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Development of a species-specific colorimetric-PCR assay for detection and species differentiation of *Mycobacterium immunogenum* and *Mycobacterium chelonae* and its comparison with quantitative real-time PCR for field metalworking fluids

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ABSTRACT

Mycobacterium immunogenum and *Mycobacterium chelonae* are closely related species associated with occupational hypersensitivity pneumonitis (HP) and nosocomial infections. There is a need to develop specific and readily adaptable methods for detection and speciation of these agents. Here we report development of a probe-based colorimetric-PCR assay involving heat shock protein (*hsp*) gene amplification (228bp) and its detection in an ELISA-like reaction. A quantitative format of this assay was developed and validated on metalworking fluids (MWF). The assay showed a minimum detection limit of 10 fg genomic DNA or 1 mycobacterial cell, albeit with variations depending on type and composition of the MWF matrix. When applied to the field MWF samples, the developed assay was found to be comparable to the real-time PCR assay, and allowed direct speciation of MWF mycobacteria without sequencing and/or restriction pattern analysis. In conclusion, the developed colorimetric PCR allows detection and quantification of MWF mycobacteria without culturing and is the first probe-based assay for unambiguous differentiation between the two phylogenetically closely related species, *M. immunogenum* and *M. chelonae*. Considering that the assay offers high throughput format involving relatively simpler instrument infrastructure, it has a potential for applications in routine assessment of MWF mycobacteria in diagnostic and industrial laboratories.

1. Introduction

Nontuberculous mycobacteria (NTM) are ubiquitously present in the environment and have emerged as one of the major causatives of occupational and recreational respiratory illnesses and nosocomial infections [1]. Particularly, the rapidly growing NTM species of the *Mycobacterium chelonae* complex (MCC) namely, *Mycobacterium immunogenum and M. chelonae* have been shown to possess the unique ability to colonize modern water-based metalworking fluids (MWFs) used in machining operations for cooling and lubrication [2,3]; the two species have been implicated in hypersensitivity pneumonitis and other respiratory illnesses in machine workers occupationally exposed to MWFs and their aerosols [3–6]. These NTM species are also prevalent in hospital water supplies and equipment [7–9] and are known to cause nosocomial infections or pseudo outbreaks in hospital environments [7,8,10–12]. Potential human health significance of these species [1] has made it important to rapidly and unambiguously identify these closely related species for effective exposure assessment and intervention in occupational exposures and for epidemiologic surveillance.

The conventional culture-based and phenotypic methods that distinguish the two species of MWF mycobacteria include tests such as citrate utilization and high performance liquid chromatography (HPLC) of mycolic acids [10]. These tests are time consuming and/or require special equipment and expertise and are not highly specific. Hence there has been an increasing interest in developing rapid DNA-based methods for detection and speciation of these agents. In this direction, we have recently developed DNA amplification assays, based on both end product PCR [13–15] and real-time PCR [16,17], for detection and/or differentiation of these *Mycobacterium* species in MWF. However, the existing PCR-based assays for these species involve cumbersome downstream confirmation of the PCR signal

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such as by gel electrophoresis combined with restriction digestion using multiple enzymes [14,15] or by ambiguous melting curve analysis [18] using advanced real-time thermal cycler with a fluorescence detector [16,17]. Hence, there is a need to develop a simpler, more specific, and direct PCR assay format for species differentiation, such as the one involving species-specific probes in a simple downstream processing strategy consistent with the skills routinely prevalent in low-tech diagnostic or analytical and in-house laboratories. Here we report the development of a simple colorimetric-PCR assay involving a PCR amplification step based on the genus-specific heat shock protein (*hsp*) gene target combined with the use of a species-specific probe for an ELISA-like colorimetric detection of the PCR amplicon.

The specific aims of this study were (i) to develop a species-specific colorimetric-PCR assay for speciation or differentiation of *M. immunogenum* and *M. chelonae* and (ii) to develop and compare a genus-specific quantitative colorimetric-PCR assay with real-time PCR assay for quantification of these agents in industrial metalworking fluid (MWF). This is the first report on a species-specific probe-based PCR assay for speciation and/or differentiation of the two closely related species of MCC, namely *M. immunogenum* and *M. chelonae*. Considering the high throughput format and relatively less infrastructure-intensive nature of the assay, it could be particularly adaptable for routine sample screening and assessment applications in in-house industrial laboratory settings and low-tech diagnostic laboratories.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The mycobacterial strains used in this study were obtained from either the American Type Culture Collection (ATCC) or our own collection of isolates. *M. immunogenum* ATCC 700506 and *M. chelonae* ATCC 35752 were used as the test strains. In order to confirm the species-specificity of the assay, representative multiple genotypes of either test species, 4 each for *M. immunogenum* (MJY-3, 4, 12, and 13) and *M. chelonae* (MJY-1, 2, 6, and 11), were used; these genotypes were isolated in our previous study [2] from field MWF samples collected from industrial plant operations located in geographically diverse regions in North America. Additionally, the remaining member species of the MCC complex, namely *Mycobacterium abscessus* (ATCC 19977 and ATCC 23006) and the reference strains and isolates of other rapidly growing NTM species, namely *Mycobacterium fortuitum* ATCC 6841, *Mycobacterium mageritense* ATCC 700351, *Mycobacterium septicum* ATCC 700731, *Mycobacterium senegalense* ATCC 35796, *Mycobacterium smegmatis* ATCC 19420, *Mycobacterium peregrinum* ATCC 14467, *Mycobacterium phlei* ATCC 11758 and *Mycobacterium wolinskyi* ATCC 700010 were included in the study to determine cross-reactivity. The cultures were grown in Middlebrook 7H9 broth supplemented with 10% OADC (Oleic acid Albumin Dextrose Catalase) (BD Biosciences, Sparks, MD) and Tween 80 (0.05%) with continuous shaking (200 rpm) at 37 °C.

