium immunogenum *in green).*

Generation of Standard Curves and Sensitivity

We generated standard curves using a plasmid carrying the target sequence (16S gene of *M. immunogenum*) (see Fig. 2) that yielded a high correlation coefficient (r^2 = 0.998). Using this method, we showed that we could effectively amplify one copy of the target sequence using our real-time PCR assay.

Optimization of the Lysis/DNA Extraction Protocol

To verify the performance of our DNA recovery protocol, specific steps were targeted and evaluated for their importance in the assay. Four different extraction procedures were performed (see Table 3): 1) Complete procedure, 2) Procedure done by omitting lysozyme digestion (without lysozyme), 3) Procedure done by omitting mechanical disruption, and finally, 4) Only the commercial columns procedure. To clearly demonstrate the differences between each procedure, these protocols were performed on MWF containing 10^6 and 10^5 *Mycobacterium* cells/ml.

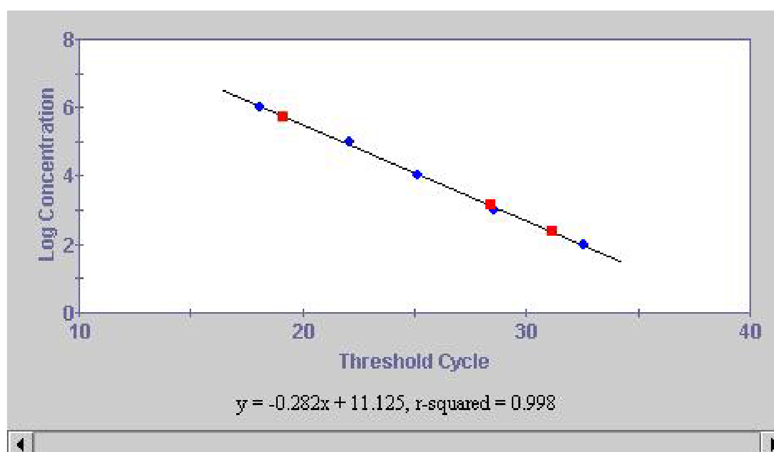

FIG. 2—*Standard curve of the real-time PCR assay using plasmid standard (from 10 to 100 000 copies of the target sequence).*

TABLE 3—*Critical steps involved in the setup of an optimized DNA extraction procedure for mycobacteria in metal working fluid.*

Steps	1-Complete Procedure	2-Without Lysozyme	3-Without Mechanical Disruption	4-Commercial Columns Only
Proteinase K Digestion	Yes	Yes	Yes	Yes
Lysozyme Digestion	Yes	No	Yes	No
Mechanical Digestion	Yes	Yes	No	No
Commercial Columns Procedure	Yes	Yes	Yes	Yes

Realtime PCR

Figure 3 shows the efficiency and importance of each step of the extraction procedure. Given that *M. immunogenum* cells contain two copies of the 16S operon, there may be an estimation of the recovery rate knowing the number of cells present in the starting MWF samples (Fig. 4). The results shown are the cumulative data of these replicates. These data demonstrate that the recovery rate is higher ($p = 0.03$) and more constant using the complete extraction procedure. The results obtained when comparing the number of copies detected when using the different extraction procedures also tend to be significantly higher when using the complete procedure ($p = 0.06$).

Discussion

The protocol presented in this paper provides an assay that is sensitive and highly specific to *Mycobacterium immunogenum* in the MWF environment. Our newly developed genus-specific primers and species-specific probe could be used with other species-specific probes designed for the detection of other *Mycobacterium* species, such as *Mycobacterium chelonae*. Various mycobacterial species could be quantified in a multiplex assay.

