RESEARCH ARTICLE

Expanding the mycobacterial diversity of metalworking fluids (MWFs): evidence showing MWF colonization by *Mycobacterium abscessus*

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Abstract

Nontuberculous mycobacteria (NTM) have been associated with hypersensitivity pneumonitis in machinists. Only two species of NTM, namely *Mycobacterium immunogenum* and *Mycobacterium chelonae*, have been reported thus far to have the ability to colonize contaminated metalworking fluids (MWFs). Here, we report, for the first time, the presence and characterization (phenotypic and genotypic) of a third species, *Mycobacterium abscessus*, colonizing these harsh alkaline machining fluids. Two *Mycobacterium* morphotypes, smooth (S) and rough (R), were isolated (two isolates each) from an in-use industrial MWFs. Biocide susceptibility analysis using triclosan as a model yielded the same minimal inhibitory concentration for the two morphotypes. PCR-restriction analysis –based speciation of the morphotypes confirmed their identity as *M. abscessus*. Genotyping based on partial DNA sequences corresponding to the variable regions of the *hsp65* gene and 16S-23S rRNA operon internal transcribed spacer region and randomly amplified polymorphic DNA-PCR analysis showed that both morphotypes belong to a single genotype. In addition, we isolated and confirmed two novel mycobacterial genotypes, one each of *M. immunogenum* and *M. chelonae* from additional in-use MWF screening. Taken together, this study expands the known mycobacterial species- and strain-diversity colonizing MWF. Furthermore, the study emphasizes the need for including *M. abscessus* species in the existing mycobacterial screening of contaminated MWF.

Introduction

Nontuberculous mycobacteria (NTM) comprising of slow-growing and rapidly growing species are ubiquitously distributed in the environment, such as in water, soil, and aerosols (Falkinham, 2002). In the past two decades, rapidly growing mycobacteria (RGM) have also been isolated from metalworking fluids (MWFs) and their aerosols. Conventionally, these fluids have been considered to support microbial growth because of their prolonged circulation and maintenance in well agitated, aerated and warm temperature in an open system (Bennette & Wheeler, 1954; Rossmoore & Holtzman, 1974). In addition, modern water-based MWFs are believed to be particularly amenable to colonization by mycobacteria owing to the repeated biocide additions and prevalence of selective harsh microenvironment. MWF-associated mycobacteria have been implicated in hypersensitivity pneumonitis (HP) and asthma in the workers exposed to the contaminated MWFs in the occupational machining environment (Muilenberg *et al.*, 1993; Shelton *et al.*, 1999; Trout *et al.*, 2003; Beckett *et al.*, 2005; Gupta & Rosenman, 2006; Suuronen *et al.*, 2007; Rosenman, 2009). According to the National Institute for Occupational Safety and Health (NIOSH), more than 1 million US machine workers are exposed to these fluids in the workplace environment (CDC, 1998).

In terms of speciation of the MWF-colonizing mycobacteria, isolates from HP-associated MWF in four of the eight automotive plants were broadly identified as those belonging to *Mycobacterium chelonae* complex or *Mycobacterium fortuitum/chelonae* complex or atypical mycobacteria group (Kreiss & Cox-Ganser, 1997). These identifications were, however, based on conventional

