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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