2.2. Metalworking fluids

Pristine (fresh, with no contamination) samples of synthetic and semi-synthetic metalworking fluids were obtained from an industrial source. The pristine synthetic and semi-synthetic fluids were diluted to a commonly practiced working concentration of 5% (v/v) and 2% (v/v), respectively, and filter sterilized (0.2 μm cellulose nitrate membrane) for use as test matrices. In addition, field samples (also designated as 'in-use' samples) of both synthetic and semi-synthetic MWF containing natural mixed microflora collected from diverse field MWF operations by the above sample source, were used.

2.3. Extraction of DNA

DNA was extracted from the mycobacterial strains using the Bactozol-based cell lysis method [16], with additional modifications. Briefly, 1 ml of the culture grown to 120 Klett reading ($\sim 10^8$ cells ml^{-1}) was centrifuged at 12,000 rpm for 20 min and the cell pellet was subjected to DNA extraction. The extraction protocol involved a cell disruption pre-step based on bead beating, using 0.1 mm glass beads (BioSpec Products, Bartlesville, OK), for 3 min, after bactozyme treatment of the cells. Following the proteinase K and DNAzol treatment, the free (unbound) DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The bead-bound DNA removed by washing the beads 5 times with 200 μl each of TE buffer (pH 8.0) was similarly extracted. The free and bead-bound DNA fractions were pooled together and precipitated with glycogen (final concentration of 1 μg/μl) (Fermentas, Glen Burnie, MD) and one volume of isopropyl alcohol at −70 °C for 30 min. The DNA pellet was dissolved in 5 μl of sterile water (Sigma Aldrich, St Louis, MO) and stored at −20 °C.

2.4. Assay development

2.4.1. Design of primers and capture probes

Genus-specific primers designed based on a 228 bp variable region of heat shock protein gene (*hsp*) of mycobacteria [19] were used for the initial amplification step. Of these, the forward primer was synthesized with a fluorescein label at the 5′ end (hsp I – F 5′/5 Fluor/CT GGT CAA GGA AGG TCT GCG 3′) whereas the reverse primer was kept unlabeled (hsp I – R 5′ GAT GAC ACC CTC GTT GCC AAC 3′). A common genus-specific capture probe able to hybridize to the 228 bp amplified product of both *M. immunogenum* and *M. chelonae* was designed based on a conserved region of the *hsp*228 gene sequence in these two MWF-prevalent mycobacterial species. The species-specific capture probes for the two species were designed based on the variable region of the 228 bp *hsp* sequence (Table 1). Each of the probes was synthesized with a biotin label at the 5′ end.

2.4.2. PCR amplification

The PCR amplification was performed in a final reaction volume of 50 μl containing 1× PCR Buffer (Takara Bio Inc., Japan), 200 μM each of the dNTPs, 40 ng of each primer, 1.25 U of Ex Taq DNA polymerase (Takara Bio Inc., Japan) and 2.5 μl of the template DNA. The amplification conditions were as follows: denaturation at 94 °C for 5 min followed by 35 cycles each at 94 °C for 15 s, 58 °C for 15 s and 72 °C for 30 s and a final extension cycle at 72 °C for 5 min.

2.4.3. Capture probe hybridization and amplicon detection

The 5′ biotinylated probes were immobilized in the streptavidin-coated wells of a microtiter plate (Pierce, Rockford, IL) using a published protocol [20] with modifications. Briefly, the wells were

Table 1
Capture probes based on the mycobacterial *hsp* gene.

