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Peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) assay for specific detection of *Mycobacterium immunogenum* and DNA-FISH assay for analysis of pseudomonads in metalworking fluids and sputum

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ABSTRACT

Specific and rapid detection and quantification of mycobacteria in contaminated metalworking fluid (MWF) are problematic due to complexity of the matrix and heavy background co-occurring microflora. Furthermore, cross-reactivity among neighboring species of *Mycobacterium* makes species differentiation difficult for this genus. Here, we report for the first time a species-specific peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) method for *Mycobacterium immunogenum*, a non-tuberculous *Mycobacterium* species prevalent in MWF and implicated in occupational lung disease hypersensitivity pneumonitis and pseudo-outbreaks. A novel species-specific 14-bp PNA probe was designed for *M. immunogenum* based on its 16S rRNA gene sequence and was validated for specificity, by testing against a panel of other phylogenetically closely related rapidly growing mycobacteria and representative species of gram-positive, gram-negative, and acid fast organisms. In addition, a DNA-FISH protocol was optimized for co-detection of *Pseudomonas*, the most predominantly co-occurring genus in contaminated MWF. Reliable quantification for both the test organisms was achieved at or above a cell density of 10^3 cells ml^{-1}, a recognized minimum limit for microscopic quantification. The mycobacterial PNA-FISH assay was successfully adapted to human sputum demonstrating its potential for clinical diagnostic applications in addition to industrial MWF monitoring, to assess MWF-associated exposures and pseudo-outbreaks.

1. Introduction

Non-tuberculous mycobacteria (NTM) have been frequently implicated in occupational pulmonary diseases [3,10] and nosocomial infections [1]. Specifically, members of the *Mycobacterium chelonae* complex (MCC), *Mycobacterium immunogenum* and *M. chelonae*, have been associated with outbreaks of occupational hypersensitivity pneumonitis (HP) in machine workers exposed to contaminated metalworking fluid (MWF) and in opportunistic lung infections and pseudo-outbreaks [1,28]. Particularly, *M. immunogenum* has been reported as the predominant species for its association with MWF [21,30] and occupational HP [25,26]. Since mycobacteria frequently evade detection by conventional culturing in MWF matrix, there is an increasing need and requirement for the development of specific and culture-independent methods for their detection and enumeration [30]. This will provide early and conclusive evidence of MWF contamination and exposure of metal workers to mycobacteria, and facilitate intervention measures. Among the MWF-associated gram-negative genera responsible for endotoxin release and build up, pseudomonads have been recognized as the dominant group and as initial colonizers [19] and have also been implicated in occupational health hazards including HP in exposed metal workers [11,15,17,18]. Considering this, an early and simultaneous detection of both mycobacteria and pseudomonads becomes critical in MWF exposures associated to occupational HP for reducing exposures and preventing HP outbreaks in occupational settings.

The conventional culture-based method used for detection and quantification of the specific MWF-associated pathogens such as mycobacteria and pseudomonads lack specificity and speed; even rapidly growing mycobacteria (RGM) take 3–7 days to yield colonies on the selective media used in this method. Most importantly, the stressed or non-culturable fraction of the population, which is equally significant in contributing to the etiological factors (antigens and endotoxin), goes undetected in cultural analysis [2]. This has led to an increasing interest in developing DNA-based

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methods particularly PCR and real-time PCR for specific detection and quantification of these agents [8,30,31]. Nevertheless, PCR-based assays require specialized skills and are still relatively expensive for certain in-house industrial analytical laboratories and low-cost diagnostic facilities. Hence, alternative DNA-based methods involving simple instrumental skills such as microscopy and probing of the whole cells instead of DNA extraction before probing or amplification will be desirable for a routine screening for assessment of exposure or infection. Fluorescence *in situ* hybridization (FISH) is one such method, which is less expensive and adaptable for routine screening applications in clinical and environmental settings [12,16,23,29].

While the conventional DNA oligonucleotide probes for FISH analysis have proved useful for the common gram-positive and gram-negative microbial genera or groups [4,5,22,24,27], these probes do not work in case of mycobacteria as they can barely penetrate mycobacterial cell wall, which contains mycolic acids. In contrast, the relative hydrophobic character of peptide nucleic acid (PNA) allows better diffusion of the PNA-based probes through the cell wall of mycobacteria [23]. Such probes have been recently applied for detection of tuberculous versus non-tuberculous groups of mycobacteria [6,12,23]. However, little is known on the species-specific PNA probes and detection protocols for individual mycobacterial species [13]. Considering the high homology at the 16S rRNA sequence level among the member species in individual NTM complexes such as among the three common species of the *M. chelonae* complex (MCC), namely *M. immunogenum*, *M. chelonae*, and *M. abscessus*, there is a need to develop highly specific PNA-FISH probes for the individual member species to overcome cross-reactivity. Moreover, the need for adapting the mycobacterial FISH analysis to clinical specimens particularly human sputum has been emphasized [6]. Here we report the development of a species-specific PNA-based FISH probe and assay conditions for detection of *M. immunogenum*, both in cultures and complex matrices, including metalworking fluid (MWF) and human sputum. We further report optimization of assay conditions for a DNA oligonucleotide probe-based FISH assay for direct and simultaneous detection of pseudomonads in the complex MWF matrix. Our aims in this study were (i) to develop a PNA-FISH assay for specific detection and quantification of *M. immunogenum* (culturable and non-culturable) in complex MWF matrix, containing other microbial co-contaminants and (ii) to optimize a DNA-FISH protocol for co-detection and quantification of pseudomonads (culturable and non-culturable) in MWF matrix. The PNA-FISH assay was also adapted for detection of mycobacteria in human sputum, to demonstrate its feasibility for potential clinical applications, such as for MWF-exposed occupational patients and nosocomial infections.