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phenotypic methods owing to the lack of molecular identification methods at that time (Khan *et al.*, 2005a, b). Later, a molecular analysis-based study in the year 2000 on mycobacterial isolates from an industrial plant with a recent HP outbreak revealed the prevalence of a single mycobacterial species in these fluids; it was designated as *Mycobacterium immunogenum* for its association with the immune-mediated disease HP (Moore *et al.*, 2000). Subsequently, in the largest reported outbreak of work-related respiratory illnesses in machining environment in the United States that occurred during October 2000–April 2001 in Ohio (CDC, 2002), analyses of the plant's MWF samples revealed predominant growth of the same species (*M. immunogenum*). Further molecular analysis of several isolates from diverse MWF samples reported the predominance of a single genotype of this species in the contaminated MWF (Wallace *et al.*, 2002), leading to the assumption that this is the predominant, if not the only, mycobacterial species that is capable of colonizing the MWF. However, the recent detailed analysis performed in our laboratory on MWF samples collected from different industrial plants located in diverse geographic regions in North America revealed the prevalence of two species, *M. immunogenum* and *M. chelonae*, in the contaminated MWF (Khan *et al.*, 2005a, b). Both these species belong to the *M. chelonae* complex (MCC). Here, we provide the evidence on the presence of another species, *Mycobacterium abscessus*, in MWF based on its phenotypic and genotypic characterization. This is the first report on the occurrence of this species in MWFs, in contrast to previous assumptions based on limited data, that only two mycobacterial species, *M. immunogenum* and *M. chelonae*, are capable of colonizing the harsh, often alkaline, microenvironment prevalent in modern MWFs.

Materials and methods

MWF samples and isolation of mycobacteria

Three samples of in-use synthetic (water-based) MWF drawn from an industrial machining plant were procured through a local MWF supplier. Mycobacterial culturing was performed under selective conditions as described previously (Yadav *et al.*, 2006). Briefly, 20-mL aliquots of the MWF samples were treated with the 'antibiotic cocktail' PANTA plus (BD Biosciences, Sparks, MD) at 36 μg mL^{-1} followed by incubation at 37 °C for 6 days. The samples were then centrifuged at 17 210 ***g*** for 30 min. The pellet suspension in normal saline was serially diluted and spread-plated on Middlebrook 7H10 (MB7H10) agar supplemented with 10% (vol/vol) oleic acid-albumin dextrose-catalase (OADC) enrichment (BD Biosciences). Following incubation (37 °C for up to 10 days), representative putative mycobacterial colonies were selected based on colony morphology.

Molecular identification of mycobacterial isolates

The mycobacterial colony isolates and three reference strains namely *M. immunogenum* ATCC 700506, *M. chelonae* ATCC 35752, and *M. abscessus* ATCC 19977T were grown in Middlebrook 7H9 (MB7H9) broth, containing 10% (vol/vol) OADC enrichment at 37 °C under shaker incubation conditions. One-milliliter aliquots from the cultures were centrifuged at 11 951 ***g*** for 10 min, and DNA was extracted from the cell pellets using our optimized Bactozol-mediated lysis protocol (Khan & Yadav, 2004a, b). The putative mycobacterial isolates were conclusively identified using the amplified *hsp*65 restriction analysis (AHSPRA) method developed for the MCC in our laboratory (Selvaraju *et al.*, 2005).

Amplicon sequencing-based genotyping

Variable regions of the *hsp65* gene (228 bp) and the internal transcribed spacer (ITS) segment (253 bp) of 16S-23S rRNA operon were amplified using the primers and conditions optimized in our earlier studies (Khan *et al.*, 2005a, b; Selvaraju *et al.*, 2005). The amplicons were purified by Montage PCR columns (Millipore Corporation, Bedford, MA) and subjected to DNA sequencing using the services of a commercial sequencing laboratory. The sequences were BLAST-analyzed against the current Genbank database sequences for homology-based identification. Multiple alignments of the *hsp* and ITS sequences using MegAlign, 1993–2002 (DNASTAR Inc., Madison, WI) and GENEDOC softwares assisted in genotyping of the isolates. Sequences of the MCC reference strains and representative genotypes of the *M. chelonae* (M-JY1, M-JY2, M-JY6, and M-JY11) and *M. immunogenum* (M-JY3, M-JY-4, M-JY12, and M-JY13), earlier isolated and characterized in our laboratory (Khan *et al.*, 2005a, b), were included for sequence comparisons.