Probe type	Base position[a]	Oligo Sequence (5′–3′)[b]
Genus-specific	425–400	TCTGCTCCTTGGTGTCGATCTCCTTG
Species-specific		
M. immunogenum-specific	385–366	GGCACTGGTAACAGCCTCCA
M. chelonae-specific	385–366	AGAGCTGGTGACGGCCTCCA

[a] Base numbering corresponds to that in the *hsp* gene sequence (Genbank accession no. AY498741) for *M. immunogenum* [2].
[b] The oligo sequences represent the non-coding strand (antisense sequence).

washed (3× 200 μl) with phosphate-buffered saline containing 0.01% Tween 20 (PBST). One hundred microliter volume of PBST containing 0.25 μM of probe was added to each well followed by incubation at 37 °C for 30 min. The coated plate was washed three times with PBST as above and cooled on ice. The PCR product was denatured by heating at 99 °C for 10 min followed by chilling on ice. An aliquot (10 μl) of the denatured PCR product was mixed with 90 μl of an ice-cold hybridization buffer [5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% BSA, 0.3% Tween 20] and added to the test well. The plate was incubated at 58 °C for 30 min. The wells were then washed twice with wash buffer (2× SSC, 0.1% SDS) and once with PBST. Hybridization of the fluorescein-labeled PCR product to the capture probe was detected colorimetrically with an anti-fluorescein horseradish peroxidase conjugate (Invitrogen, Eugene, Oregon) and o-phenylenediamine (OPD) substrate (Sigma Aldrich, St Louis, MO). The conjugate was diluted (1 in 1000) in hybridization buffer and 100 μl of the diluted conjugate was added to each well. The plate was incubated at 37 °C for 30 min and washed twice with wash buffer and once with PBST. A 100 μl volume of OPD solution per well was added, and the yellow color was allowed to develop for 15 min. The reaction was stopped by adding 50 μl of 3 M HCl, and optical density (OD) (490 nm) was measured with a microtiter plate reader (Wallac Victor 2, PerkinElmer, Waltham, MA). All the PCR-hybridization assays were performed using a positive control (*M. immunogenum* ATCC 700506 DNA) and a negative (no template) control.

2.5. Evaluation of the developed colorimetric-PCR assay on MWF

The effect of MWF matrix on the performance of the developed colorimetric-PCR assay was determined by using MWF samples spiked with test mycobacteria species. The pristine (no background flora) synthetic and semi-synthetic MWF samples were spiked with serial dilutions of *M. immunogenum* and *M. chelonae* cultures (120 Klett; ~10^8 cells ml^{-1}) to achieve a final concentration of 10^6–10^0 cells ml^{-1}. Likewise, in order to assess the potential interfering effect of the co-occurring background microflora on the quantification efficiency, the colorimetric-PCR assay was evaluated using background-simulated MWF. The samples were prepared by spiking the pristine MWF matrix with defined background microflora comprising of 10^6 cells each of *Pseudomonas fluorescens* ATCC 13525 and *Bacillus* sp., the most common Gram-negative and Gram-positive species, respectively found in contaminated MWF, along with increasing number (10^0–10^6) of *M. immunogenum* cells. The counts (cells ml^{-1}) detected were calculated from the OD values using the standard curve generated by performing the assay on DNA extracted from the increasing known number of *M. immunogenum* cells.

2.6. Comparison of the developed colorimetric-PCR with real-time PCR, culturing, and microscopy on field MWF samples

The developed colorimetric-PCR assay and the real-time PCR assay protocol optimized earlier in our laboratory [16] were compared using field samples of synthetic and semi-synthetic 'in-use' MWF containing a natural mixed background microflora (total count 10^8–10^9 cells ml^{-1}). The developed assay used the common capture probe for detection and quantification and species-specific probes for speciation of MWF mycobacteria in this evaluation. Cultural analysis for mycobacterial assessment in the samples was performed using Middlebrook 7H10 (MB7H10) agar supplemented with 10% OADC (BD Biosciences, Sparks, MD). Microscopy using Modified Auramine-O stain set (Scientific Device Lab, Des Plaines, IL), originally meant for clinical mycobacterial detection, was applied for assessment of total mycobacteria in the MWF samples.

2.7. Statistical analysis

Cell counts (cells ml^{-1}) of *M. immunogenum* and *M. chelonae* detected in synthetic and semi-synthetic MWF samples were compared by analysis of variance by mixed model with SAS software version 9.1 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Assay development and specificity

A colorimetric-PCR assay was developed for *M. immunogenum* and *M. chelonae* based on the heat shock protein (*hsp*) gene amplification coupled with downstream detection of the amplicon using a colorimetric reaction strategy. Among the genomic regions most often targeted for identification of individual species of mycobacteria, the *hsp* gene, has shown more polymorphism than the 16S rRNA gene [10] and thus has proved to be a more useful target for the species differentiation assays [21,22]. A schematic representation of the ELISA-like detection strategy is given in Fig. 1. This strategy for amplicon detection combined the principles of enzyme-linked immunosorbent assay (ELISA) and enzyme-linked oligonucleotide-sorbent assay (ELOSA). Briefly, the amplicon is immobilized to the microtiter wells via streptavidin-biotin interaction of the biotin-labeled oligonucleotide probe. The free fluorescein-labeled end of the amplicon forms the basis for detection by colorimetric reaction with anti-fluorescein HRP conjugate.