2. Materials and methods

2.1. Bacterial species

M. immunogenum ATCC 700506 (metalworking fluid isolate) and a representative of the MWF pseudomonads, *Pseudomonas fluorescens* ATCC 13525 (pre-filter tanks isolate) were used in this study. In addition, three different genotypes of *M. immunogenum*, isolated in our previous study [9] from MWF samples obtained from different occupational settings located in different regions of the country, were included in the study. To test the cross-reactivity of the designed PNA probe and to validate the PNA-FISH protocol, other member species of the *M. chelonae* complex (MCC) *viz. M. chelonae* ATCC 35752^T (tortoise), and *M. abscessus* ATCC 19977^T (knee abscess) and ATCC 23006 (human sputum) were used. We also used the reference species and isolates of other non-pigmenting rapidly growing mycobacteria (RGM) including *Mycobacterium fortuitum* ATCC 6841^T (cold abscess), *Mycobacterium mageritense* ATCC 700351^T (human sputum), *Mycobacterium peregrinum* ATCC 14467^T (bronchial aspiration), *Mycobacterium mucogenicum* ATCC 49650^T (infected thyroglossal duct cyst), *Mycobacterium senegalense* ATCC 35796^T (bovine farcy lesion), *Mycobacterium smegmatis* ATCC 19420^T (endothelial cells), *Mycobacterium wolinskyi* ATCC 700010^T (human facial wound), and *Mycobacterium septicum* ATCC 700731^T (venous catheter tip), and the pigmenting RGM species including *Mycobacterium phlei* ATCC 11758^T and *Mycobacterium vaccae* ATCC 15483^T (cow milk). In addition, the specificity of the PNA probe was tested against other gram-negative, gram-positive and acid fast organisms *viz. P. fluorescens* ATCC 13525 (pre-filter tanks), *Bacillus* sp. B22 (MWF), *Escherichia coli* DH5α, *Streptomyces griseus* subsp. *griseus* ATCC 11746 (soil) and *Legionella pneumophila* subsp. *pneumophila* ATCC 33215 (human lung biopsy).

2.2. Metalworking fluids

Four different types of MWF, including pristine synthetic, pristine semi-synthetic, in-use synthetic and in-use semi-synthetic, were used in this study. Pristine synthetic and semi-synthetic fluids obtained from an industrial source were diluted to 5% (vol/vol), a commonly used working concentration and 2% (vol/vol), an estimated non-inhibitory concentration for microorganisms, respectively (data not shown). The diluted MWF preparations were filter sterilized and used as the test matrices. Additionally, in-use (real) MWF samples, two each of synthetic and semi-synthetic composition, contaminated with mycobacteria and pseudomonads were obtained from the same source.

2.3. PNA or DNA oligonucleotide probes (design and synthesis)

A peptide nucleic acid (PNA) probe specific to *M. immunogenum* was designed based on the antisense sequence of its 16S rRNA gene to target the 16S rRNA molecules in the cell. Sequence analysis was carried out using the DNASTAR software (DNASTAR, Inc., Madison, WI, USA). The 16S rRNA sequences of various rapidly growing mycobacteria and three slow growing mycobacteria (*Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium avium*) were downloaded from the GenBank and aligned using the Megalign version 5.05 software. PNA probe was designed with regard to secondary structures and validated by PNA probe designer (Applied Biosystems, Foster City, CA, USA). A 14-base length PNA probe sequence (5′-CAT GCG GTC CTA TC-3′) targeting the 16S rRNA gene was selected and obtained (custom-synthesized) from Biosynthesis Inc., Lewisville, TX, USA. The PNA oligomer contained a Fluorescein 5-isothiocyanate (FITC) label at the N-terminus and a lysine residue at the C-terminus. A *Pseudomonas*-specific DNA oligonucleotide probe-based on 16S rRNA gene sequence was custom-synthesized by Molecular probes Inc. (Eugene, OR, USA) and used, as described earlier [4,27].