Randomly amplified polymorphic DNA (RAPD)-PCR-based genotyping

Mycobacterium abscessus isolates were further characterized for genotype by RAPD analysis using the protocol described elsewhere (Zhang *et al.*, 1997), based on selected primers OPA2, OPA18, INS-2, and IS986-FP. Briefly, the amplification reaction mixture (50 μL) contained 250 μM dNTPs, 100 pmol of primer, 1.25 U of Ex Taq DNA polymerase (Takara Bio Inc, Japan), and 100 ng of DNA. The PCR amplification conditions

included 40 cycles of 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min. The PCR products were resolved on a 2% agarose gel using ethidium bromide staining. The band profiles were compared visually, and strain relatedness was determined using the proposed criteria (Zhang *et al.*, 1997). *Mycobacterium abscessus* reference strains (ATCC 19977T and ATCC 23006) were included for comparison.

Biocide susceptibility of *M. abscessus* isolates

Mycobacterium abscessus isolates were phenotypically characterized for their susceptibility to the model test biocide triclosan in terms of minimal inhibitory concentration (MIC) determined using broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2011) with slight modifications in temperature (we used 37 °C as against 30 °C advocated in the M24A and M24A2 documents). Briefly, triclosan stock solution prepared in DMSO was serially diluted 2-fold in cation-adjusted Mueller Hinton broth to a final concentration range 64.0—0.031 μg mL^{-1}. Aliquots (100 μL each) of the biocide dilutions were dispensed into microtiter plate wells. Mycobacterial cells for each isolate were swabbed from a freshly grown agar plate into sterile distilled water (4.5 mL) to obtain a cell suspension matching 0.5 McFarland standard (1.5×10^8 CFU mL^{-1}). The suspension was serially diluted in sterile water to obtain the desired cell concentration of 1.5×10^6 CFU mL^{-1}; 10 μL of this suspension was added to the biocide-containing wells. The plates were incubated at 37 °C in ambient air and examined at 72 h for growth inhibition. The MIC was defined as the lowest concentration of biocide that completely inhibited the macroscopic growth.

Nucleotide sequence accession numbers

The partial *hsp65* gene sequences of the mycobacterial isolates determined in this study have been deposited in the Genbank database under the following accession numbers: GQ856678–GQ856681 (*M. abscessus*), HM849732 (*M. chelonae*), and HM849733 (*M. immunogenum*). The ITS sequences have been deposited under Genbank accession numbers GQ856682–GQ856685 (*M. abscessus*), HM849730 (*M. chelonae*), and HM849731 (*M. immunogenum*).

Results

Isolation of mycobacteria

Culturing of the three PANTA-treated MWF samples on MB7H10 agar plates yielded the bacterial counts 1.3×10^4, 2.0×10^4, and 6.4×10^5 CFU mL^{-1}, respectively. The colonies from sample 1 showed smooth morphotype, while those from sample 2 showed rough morphotype. In contrast, the colonies from sample 3 displayed two different morphotypes; one morphotype represented smooth and shiny colonies, whereas the other represented larger, rough, and markedly waxier colonies. All isolated colony morphotypes were stable during subculturing for six consecutive generations. Two representative colonies of either morphotype were chosen for further characterization.

Molecular identification and speciation of mycobacterial isolates

AHSPRA on the MWF isolates yielded the expected 667-bp amplicons and *Bbv*I restriction patterns identical to those of the following reference strains: *M. chelonae* for sample 1 isolate, *M. immunogenum* for sample 2 isolate, and *M. abscessus* for sample 3 isolates (Fig. 1), confirming the identity of the MWF isolates from the three samples as *M. chelonae*, *M. immunogenum,* and *M. abscessus*, respectively.