The assay development strategy involved the use of a genus-specific PCR primer pair based on the variable 228 bp region of the *hsp* gene for the initial amplification reaction. A common capture probe was designed and utilized in the colorimetric detection step to detect total MWF mycobacteria, belonging to either or both of the MWF species, *M. immunogenum* and *M. chelonae*. Subsequently, a species-specific format of the colorimetric-PCR assay for either

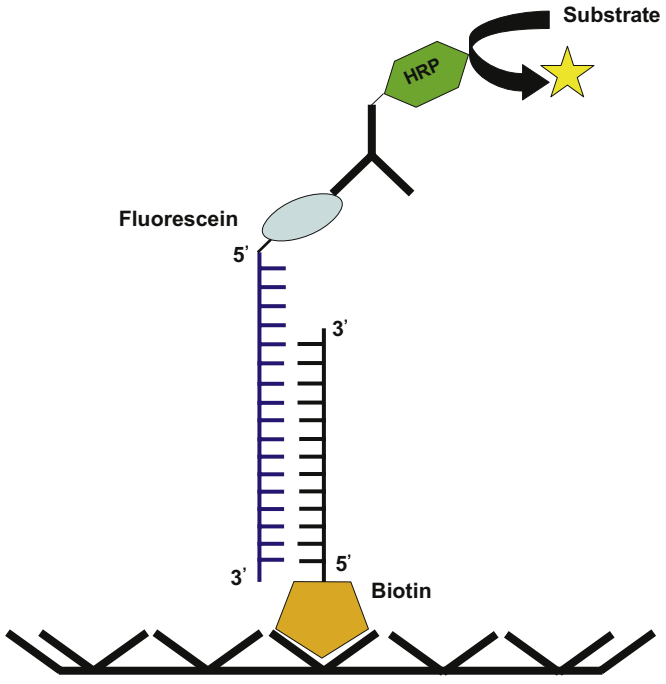

Fig. 1. Schematic representation of the developed ELISA-like colorimetric strategy for the downstream detection of PCR amplicon. The denatured amplicon is hybridized to the biotin-labeled probe immobilized to the streptavidin-coated microtiter wells. Subsequent to the hybridization reaction, the 5′ fluorescein label on the PCR amplicon is reacted with anti-fluorescein HRP conjugate and the substrate (o-phenylenediamine) for the colorimetric detection and quantification.

species was standardized by designing and using the species-specific capture probes. Specificity of the species-specific probes was tested against other closely related and unrelated environmental and clinical NTM species and genotypes. The *M. immunogenum*-specific probe and the *M. chelonae*-specific probe hybridized only with their respective genotypes. The two species-specific probes did not show cross hybridization with each other and with any of the other test NTM species, including *M. abscessus*, *M. fortuitum*, *M. mageritense*, *M. peregrinum*, *M. phlei*, *M. senegalense*, *M. septicum*, *M. smegmatis* and *M. wolinskyi* (Table 2).

3.2. Sensitivity and minimal detection limit of the developed quantitative colorimetric-PCR assay

A quantitative format of the colorimetric-PCR assay was developed based on a common capture probe because it was able to detect both species (*M. immunogenum* and *M. chelonae*) of mycobacteria prevalent in metalworking fluids thereby allowing assessment of total mycobacteria load in a sample. Quantification efficiency was determined based on the use of the template DNA as well as the whole cells as the starting material, using *M. immunogenum* as the test species. The quantitative colorimetric-PCR assay could detect as low as 10 fg of the chromosomal DNA (Fig. 2B). Comparison of the results with those obtained by the conventional agarose gel electrophoresis analysis of the same samples is presented in Fig. 2A. For whole cell-based quantification, a standard curve ($r^2 = 0.99$) generated using DNA extracted from increasing numbers of *M. immunogenum* (10^0–10^6 cells) is presented in Fig. 2C. The OD values lower than 0.225 were considered negative. This cut-off value was the mean of negative controls plus 3 times the standard deviation. The OD value of the reaction performed for the lowest cell number (1 cell) was above this value indicating a minimal detection limit of 1 cell.

3.3. Effect of MWF sample matrix

The two species of MWF mycobacteria, *M. immunogenum* and *M. chelonae*, were detectable in the simulated MWF matrices spiked at varying levels (10^6–10^1 cells ml^{-1}), at the expected efficiency within experimental limits. Nevertheless, for the same spiked initial count, *M. chelonae* yielded relatively higher deduced counts as compared to *M. immunogenum*, in both the test matrices (Fig. 3A and B) although the difference in counts was not statistically significant (p value > 0.05). Between the two matrices, the semi-synthetic fluid matrix yielded higher deduced counts for both the test organisms as compared to the synthetic MWF matrix but the difference was not statistically significant (p value > 0.05). As low as 10 cells of either species (*M. immunogenum* or *M. chelonae*) were detectable in the pristine synthetic and semi-synthetic MWF matrices. However, in the presence of spiked background microflora (constituted of 10^6 cells ml^{-1} each of *P. fluorescens* and *Bacillus* sp.) *M. immunogenum* detection limit was lowered to 100 cells ml^{-1} in the low-count (100 cells to 1 cell ml^{-1}) samples (Fig. 4).