2.4. Fluorescence in situ hybridization (FISH) assay conditions

A loopful from an isolated colony of *M. immunogenum* grown on M7H10 agar medium was suspended in 250 μl of phosphate-buffered saline (pH 7.2) and a 25 μl aliquot from the bacterial suspension was placed on a Teflon-coated diagnostic microscope slide (Erie Scientific Company, Portsmouth, NH, USA). The smear was air dried and fixed with 80% (vol/vol) ethanol for 15 min. The FISH hybridization and washing protocol for *M. immunogenum* was adapted from that reported earlier [23], using appropriate modifications. Briefly, the slide with smear was pre-heated to 40 °C on a heat block and covered with 25 μl of the PNA probe (1 μM) in a hybridization solution containing 10% (wt/vol) Dextran sulfate (Sigma Chemical Co., St. Louis, MO, USA), 10 mM NaCl (Fisher

Scientific, Pittsburgh, PA, USA), 30% (vol/vol) Formamide (Fisher Scientific, Pittsburgh, PA, USA), 0.1% (wt/vol) Sodium pyrophosphate (Sigma Chemical Co., St. Louis, MO, USA), 0.2% (wt/vol) Polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, MO, USA), 0.2% (wt/vol) Ficoll (Sigma Chemical Co., St. Louis, MO, USA), 5 mM Disodium EDTA (Sigma Chemical Co., St. Louis, MO, USA), 0.1% (vol/vol) Triton-X-100 (Fisher Scientific, Pittsburgh, PA, USA), and 50 mM Tris–HCl (pH 7.5; Sigma Chemical Co., St. Louis, MO, USA) using a cover slip. After hybridization for 120 min at 40 °C, the slide was washed at 55 °C by immersing in a prewarmed washing solution containing 5 mM Tris base (pH 10; Sigma Chemical Co., St. Louis, MO, USA), 15 mM NaCl and 0.1% (vol/vol) Triton-X-100 (Sigma Chemical Co., St. Louis, MO, USA). The washed slide was immersed in double distilled water for 5 min and allowed to air dry. The smear was covered with a drop of Dako Cytomation fluorescent mounting medium (DAKO Cytomation, Carpentaria, CA, USA), using a cover slip and incubated at 40 °C for 30 min, before examination by microscopy.

The DNA-FISH assay for pseudomonads was performed using the Texas red-labeled pseudomonads-specific oligonucleotide probe and the assay parameters as described earlier [27], with appropriate modifications for the test matrix (MWF or sputum). Briefly, *P. fluorescens* cells were resuspended in metalworking fluid or sputum and the bacterial suspension was applied as a smear on a microscopic slide, as described above. Following air drying, the smear was hybridized with a prewarmed (48 °C) hybridization buffer containing 0.5 pmol μl^{-1} DNA oligonucleotide probe by incubating at 48 °C for 90 min. The hybridization reaction was stopped by immersing the slide in cold phosphate-buffered saline (pH 8.0) for 5 min and washing with double distilled water followed by air drying. A drop of the fluorescent mounting medium was applied to the smear before examination by microscopy.

2.5. Quantification efficiency

Test samples for either test organism (*M. immunogenum* or *P. fluorescens*) were generated by making serial dilutions (10^0 through 10^6 CFU ml^{-1}) in pristine synthetic or semi-synthetic MWF fluid. The *M. immunogenum* samples were prepared either in an MWF fluid pre-spiked with 10^6 CFU ml^{-1} each of *P. fluorescens* and *Bacillus* sp. or in an in-use contaminated synthetic or semi-synthetic MWF containing an existing mixed microflora. One millilitre each of the simulated test samples was centrifuged at 14,000 rpm for 15 min. The pellet was resuspended in 100 μl of the respective MWF supernatant for the 'high count' samples (10^5 and 10^6 CFU ml^{-1}) and in 10 μl for the 'low count' samples (10^0 through 10^4 CFU ml^{-1}) and an aliquot of the resulting suspension (10 μl) in either category was used directly for the microscopic counting. Appropriate multiplication factors were used to calculate the number of cells ml^{-1} of the original MWF sample.

2.6. Non-culturable cells

The optimized FISH protocols for the two test organisms (*M. immunogenum* and *Pseudomonas*) were evaluated for their ability to detect non-viable (non-culturable) cells. A viable cell suspension for either test species was prepared by centrifugation (10,000 rpm for 10 min) of its freshly grown culture followed by resuspension of the pelleted cells in sterile water, as described above. A non-viable cell suspension for either test organism was prepared by treating its above prepared fresh cells with 70% isopropyl alcohol at room temperature for 1 h, followed by centrifugation, washing and resuspension in sterile water. The two test suspensions (the culturable and non-culturable) were compared for the detection efficiency by the FISH assays.

2.7. Assay evaluations with in-use (field) MWF samples

The optimized PNA-FISH (*M. immunogenum*) and DNA-FISH (*Pseudomonas* sp.) protocols were evaluated with in-use field MWF samples. For both the protocols, a 10 ml volume of the field MWF sample was centrifuged at 12,000 rpm for 15 min and the pellet washed with either PBS (pH 7.2; for synthetic) or 70% ethanol (for semi-synthetic) and resuspended in 100 μl of PBS (pH 7.2.) For detection of *M. immunogenum*, a 10 μl aliquot of the suspension was tested using the optimized PNA-FISH protocol and counterstained with DAPI stain before microscopy. For detection of pseudomonads in in-use MWF samples, the DNA-FISH protocol was used with some modifications. Briefly, cells from a 100 μl aliquot of the cell suspension were fixed using three volumes of the fixing solution (4% paraformaldehyde prepared in PBS pH 7.2) at 4 °C for 16 h. The fixed cells were washed with PBS and hybridized using 25 μl of a prewarmed hybridization solution using the optimized conditions. The hybridization reaction was stopped by adding 250 μl of the ice-cold PBS (pH 8.4). An aliquot (25 μl) of the suspension was smeared on a Teflon-coated microscopic slide. The smear was air dried and counterstained with DAPI stain before performing the fluorescence microscopy.