Amplicon sequencing-based genotyping of the isolates

The MWF mycobacterial isolates yielded the expected amplicons of *hsp65* gene and ITS region (228 and 253 bp, respectively). BLAST homology searches on the two amplicon sequences revealed the highest homology with *M. chelonae* for sample 1 isolate (95–97% and 91–93%, respectively), with *M. immunogenum* for sample 2 isolate

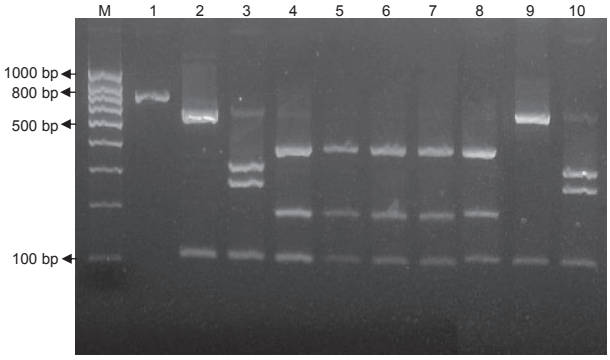

Fig. 1. Molecular identification and speciation of the mycobacterial isolates based on AHSPRA method. Comparison of amplified hsp65-BbvI restriction patterns of MWF isolates with those of MCC reference species is presented. Lane 1: undigested amplicon (667 bp), Lane 2: *Mycobacterium immunogenum* ATCC 700506, Lane 3: *Mycobacterium chelonae* ATCC 35752, Lane 4: *Mycobacterium abscessus* ATCC 19977T, Lane 5–10: MWF isolates M-JY23, M-JY24, M-JY25, M-JY26, M-JY22, and M-JY20.

(96% and 99%, respectively), and with *M. abscessus* for sample 3 isolates (96–98% and 98–99%, respectively). Multiple sequence alignment revealed a single-base pair difference in the *hsp65* gene amplicon and 2-bp difference in the ITS region of the sample 1 isolate (M-JY20) compared to the known representative MWF genotypes of *M. chelonae* (Figs 2 and 3), indicating the isolation of a novel genotype in this study. The isolate from sample 2 (M-JY22) showed 100% identity with the *hsp65* gene sequence but a 3-bp difference in the ITS sequence compared to the known *M. immunogenum* MWF genotypes (Figs 2 and 3), indicating the isolation of a novel genotype in this study. The *hsp65* and ITS sequences of the four isolates, two rough (M-JY23 and M-JY24) and two smooth (M-JY25 and M-JY26), from sample 3 showed 100% identity among themselves (Figs 2 and 3), suggesting that these isolates originated from the same genotype. However, this MWF genotype differed from the reference genotype (ATCC strain 19977T) by 2 bp in terms of the ITS sequence, indicating novel nature of the *M. abscessus* genotype isolated from MWF in this study.

RAPD-based genotyping

The four *M. abscessus* isolates (representing the two morphotypes) showed a common RAPD pattern that differed from those of the two reference strains (ATCC 19977T and ATCC 23006), with each of the four primers tested (Fig. 4). This confirmed that all the *M. abscessus* isolates belonged to a single novel genotype.

Triclosan susceptibility

All *M. abscessus* isolates as well as the ATCC reference strains showed complete inhibition of growth at 8–64 μg mL^{-1} concentration range (data not shown), meaning a common MIC value of 8 μg mL^{-1}.

Discussion

According to the current state-of-knowledge, only two species of mycobacteria, namely *M. immunogenum* and *M. chelonae*, are known to have the ability to colonize