3.4. Comparison of the developed colorimetric PCR with the real-time PCR on field MWF samples

Comparison of the mycobacterium-specific colorimetric-PCR and real-time PCR assays showed that the field MWF samples contained mycobacteria in log range of 2–6 (Table 3). The detection efficiency observed with the developed colorimetric assay was comparable to that of the real-time PCR assay. In addition, the species-specific colorimetric PCR allowed the culture-independent speciation of mycobacteria. Interestingly, it showed the presence of *M. immunogenum* in all the samples (Table 3), drawn from geographically diverse industrial plants, implying the frequent occurrence of this species in the field MWF operations. Cultural analysis of the fluid samples yielded a mycobacterial isolate that was independently identified as *M. immunogenum* (data not shown) using our previously developed DNA-based assay [14,15]. While the cultural analysis yielded no mycobacterial count in 11 of the 12 field MWF samples, microscopy showed presence of high mycobacterial counts (Table 3) as observed in case of PCR analyses in these samples.

4. Discussion and conclusions

4.1. Species differentiation

In this study, we developed a colorimetric-PCR assay for detection and speciation of MWF mycobacteria based on the use of

Table 2
Evaluation of the species-specific probes for cross-reactivity with other closely related species of nontuberculous mycobacteria using the developed colorimetric-PCR assay.

Mycobacterial species	Genus-specific amplification step (*hsp*228 amplicon)	Species-specific hybridization step	
		Mi-specific probe[a]	Mc-specific probe[a]
M. immunogenum ATCC 700506	+	+	−
MJY-3	+	+	−
MJY-4	+	+	−
MJY-12	+	+	−
MJY-13	+	+	−
M. chelonae ATCC 35752	+	−	+
MJY-1	+	−	+
MJY-2	+	−	+
MJY-6	+	−	+
MJY-11	+	−	+
M. abscessus ATCC 19977	+	−	−
M. abscessus ATCC 23006	+	−	−
M. fortuitum ATCC 6841	+	−	−
M. mageritense ATCC 700351	+	−	−
M. septicum ATCC 700731	+	−	−
M. senegalense ATCC 35796	+	−	−
M. smegmatis ATCC 19420	+	−	−
M. peregrinum ATCC 14467	+	−	−
M. phlei ATCC 11758	+	−	−
M. wolinskyi ATCC 700010	+	−	−

+: Positive amplification or hybridization reaction.
−: Negative hybridization reaction.
[a] Mi = *M. immunogenum*; Mc = *M. chelonae*.