2.8. Human sputum

Human sputum specimen collected from a healthy volunteer was decontaminated using the modified Petroff's method [7]. The sputum was spiked with either of the two test organisms *M. immunogenum* or *P. fluorescens* (each at 10^6 cells ml^{-1}) to generate the simulated test samples. PNA-FISH and DNA-FISH analyses were performed to detect the spiked test organisms using optimal hybridization conditions described above.

2.9. Epifluorescence microscopy

A Zeiss Axioplan 2 or Nikon eclipse TE 300 Microscope System was used. The epifluorescence microscopic counting was performed using 100-W mercury light source, with FITC green or Texas red filter cubes using 100× (UV-F) objective. Ten randomly selected microscopic fields were counted and the number of bacteria per ml of the original sample was calculated based on the following formula: bacterial count (cells ml^{-1}) $= N_b \times M_f \times 1/V \times 1/C$, where, N_b is mean number of bacteria per field, M_f (microscopic factor) is number of fields per cm^2 area on the slide, V is volume of the stained sample suspension applied to the slide (0.01 ml), and C is the concentration factor (fold-concentration of the original sample to prepare the staining sample suspension). The value of C was 10 for high-count samples (concentrated 10-fold) and 100 for low-count samples (concentrated 100-fold).

3. Results

3.1. Probe design and specificity

A 14-bp probe sequence specific for the MWF *Mycobacterium* species *M. immunogenum* was identified based on alignment of all available 16S rRNA gene sequences from various rapidly growing mycobacteria (RGM). The selected probe sequence showed a minimum of 2 bp variation (as with *Mycobacterium kansassi*) and a maximum of 8 bp variation (as with *Mycobacterium goodii*) from the RGM species. The closely related species of *M. chelonae* complex, *M. chelonae* and *M. abscessus,* showed a 3-bp variation. A global BLAST search for the identified probe sequence did not detect any homology with other bacterial rRNA sequences.

Using optimized FISH assay conditions, specificity of the designed PNA probe was tested by hybridization against a number

Table 1
Evaluation of the genus- and species-specificity of the *M. immunogenum*-specific PNA probe against different test organisms

Test organism	FISH signal
Mycobacterium immunogenum ATCC 700506	+
M. immunogenum – MJY-3[a]	+
M. immunogenum – MJY-10[a]	+
M. immunogenum – MJY-13[a]	+
M. chelonae ATCC 35752	–
M. abscessus ATCC 19977	–
M. abscessus ATCC 23006	–
M. mucogenicum ATCC 49650	–
M. fortuitum ATCC 6841	–
M. mageritense ATCC 700351	–
M. peregrinum ATCC 14467	–
M. senegalense ATCC 35796	–
M. smegmatis ATCC 19420	–
M. wolinskyi ATCC 700010	–
M. septicum ATCC 700731	–
M. phlei ATCC 11758	–
M. vaccae ATCC 15483	–
Pseudomonas fluorescens ATCC 13525	–
Bacillus sp. B22	–
E. coli DH5α	–
Streptomyces griseus subsp. *griseus* ATCC 11746	–
Legionella pneumophila subsp. *pneumophila* ATCC 33215	–

+: Positive reaction of the FISH assay.
–: Negative reaction of the FISH assay.
[a] *M. immunogenum* genotypes isolated from field MWF samples [9].

of reference species (selected as phylogenetic representatives of the *Mycobacterium* genus) and the clinically relevant species of *Mycobacterium* and other genera. The results presented in Table 1 show that the *M. immunogenum* PNA probe did not cross-hybridize with the other RGM species. Specificity of the designed probe was further evidenced by the lack of cross-reactivity with any of the tested representatives of gram-positive and gram-negative bacteria and actinomycetes (*S. griseus*). To further ensure the species-specificity of the designed probe to *M. immunogenum* (Fig. 1), we tested other genotypes of this species, isolated from geographically diverse MWF samples obtained from various parts of the country in our recent study [9]. The reference genotype/strain of *M. immunogenum* (ATCC 700506) is presented in panel A of Fig. 1, whereas its other genotypes namely, MJY-3, MJY-10, and MJY-13 are shown in panels B, C and D, respectively. The observed fluorescence intensities for the four genotypes were similar.

Specificity of the pseudomonads probe used in this study was confirmed by testing against a selected representative each from gram-positive bacteria (*Bacillus* sp.) and gram-negative bacteria (*E. coli*) and, as expected, no cross-reactivity with either of these phylogenetic out groups was observed.