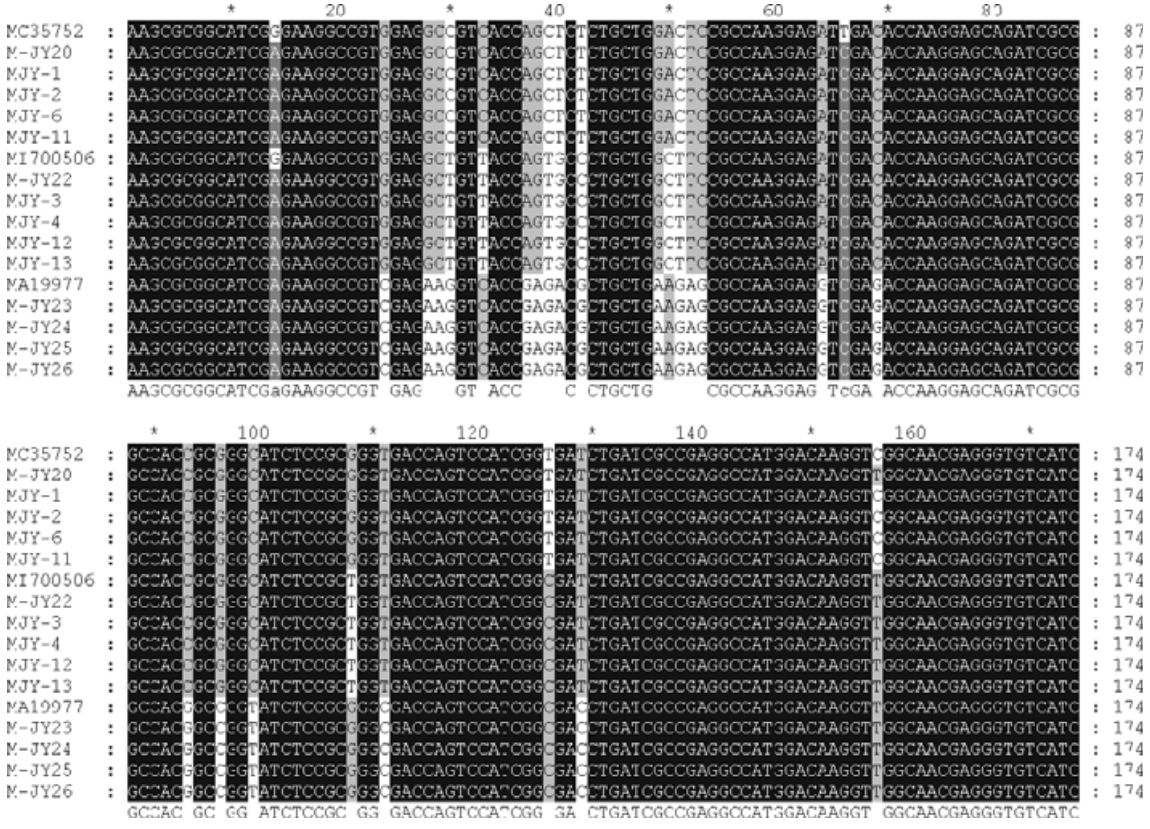

Fig. 2. Multiple alignment of *hsp65* amplicon sequences of the MWF isolates (M-JY20, M-JY22, and M-JY23–M-JY26). The sequences of the MCC reference species *Mycobacterium chelonae* ATCC 35752, *Mycobacterium immunogenum* ATCC 700506, and *Mycobacterium abscessus* ATCC 19977T and representative genotypes of *M. chelonae* (M-JY1, M-JY2, M-JY6, and M-JY11) and *M. immunogenum* (M-JY3, M-JY4, M-JY12, and M-JY13) are aligned for comparison. The sequence variations are shown in white background, whereas the conserved sequences are shown in dark background.