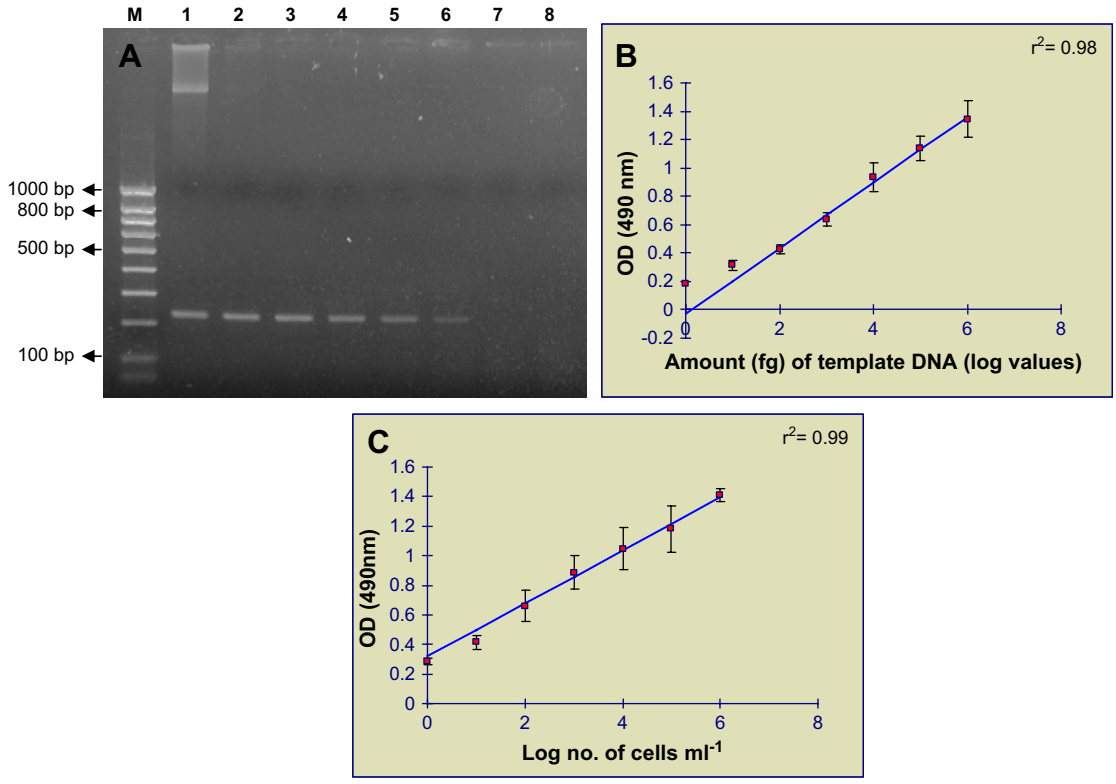

Fig. 2. Detection limit of the developed colorimetric-PCR assay in comparison with the conventional PCR-gel electrophoresis assay for *M. immunogenum* using DNA extract or whole cell suspension. Ten-fold serial dilutions of *M. immunogenum* DNA were subjected to PCR; the resulting products were analyzed by (A) agarose gel electrophoresis and (B) colorimetric ELISA-like assay (plot of optical density versus amount of template DNA). The amplicons were derived from the following varying amounts of template DNA: 1000 pg (lane 2), 100 pg (lane 3), 10 pg (lane 4), 1000 fg (lane 5), 100 fg (lane 6), 10 fg (lane 7). Lane 1 (positive control), lane 8 (no template control) and lane M (100 bp DNA ladder) were used for comparison. (C) Ten-fold serial dilutions of *M. immunogenum* cell suspension (10^6–1 cell ml^{-1}) were subjected to DNA extraction followed by PCR and the amplicon analyzed by the developed colorimetric assay. The OD (490 nm) was plotted against the number of cells. This plot based on known number of cells served as the standard curve for quantification of the simulated and field MWF samples in subsequent experiments.

newly designed genus- and species-specific probes. The probes were designed such that the sequence mismatches were present somewhere in the middle region of the oligonucleotide probe. In this context, it has been shown earlier that mismatches in the center of the sequence are more likely to confer specificity to the probe [23]. Although to date, only these two species of mycobacteria have been reported to have the ability to colonize metal-working fluids, the probes were still validated for specificity against other phylogenetically-related environmental and clinical species of rapidly growing mycobacteria (RGM); the probes showed no cross-reactivity with other species and were found to be highly specific to the respective MWF-associated mycobacterial species (*M. immunogenum* and *M. chelonae*) when tested on multiple genotypes. Although the specific probes were developed for colorimetric-PCR assay in this study, they have the potential for broader applications in other assay platforms such as Taqman real-time PCR [24].

4.2. Minimum detection limit

Sensitivity of detection of the developed colorimetric-PCR assay was better than that of the conventional PCR-agarose gel electrophoresis analysis. The latter had a detection limit of 100 fg of genomic DNA, while the colorimetric-PCR assay could detect up to 10 fg of genomic DNA, both derived from *M. immunogenum*. Hence, sensitivity of detection by probe hybridization-based assay was 10 times higher than what could be achieved by agarose gel electrophoresis-based assay. This is consistent with the reported [25,26] minimal detection limit of 10–125 fg for similar assays for slow-growing mycobacteria (*Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium leprae*). A detection limit of 10 fg for mycobacterial DNA is equivalent to 1 bacterial cell, given that genome size of mycobacterial species ranges from 3.3×10^6 bp to 7×10^6 bp [27,28]. A similar ideal detection limit (equivalent to 1 cell) was obtained for the developed colorimetric-PCR assay when we actually tested increasing amounts of DNA extracted from serially diluted cells.

4.3. Quantification of mycobacteria in MWF

A logarithm-linear relationship between the amount of template DNA and the PCR product optical density (OD) in the colorimetric assay suggested that the assay could be used for quantitative determination of the mycobacterial load in MWF. In this context, our results showed that the assay could detect up to 10 cells of the target organism in pristine synthetic and semi-synthetic MWF samples and up to 100 cells of the target organism in the MWF matrix containing the background microflora consisting of a mixture of the common Gram-negative (*Pseudomonas* sp.) and Gram-positive (*Bacillus* sp.) bacteria. The assay showed greater sensitivity with pure DNA template of *M. immunogenum* as compared to the mixed DNA template derived from *M. immunogenum* and mixed bacterial population. This difference in the detection limit could be attributed to the cluttering effect of co-occurring DNA of the other Gram-positive and Gram-negative organisms. Although the minimal detection limit of the test species