3.2. Quantification efficiency in MWF matrix containing background microflora

The PNA-FISH protocol for *M. immunogenum* was evaluated for its quantification efficiency in various MWF fluid types, spiked with an increasing known number of *M. immunogenum* cells (10^0 through 10^6 cells ml^{-1}) along with the test background co-contaminants. The quantitative detection limit was consistent with the microscopic quantitation limit ($\geq 10^3$ cells ml^{-1}). However, the quantification efficiency varied with the fluid type and the background microflora (defined versus undefined mixed flora) as follows: pristine synthetic (3.5×10^3 cells ml^{-1}), semi-synthetic fluids (5.2×10^3 cells ml^{-1}) and in in-use synthetic fluid (1.24 ×

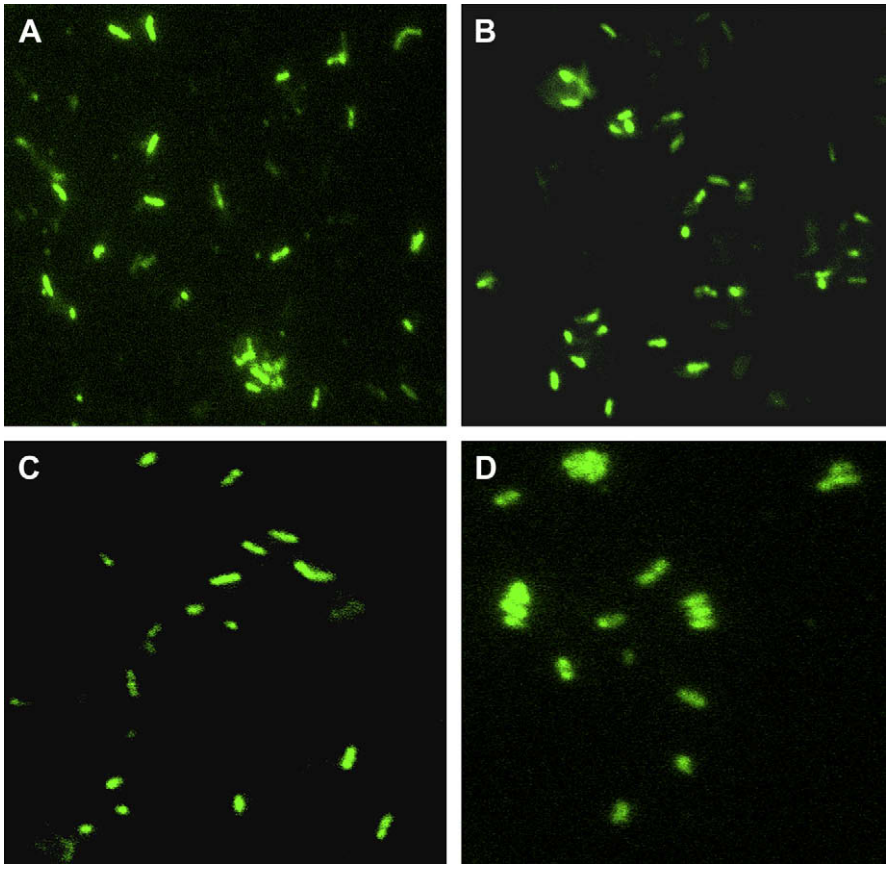

Fig. 1. Evaluation of the peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) assay for specific detection of *M. immunogenum* genotypes. Panel A: *M. immunogenum* ATCC 700506. Panel B: *M. immunogenum* MJY-3. Panel C: *M. immunogenum* MJY-10. Panel D: *M. immunogenum* MJY-13.