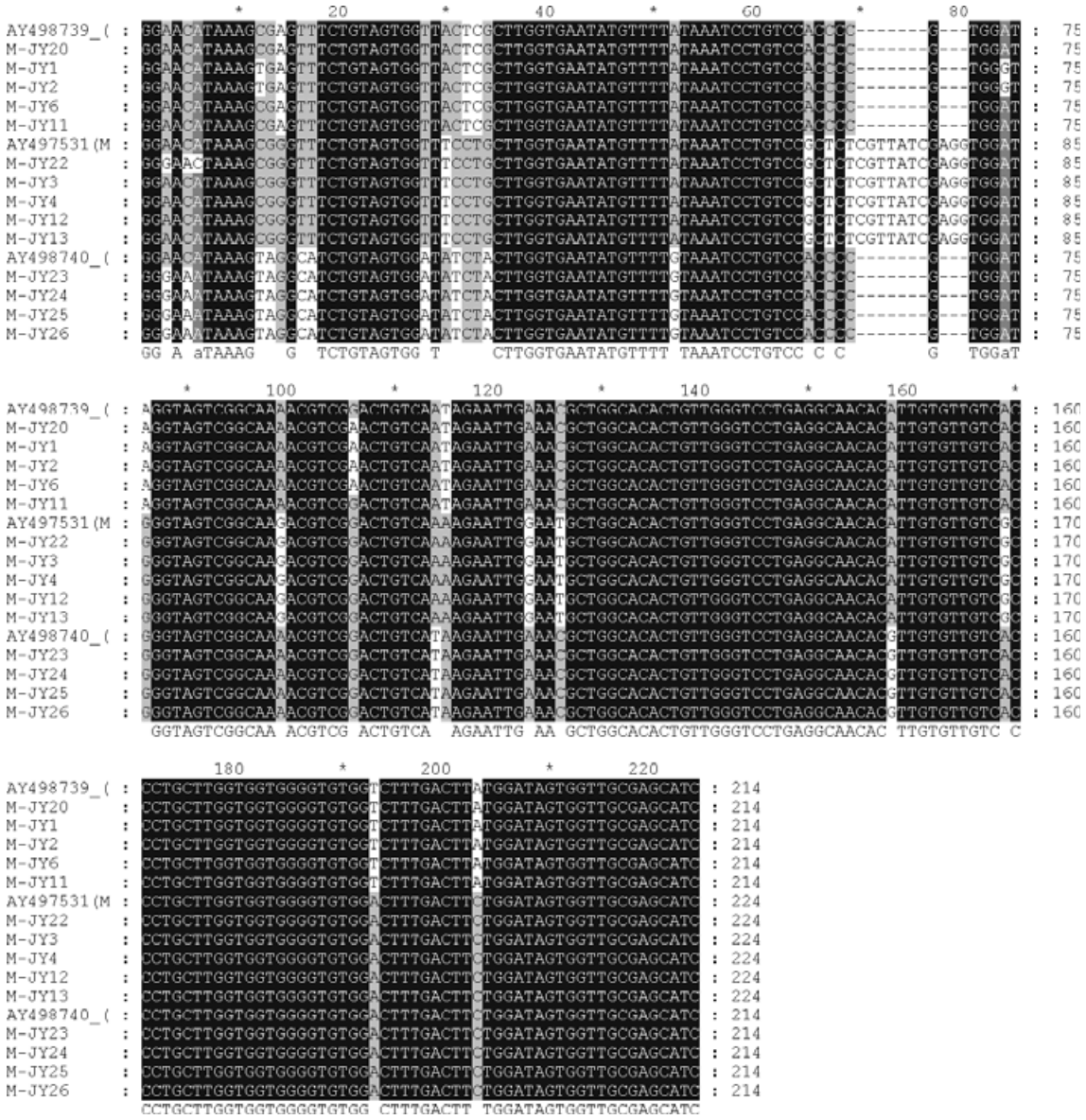

Fig. 3. Multiple alignment of 16S-23S rRNA operon ITS sequences of the MWF isolates (M-JY20, M-JY-22, M-JY23–M-JY26). The sequences of MCC reference species *Mycobacterium chelonae* ATCC 35752 (AY498739), *Mycobacterium immunogenum* ATCC 700506 (AY497531), and *Mycobacterium abscessus* ATCC 19977T (AY498740) and representative genotypes of *M. chelonae* (M-JY1, M-JY2, M-JY6, and M-JY11) and *M. immunogenum* (M-JY3, M-JY4, M-JY12, and M-JY13) were used for comparison. The sequence variations are shown in white background, whereas the conserved sequences are shown in dark background.