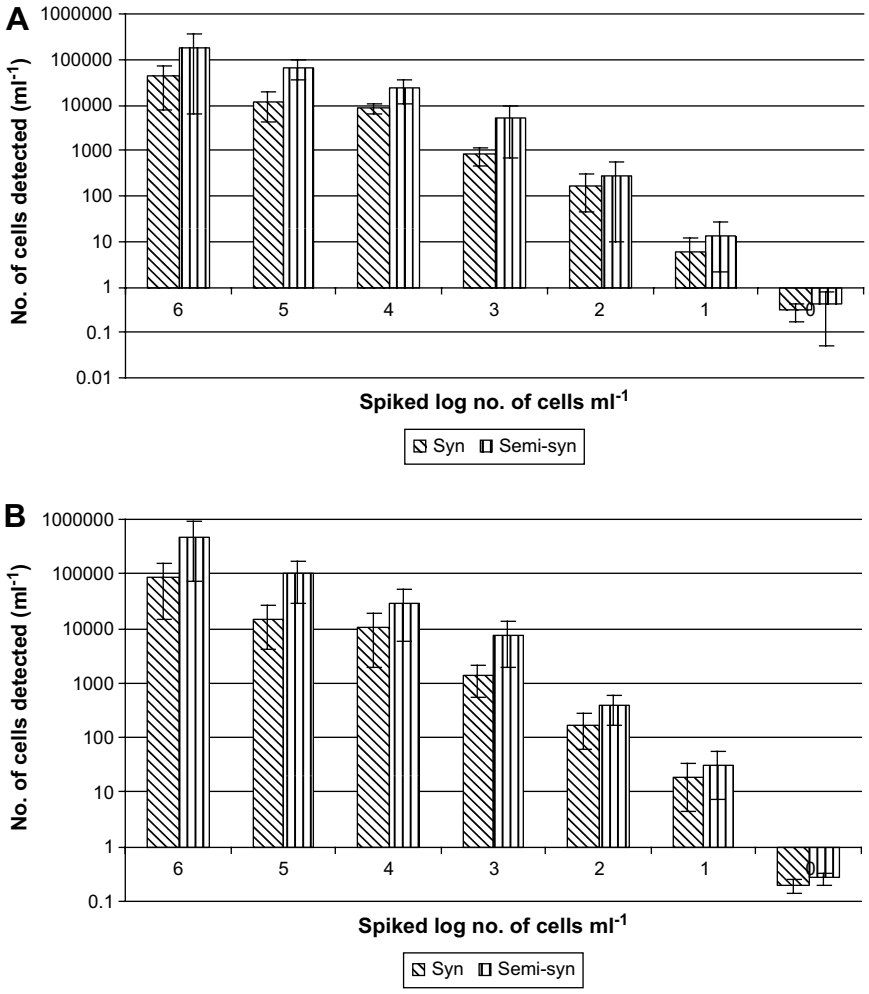

Fig. 3. Effect of MWF matrix and type on the quantification limit of the developed species-specific colorimetric-PCR assay to assess (A) *M. immunogenum* and (B) *M. chelonae* in pristine synthetic and semi-synthetic MWF samples.

(based on the initial numbers) was the same in both synthetic and semi-synthetic MWF samples, the actual minimal quantification levels were found to be higher in the latter as compared to the former sample matrix. This difference could be due to the intrinsic difference in the composition of the two types of metalworking fluids.

A major limiting step in the PCR-based detection of microorganisms in complex matrices is the extraction of amplifiable quality

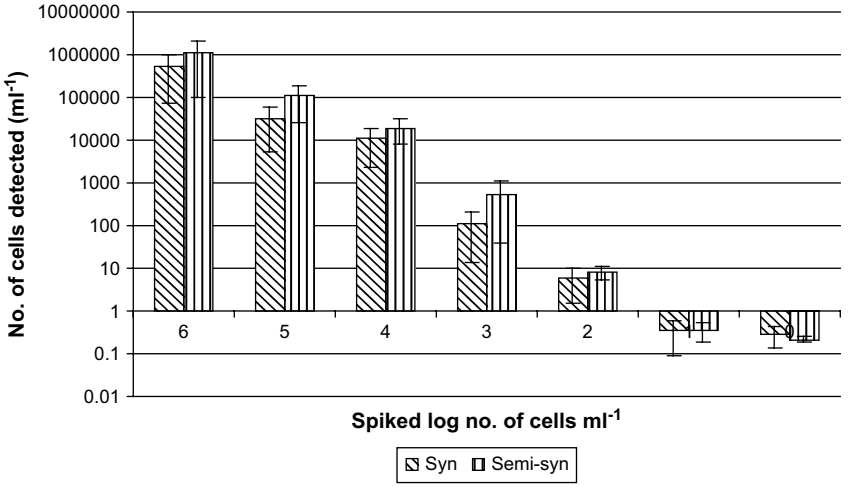

Fig. 4. Effect of background microflora on the quantification limit of the developed species-specific colorimetric-PCR assay to assess *M. immunogenum* in synthetic and semi-synthetic MWF. The pristine MWF samples of either type were spiked to simulate background microflora comprising of *P. fluorescens* (10^6 cells ml^{-1}) and *Bacillus* sp. (10^6 cells ml^{-1}).

Table 3
Evaluation of colorimetric-PCR assay on field MWF samples for quantification and speciation of mycobacteria and comparison with quantitative Real-time PCR assay.