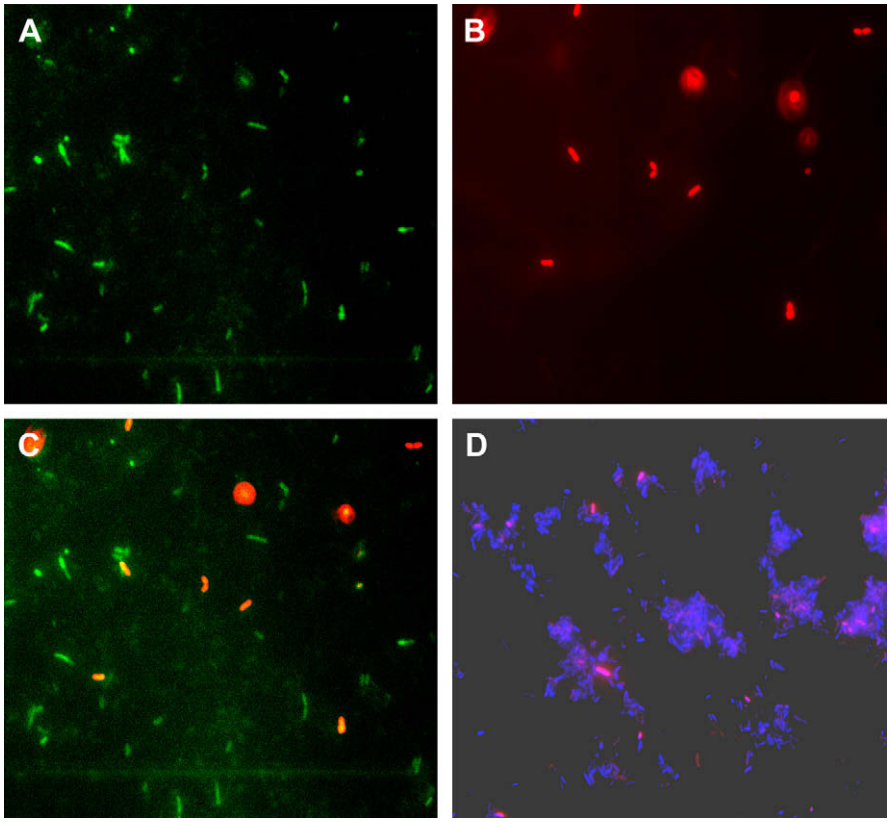

Fig. 2. Evaluation of the 16S rRNA gene-based fluorescence *in situ* hybridization (FISH) assays for differential detection of *M. immunogenum* (PNA-FISH) and *P. fluorescens* (DNA-FISH) in synthetic MWF. Panel A: *M. immunogenum* ATCC 700506. Panel B: *P. fluorescens.* Panel C: superimposed images of *M. immunogenum* (green cells) and *P. fluorescens* (red cells) for differential detection of the two sub-populations. Panel D: superimposed images of *P. fluorescens* (red cells) and DAPI-stained background flora (blue cells) for specific detection of *P. fluorescens* in a mixed microflora in metalworking fluid.

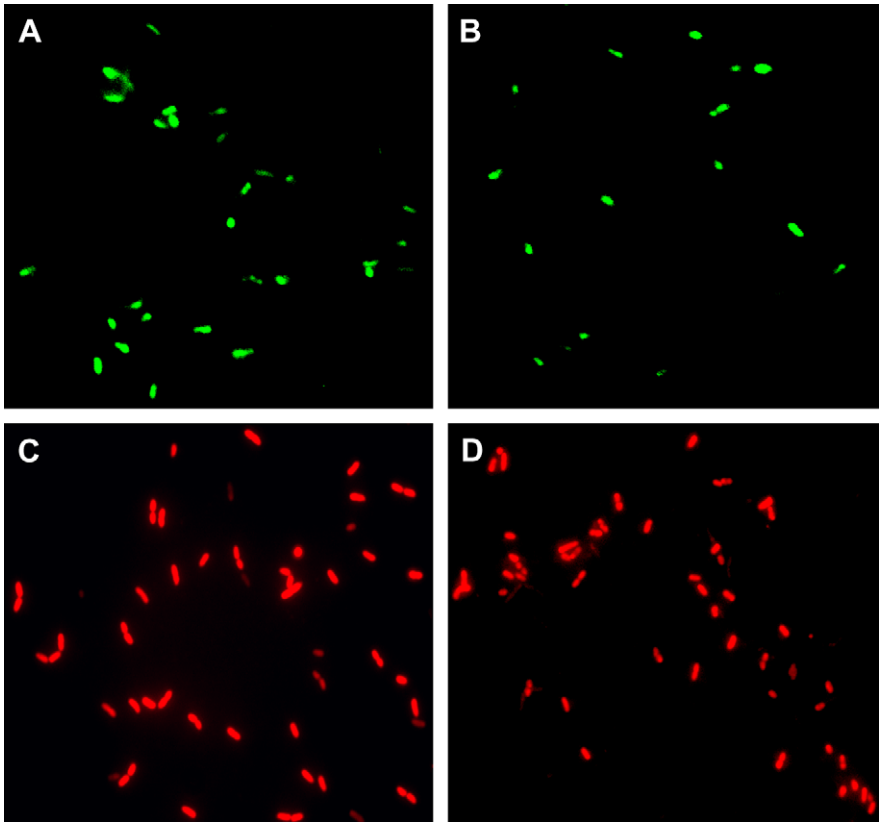

Fig. 3. Evaluation of the fluorescence *in situ* hybridization (FISH) assays for detection of culturable versus non-culturable cells of *M. immunogenum* (Panel A versus B) and *P. fluorescens* (Panel C versus D).

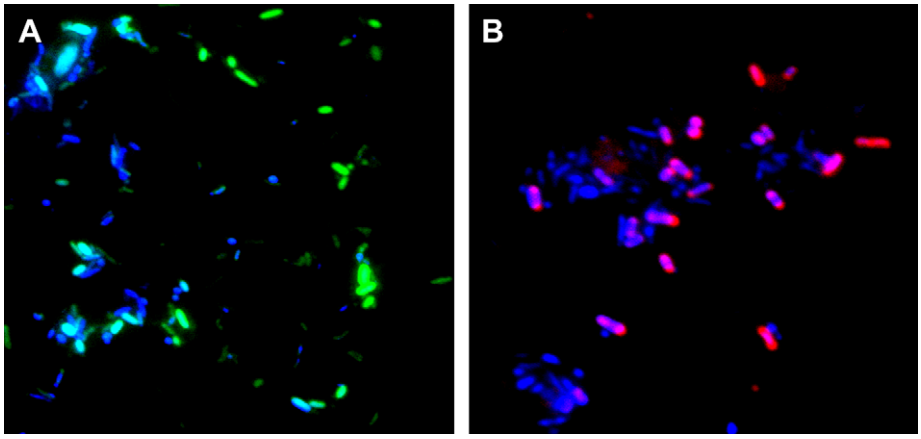

Fig. 4. Evaluation of fluorescence *in situ* hybridization (FISH) assays for detection of *M. immunogenum* (Panel A) and pseudomonads (Panel B) in field MWF samples contaminated with mixed background microflora. The staining color scheme is the same as in Fig. 2.