MWF (Kreiss & Cox-Ganser, 1997; Moore *et al.*, 2000; Wilson *et al.*, 2001; CDC, 2002; Wallace *et al.*, 2002; Veillette *et al.*, 2004; Beckett *et al.*, 2005; Khan *et al.*, 2005a, b; Gupta & Rosenman, 2006). Hence, in the 15-year history of mycobacterial profiling of industrial MWF, it is significant that the current study revealed the presence of a third species of mycobacteria, *M. abscessus*, in these fluids. Interestingly, these three species prevalent in these fluids belong to the MCC. This may imply that, unlike other groups of environmental mycobacteria that are prevalent in water and related aqueous environments, this group of mycobacteria has the inherent and/or acquired competence to colonize the harsh microenvironment prevalent in the in-use MWFs (alkaline pH, elevated temperature, and the presence of heavy metals). Such microenvironment may favor the development of a microbial population able to survive and grow in conditions generally considered hostile for microorganisms.

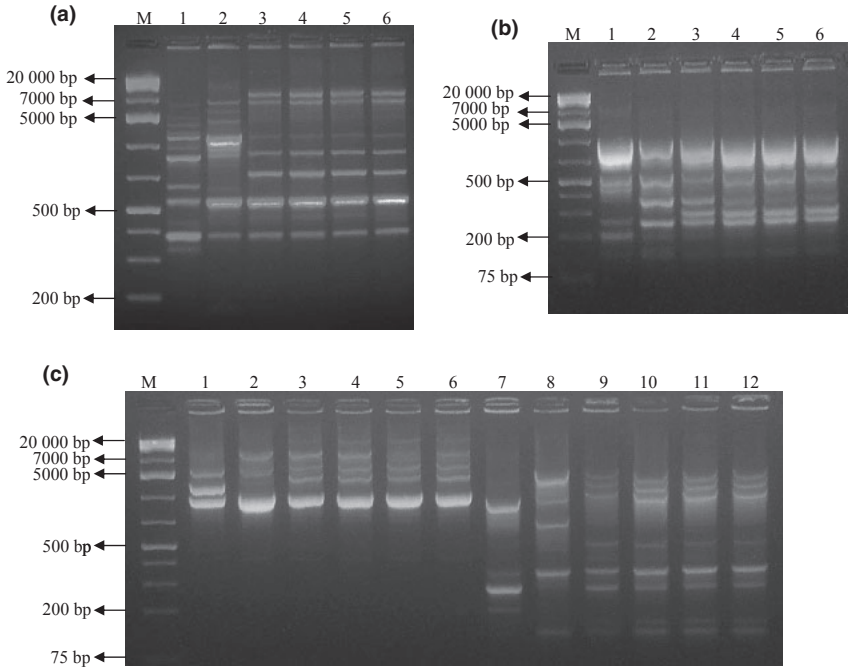

Fig. 4. RAPD-PCR patterns of the MWF isolates of *Mycobacterium abscessus* (M-JY23–M-JY26) generated using different primers. Panel a: OPA2 primer-based patterns; Panel b: IS986-FP primer-based patterns; and Panel c: OPA18 primer-based patterns (lanes 1–6) and INS-2 primer-based patterns (lanes 7–12). The following DNA templates were used: lanes 1 and 7: *M. abscessus* ATCC 23006, lanes 2 and 8: *M. abscessus* ATCC 19977T, lanes 3 and 9: M-JY23, lanes 4 and 10: M-JY24, lanes 5 and 11: M-JY25, and lanes 6 and 12: M-JY26. Lane M represents 1-kb plus DNA ladder.

The routine practice of periodic indiscriminate addition of biocides to manage microbial growth in these fluids may further result in the emergence of only those microbial species that can resist microbicidal conditions (Kreiss & Cox-Ganser, 1997; CDC, 2002). Such selection may be the basis of the prevalence of only selected species of mycobacteria in these fluids.

Mycobacterium abscessus has evaded detection in MWF in earlier attempts involving conventional culturing strategy. In this study, detection of *M. abscessus* in MWF became possible because of the use of a specialized improved method of mycobacterial recovery from these fluids, recently developed in our laboratory (Yadav *et al.*, 2006). In this method, addition of an antibiotic mixture PANTA to the growth medium circumvents the problem of overgrowth of co-contaminants as well as stimulates the growth of mycobacterial species (Yadav *et al.*, 2006).