MWF Sample ID	Quantification (Mycobacterial counts ml^{-1})[a]				Speciation	
					Colorimetric PCR	
	Microscopy[b]	Culturing method[c]	Real-time PCR assay	Colorimetric-PCR assay	Mi-specific	Mc-specific
Synthetic						
S19	$10.8 (\pm 1.94) \times 10^3$	0	$2.49 (\pm 0.65) \times 10^3$	$6.06 (\pm 1.55) \times 10^3$	+	−
S21	$4.90 (\pm 3.83) \times 10^3$	0	$2.35 (0.09) \times 10^2$	$5.42 (\pm 3.46) \times 10^2$	+	−
S22	$9.40 (\pm 3.38) \times 10^3$	0	$1.06 (\pm 0.08) \times 10^2$	$4.42 (\pm 2.70) \times 10^2$	+	−
S23	$18.4 (\pm 3.68) \times 10^3$	0	$8.04 (\pm 2.0) \times 10^3$	$1.17 (\pm 0.46) \times 10^3$	+	−
S24	$8.93 (\pm 2.62) \times 10^4$	0	$7.19 (\pm 3.07) \times 10^2$	$5.75 (\pm 1.97) \times 10^2$	+	−
S26	$5.92 (\pm 2.76) \times 10^4$	0	$2.23 (\pm 0.93) \times 10^3$	$4.17 (\pm 3.28) \times 10^3$	+	−
706170	$2.63 (\pm 0.65) \times 10^6$	$4.60 (\pm 0.14) \times 10^3$	$4.71 (\pm 0.54) \times 10^5$	$6.56 (\pm 3.89) \times 10^5$	+	−
Semi-synthetic						
SS10	$3.15 (\pm 2.16) \times 10^5$	0	$9.84 (\pm 0.33) \times 10^4$	$3.98 (\pm 2.36) \times 10^4$	+	−
SS11	$1.84 (\pm 1.31) \times 10^7$	0	$1.68 (\pm 0.09) \times 10^6$	$1.74 (\pm 1.52) \times 10^6$	+	−
SS12	$5.89 (\pm 2.91) \times 10^6$	0	$8.72 (\pm 0.03) \times 10^5$	$1.95 (\pm 0.65) \times 10^5$	+	−
SS13	$11.7 (\pm 4.34) \times 10^3$	0	$2.19 (\pm 0.92) \times 10^3$	$4.71 (\pm 1.67) \times 10^3$	+	−
SS20	$5.21 (\pm 3.18) \times 10^4$	0	$5.06 (\pm 0.46) \times 10^3$	$2.27 (\pm 1.18) \times 10^3$	+	−

+: Positive hybridization signal in the colorimetric-PCR assay.
−: Negative hybridization signal in the colorimetric-PCR assay.
[a] Values presented are the mean (±SD) of triplicates.
[b] Modified Auramine-O Stain Set (Scientific Device Lab, Des Plaines, IL) was used for microscopic counting.
[c] Middlebrook 7H10 (M7H10) agar supplemented with 10% OADC (BD Biosciences, Sparks, MD) was used for the mycobacterial culturable counts.

of genomic DNA. The sensitivity of a PCR-based method for direct detection (without culturing) of MWF mycobacteria is therefore limited by the efficiency of DNA extraction protocol. Due to the complexity and rigidity of the mycobacterial cell wall and the nature of MWF matrix, several efforts have been reported for the isolation of mycobacterial DNA by using physical, chemical, or enzymatic strategies or combinations of these methods. The Bactozol-mediated cell lysis method previously optimized in our laboratory [15] for the extraction of DNA from mycobacteria in MWF was further modified in this study by inclusion of bead beating as an additional step for cell lysis and by use of glycogen for efficient precipitation of small quantities of DNA, particularly for low-count samples (100 cells ml^{-1} to 1 cell ml^{-1}). These modifications improved efficiency of the DNA extraction protocol leading to about 1.5-folds higher yield of the genomic DNA.

When applied to 12 field ('in-use') MWF samples, the developed colorimetric-PCR assay yielded mycobacterial counts comparable to those detected by real-time PCR. The PCR assays allowed assessment of the total mycobacterial load (culturable and non-culturable) as against the conventional mycobacterial culturing method (MB7H10 agar) that yields only culturable count. Since 11 of the 12 samples showed no culturable counts and the developed PCR assay showed high total mycobacterial counts (4.42×10^2 to 1.74×10^6 cells ml^{-1}), majority of the population in these samples was considered non-culturable. Presence of non-culturable mycobacteria in these samples was independently corroborated by the fact that microscopy (Modified Auramine-O staining) showed mycobacterial counts (4.9×10^3 to 1.84×10^7 cells ml^{-1}) comparable to the range observed in the PCR assay; somewhat higher absolute values of microscopy count could be due to the relatively less-specific and semi-quantitative nature of the microscopy technique used in this analysis. Occurrence of high non-culturable population of mycobacteria in field metalworking fluids observed in this study is consistent with the previous studies by us and others [13,16,29] and may be ascribed to the harsh conditions and biocides prevalent in these fluids. In this context, it has been well recognized that both culturable and non-culturable microbial populations are equally significant in contributing to the antigen load in MWF associated with immune-mediated respiratory diseases such as hypersensitivity pneumonitis in exposed machine workers [30,31].

In addition, it allowed definitive direct speciation of mycobacteria in these samples that corroborated with our independent species identification outcome on a cultural isolate from these samples. Moreover, the colorimetric-PCR assay is not as skill-intensive and requires simpler instrument infrastructure as compared to the real-time PCR assay. Thus the developed colorimetric-PCR assay provides a readily adaptable alternative for the culture-independent and specific detection and species differentiation of *M. immunogenum* and *M. chelonae*, particularly in low-tech or in-house analytical and diagnostic laboratories.

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