10^5 cells ml^{-1}). The spiked test organism was not quantifiable in low-count samples (1–100 cells ml^{-1}) due to the known limitation of the microscopic technique for samples containing $<$1000 cells ml^{-1}.

The DNA-FISH protocol showed detection efficiency for *P. fluorescens* similar to that of PNA-FISH for *M. immunogenum*, in all test fluid matrices (data not shown). Fig. 2 shows the detection ability for these test organisms by the optimized FISH assays in pristine synthetic MWF, with panel A showing detection of *M. immunogenum* whereas panel B showing *P. fluorescens* detection. Co-detection of the two test organisms was possible by following the respective protocols and superimposing the images as shown in Fig. 2C. In synthetic in-use MWF matrix (field sample), the detection of *P. fluorescens* (red cells) was demonstrated in presence of the co-existing complex microflora cells, which were stained with the general nucleic acid stain DAPI (Fig. 2D).

3.3. Detection of non-culturable cell population

The optimized FISH protocols were evaluated on alcohol-inactivated *M. immunogenum* and *P. fluorescens* cells in pristine synthetic and semi-synthetic fluid matrices. The freshly grown live cells (Fig. 3A and C) and the inactivated cells (Fig. 3B and D) of both *M. immunogenum* and *P. fluorescens* showed comparable detection efficiency without any loss of fluorescence intensity due to inactivation.

3.4. Detection of the target organisms in field MWF samples

The *M. immunogenum*-specific PNA-FISH assay detected the presence of this organism in in-use real synthetic and semi-synthetic MWF samples collected from the field operations (Fig. 4A). Similarly, the presence of pseudomonads in field samples was detected by the optimized DNA-FISH assay as well (Fig. 4B). The microscopy counts for *M. immunogenum* and pseudomonads in these real MWF samples ranged from 1.76×10^4 through 3.30×10^5 cells ml^{-1} and 1.80×10^5 through 1.05×10^6 cells ml^{-1}, respectively (Table 2).

3.5. Adaptation of the FISH assays for human sputum

Adaptability of the PNA-FISH and DNA-FISH assays to human sputum was evaluated, using sputum samples spiked with *M. immunogenum* (Fig. 5A) or *P. fluorescens* (Fig. 5B). The results showed the same fluorescence intensity of the sputum-spiked cells as that of fresh live cells in liquid suspension, demonstrating the feasibility of clinical applications of the developed FISH assays.

4. Discussion and conclusions

Fluorescence *in situ* hybridization (FISH) assay for bacteria is based on specific binding of antisense nucleic acid probe to the 16S rRNA [20]. Lately, use of probes based on peptide nucleic acid backbone, that offers the desired hydrophobic characteristics needed to cross the hydrophobic cell wall, has been investigated for the mycobacterial FISH assays [23,32]. However, there is need for development of species-specific probes for this phenotypically diverse but phylogenetically conserved group of pathogens. Considering that the PNA probes are based on the relatively phylogenetically conserved 16S rRNA gene, the probe composition and location of mismatches can limit their utility for species differentiation [13], particularly in closely related species of individual mycobacterial groups/complexes. A mismatch located in the middle of the probe has a better discriminative effect than a mismatch close to the 5′ or 3′ end [12]. These criteria were taken into consideration while designing the specific-probe for *M. immunogenum* in this study. Consequently, the PNA probe showed lack of any cross-reactivity against other test species of mycobacteria, gram-positive bacteria and gram-negative bacteria. Furthermore, equal hybridization signal intensities with different genotypes of *M. immunogenum* demonstrated the authenticity of the probe for this species. The probe was able to discriminate *M. immunogenum* from *M. chelonae*, the other species of *Mycobacterium* known to be prevalent in metalworking fluids [9]. Likewise, the PNA probe showed no cross-reactivity with *M. abscessus*, the other closely related member species of the *M. chelonae* complex often causing cross-identification problems in clinical specimens [28].