Several molecular methods specially designed to detect and/or identify mycobacteria prevalent in MWF have been developed in the recent years by us and others (Khan *et al.*, 2005a, b; Selvaraju *et al.*, 2005, 2008; Rhodes *et al.*, 2008; Veillette *et al.*, 2008; Kapoor & Yadav, 2009). In particular, our PCR-restriction enzyme analysis–based methods (Khan *et al.*, 2005a, b; Selvaraju *et al.*, 2005) were developed for definitive species differentiation of the members of the MCC. These specialized methodologies for the MCC enabled an unambiguous identification and speciation of the MWF isolates in this study.

In general, *M. abscessus* has been shown to exist in two colony morphotypes, rough and smooth, and can naturally switch from one to the other morphotype (Byrd & Lyons, 1999; Howard *et al.*, 2006; Jönsson *et al.*, 2007). In contrast, *M. abscessus* isolates in this study did not show reversion to their alternative form and were found to retain their original colony morphotype upon several subcultures on MB7H10 agar medium. Genotyping based on DNA sequencing of the variable regions of *hsp* gene and ITS and genome-wide RAPD-PCR analysis confirmed that the *M. abscessus* isolates share the same genotype. This implies that the isolates originated from the same clone, which is consistent with that recently reported for the clinical variants of *M. abscessus* (Jönsson *et al.*, 2007).

Triclosan is a model test biocide that is widely used in hand disinfectants and other hygiene products; susceptibility to this biocide is used as an index to assess antimicrobial susceptibility of bacteria (Schweizer, 2001; Yazdankhah *et al.*, 2006), including mycobacteria (Fraser *et al.*, 1992). Considering this, relative triclosan susceptibility of *M. abscessus* isolates was compared in terms of MIC. All *M. abscessus* isolates (representing the two morphotypes) showed the same extent of susceptibility to triclosan, an observation corroborating with their common genotypic makeup. However, in this context, the absolute MIC values for triclosan may be treated with caution as there may be possible limitations of applying the CLSI guidelines (originally meant for antibiotics) to biocides.

Isolation of *M. abscessus* from MWF may have environmental health implications. In this context, it is noteworthy that *M. abscessus* has emerged as one of the most pathogenic RGM species of NTM over the past decade. It

is responsible for more than 80% of all pulmonary infections owing to RGM in the United States and is associated with a much higher fatality rate than any other RGM species (Griffith *et al.*, 1993, 2007). It is also one of the most antibiotic-resistant species of the RGM subgroup (Fraser *et al.*, 1992). *Mycobacterium abscessus* has been involved in several nosocomial outbreaks and pseudo-outbreaks caused by municipal- and hospital water supplies (Wenger *et al.*, 1990; Fraser *et al.*, 1992; Maloney *et al.*, 1994; Wallace *et al.*, 1998; Brown-Elliott & Wallace, 2002) and can potentially cause similar pseudo-outbreaks and disease outbreaks in metalworkers exposed to contaminated MWF. On the other hand, identification of hitherto unknown novel genotypes of *M. chelonae* and *M. immunogenum* from MWF in this study further expands the mycobacterial genotypic diversity characterized from this harsh microenvironment.

In conclusion, this is the first report on the occurrence of *M. abscessus* in MWFs. The findings are significant considering that *M. abscessus* is potentially more pathogenic and is one of the most common causes of respiratory illness caused by RGM in the United States. Therefore, the findings have clinical implications and point to the need for inclusion of this species in routine screening of machining fluids for mycobacterial contamination. This would aid in better understanding of the worker health-related problems and antimicrobial efficacy and performance of the biocides commonly applied for fluid management. On the other hand, the study reports additional novel genotypes of *M. immunogenum* and *M. chelonae* from MWF. The findings collectively expand the known mycobacterial diversity colonizing these fluids. Dissection of mycobacterial genetic diversity is expected to provide tools to understand the antigenic diversity of these suspected etiologic agents of occupational HP in machining environments.

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