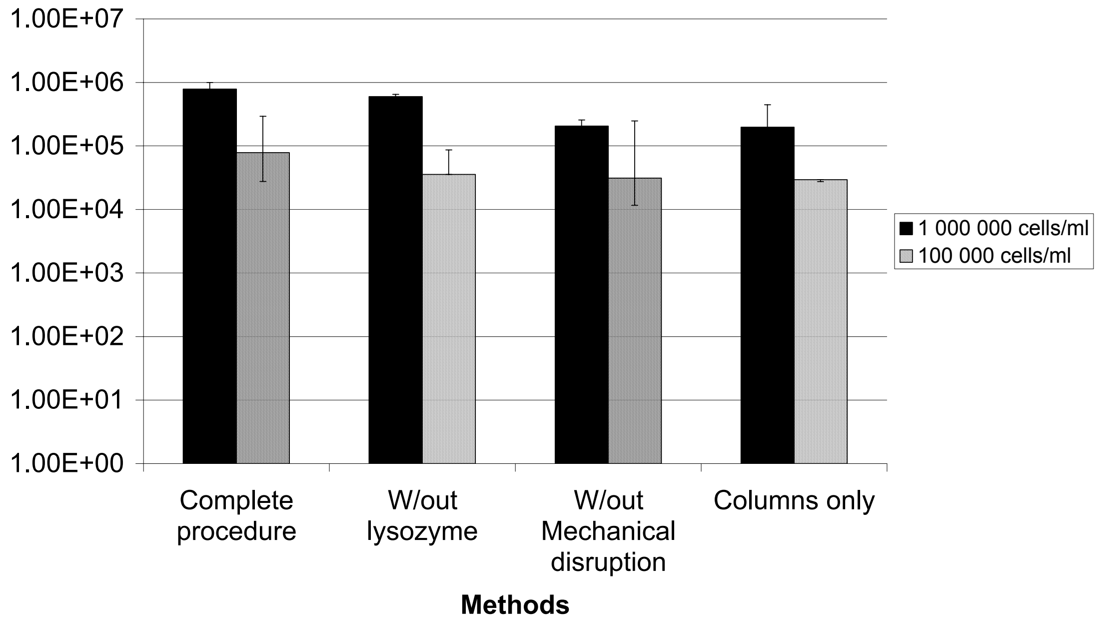

FIG. 3—*Evaluation of the number of copies of the target gene (16s) per ml of each MWF sample.*

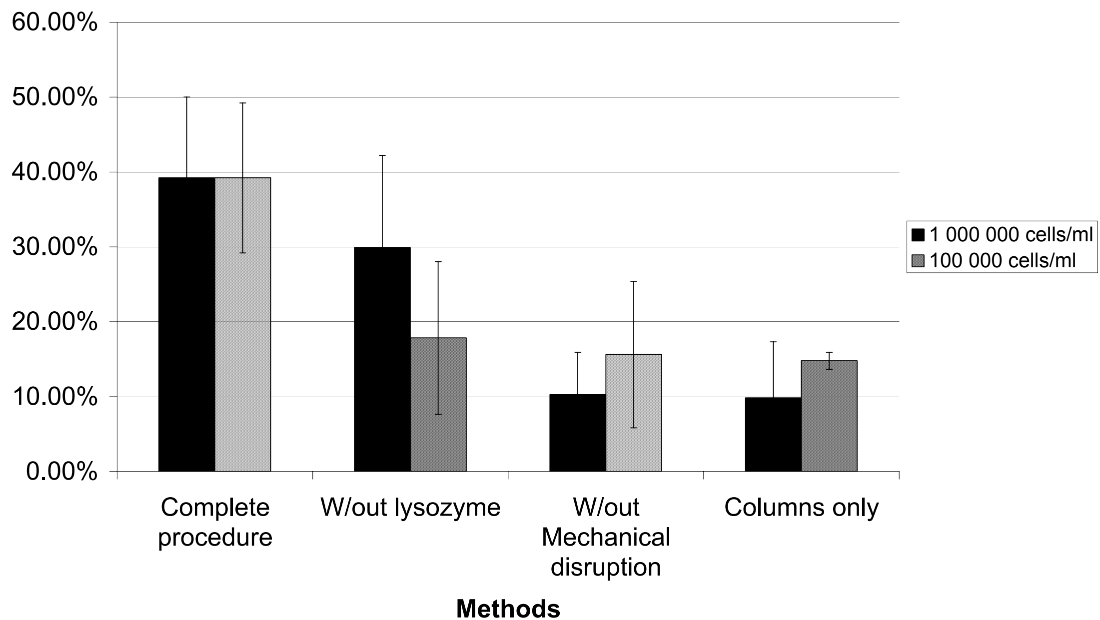

FIG. 4—*Recovery rate for each extraction procedure.*

A previously published paper proposed an approach for the quantification of *M. immunogenum* using real time PCR with specific primer set coupled with SYBR green approach [15]. This paper did not allow the determination of DNA extraction efficiency (the PCR efficiency was evaluated using pure genomic DNA from *M. immunogenum*). Our paper describes a quantification approach using two specificity levels: primers and probe and DNA extraction efficiency evaluated with spiked whole cells, allowing the determination of the best DNA extraction and purification protocol.

Our DNA extraction protocol from spiked mycobacteria in MWF is performed in a single tube, minimizing DNA loss, and thus increasing efficiency. This approach could be used on a routine basis for MWF quality control procedures. The very good sensitivity suggests that very little inhibition of the PCR occurs, and the extracted DNA is pure. However, there might be internal error in our protocol in the concentration evaluation of the stock suspension used for the spiked fluids. Mycobacteria are more difficult to quantify due to their tendency to clump together, and they are not regularly shaped. The minor lack of precision in the estimation of the spiked cells influences the recovery rate when compared to the initial number of genomes, but the comparison of extraction protocols is adequate because the same dilutions were used for each approach.

The generated standard curves using plasmidic DNA with the *M. immunogenum* insert allowed the achievement of an accurate quantification of *M. immunogenum* in a wide range of concentrations from ten copies of the bacteria/ml of MWF to concentrations higher than 1×10^7 bacteria/ml.

We demonstrated that the efficiency of the cell lysis and DNA extraction procedure is a very important step to accurately estimate *M. immunogenum* concentration present in a MWF sample. Cell lysis/DNA extraction did not reach 100 % efficiency because of possible errors in microscopic quantification of the stock suspension, and the use of multiple steps in the extraction protocol required for the waxy and very resistant cell wall of mycobacteria, preventing the access to genomic DNA. Our paper proposes a new approach for *Mycobacterium* quantification and shows the importance of having a complete lysis and extraction/purification protocol. The application of the proposed protocol is now being applied in field samples.

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