Table 2
Evaluation of the PNA-FISH and DNA-FISH assays for quantification of *M. immunogenum* and pseudomonads in in-use field MWF samples

MWF samples	Counts (cells ml^{-1})
M. immunogenum (*PNA-FISH*)	
Synthetic MWF sample 1	$9.64\ (\pm 0.61) \times 10^4$
Synthetic MWF sample 2	$3.30\ (\pm 1.04) \times 10^5$
Semi-synthetic MWF sample 1	$1.76\ (\pm 0.92) \times 10^4$
Semi-synthetic MWF sample 2	$6.63\ (\pm 2.22) \times 10^4$
Pseudomonads (*DNA-FISH*)	
Synthetic MWF sample 1	$1.80\ (\pm 0.74) \times 10^5$
Synthetic MWF sample 2	$3.40\ (\pm 1.92) \times 10^5$
Semi-synthetic MWF sample 1	$1.05\ (\pm 0.45) \times 10^6$
Semi-synthetic MWF sample 2	$8.75\ (\pm 5.04) \times 10^5$

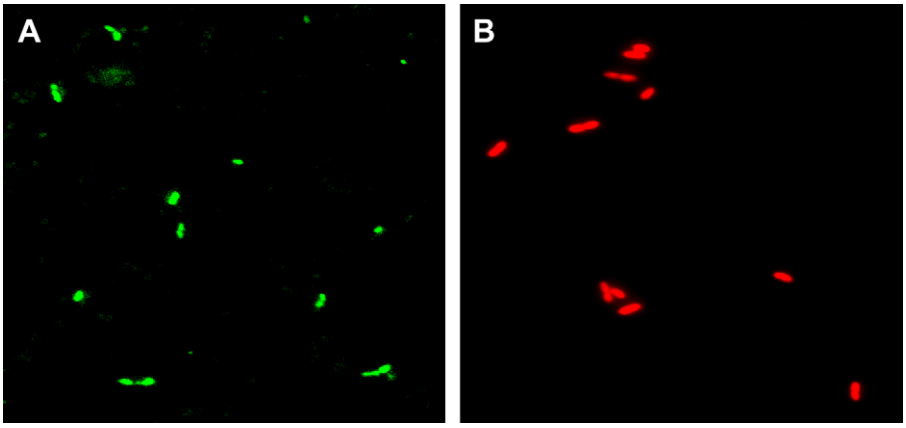

Fig. 5. Application of the fluorescence *in situ* hybridization (FISH) assays for specific detection of *M. immunogenum* (Panel A) and *P. fluorescens* (Panel B) in simulated human sputum.

FISH has inherent advantages over nucleic acid amplification-based methods such as in terms of avoiding possible false-positive signal due to DNA contamination and false-negative results caused by amplification inhibitors, in the latter [16]. Background interference in complex samples may be much higher in amplification methods as compared to FISH. In this context, our results showed that both PNA-FISH and DNA-FISH assays, targeting *M. immunogenum* and *P. fluorescens*, respectively, were readily adaptable to complex sample matrix, as in MWF and sputum that often causes interfering effect in microscopy. The selective conditions used in FISH hybridization reaction (use of selective hybridization buffer under stringent conditions) may be responsible for this observed circumventing effect toward the matrix background. However, in contrast to nucleic acid amplification-based methods, the quantitative efficiency of FISH is limited to about $\geq 10^3$ cells ml^{-1} due to inherent limitations of microscopy for quantification.

The PNA-FISH assay developed in this study is rapid considering a time-to-result of about 3.0 h with only 45 min of hands-on time (including fixation, hybridization and microscopy). This is consistent with the earlier report of PNA-FISH assay validated for detection of the members of *M. tuberculosis* complex in clinical specimens [12]. Moreover, the PNA-FISH assay for *M. immunogenum* showed no or little autofluorescence, unlike the reported autofluorescence problem with *M. avium* subsp. *paratuberculosis* [13].

Considerable non-culturable microbial population is invariably present in metalworking fluids as a result of the prevailing harsh conditions [15,31]. Despite being non-culturable (viable-but non-culturable or non-viable), this fraction has been considered as a potential contributor to the antigenic load in MWF that is responsible for immunogenic reactions underlying HP in the exposed machine workers [2,14,30]. The FISH assays successfully allowed the detection of even non-culturable population of *M. immunogenum* or *P. fluorescens*. Considering that the PNA-FISH probes target the rRNA molecules [23], their use may be considered questionable in case of detecting non-viable cells. However, the results showed comparable detection for the live versus non-culturable cells in the fluid matrix, indicating the usefulness of the 16S rRNA-based PNA-FISH analysis for assessing the total mycobacterial load.

In conclusion, we have developed a highly specific PNA probe for *M. immunogenum*. Our validated FISH assays based on *M. immunogenum*-specific PNA probe and pseudomonads-specific DNA oligo probe are adaptable for the complex matrices of the metalworking fluids and human sputum specimens. Being microscopy-based, the PNA-FISH assay is less skill-intensive and an economical way to screen for *M. immunogenum* in these matrices as compared to other DNA amplification-based methods. Considering these advantages, the FISH assays offer a potentially simple and rapid alternative to amplification-based methods for timely identification of the targeted microbial species/groups in industrial analytical laboratories for MWF analysis, and in clinical diagnostic laboratories for clinical specimens. Future research may involve further evaluation and application of these FISH assays in epidemiological studies on the etiological role of *M. immunogenum* in causing hypersensitivity pneumonitis in MWF-exposed machine workers and nosocomial infections in hospital settings and in developing antimicrobial control strategies.

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