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ORIGINAL ARTICLE

Specific detection and quantification of culturable and non-culturable mycobacteria in metalworking fluids by fluorescence-based methods

S.B. Selvaraju*, I.U.H. Khan† and J.S. Yadav

Microbial Pathogenesis and Toxicology Laboratory, Department of Environmental Health, College of Medicine, University of Cincinnati, Cincinnati, OH 45267-0056, USA

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Correspondence
Jagjit S. Yadav, Environmental Genetics and Molecular Toxicology Division, Department of Environmental Health, College of Medicine, University of Cincinnati, Cincinnati, OH 45267-0056, USA.
E-mail: jagjit.yadav@uc.edu

Present address
*S.B. Selvaraju, Children's Mercy Hospitals and Clinics, 2401 Gillham Road, 3719.00 Research, Kansas City, MO 64108, USA.

†I.U.H. Khan, Environment Canada, National Water Research Institute (NWRI), 867 Lakeshore Rd, Burlington, ON L7R 4A6, Canada.

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Abstract

Aims: To optimize and evaluate fluorescence microscopy assays for specific assessment of mycobacteria and co-contaminants, including culturable and non-culturable sub-populations, in metalworking fluids (MWF).

Methods and Results: Auramine-*O*-rhodamine (AR) staining and LIVE/DEAD *Bac*Light™ Bacterial Viability staining (L/D staining) were adapted and evaluated for detection/quantification and differentiation (viable *vs* non-viable) of the MWF-associated mycobacteria and the background bacterial flora, respectively. The AR staining method was found to be specific to MWF mycobacteria with a minimum detection limit of 10 cells ml^{-1} and was comparable to the QPCR in quantification efficiency in MWF matrix. The L/D staining-based microscopy allowed differential quantification of viable *vs* non-viable cells. In general, a 3-log difference was observed between the L/D microscopy count and culture count accounting for the presence of non-culturable fraction in the bacterial population in in-use MWF.

Conclusions: The optimized AR staining- and the L/D staining-based microscopy methods have the potential for rapid, specific and differential assessment (viable *vs* non-viable) of MWF-associated mycobacteria and co-contaminants in field MWF.

Significance and Impact of the study: Early detection of MWF mycobacteria by rapid, low-cost, less-skill intensive and culture-independent fluorescence-based microscopy methods will facilitate timely intervention to protect the machine workers from occupational hazards.

Introduction

Non-tuberculous mycobacteria of the *Mycobacterium chelonae* complex (MCC) colonizing water-based metalworking fluids (MWF) have been associated with hypersensitivity pneumonitis and other respiratory illnesses in exposed machine workers (CDC 2001). Particularly, two MCC species, *Mycobacterium immunogenum* and *Myco. chelonae*, have been identified in diverse MWF operations (Yadav *et al.* 2003; Selvaraju *et al.* 2005a). In addition, microbial populations in in-use MWF contain large amounts of non-culturable (viable or non-viable) cells (Yadav *et al.* 2003; Khan and Yadav 2004a) and these sub-populations of microbes and their products collectively contribute to the MWF-associated respiratory health hazards (Kreiss and Cox-Ganser 1997; Besnard *et al.* 2000). In view of these facts, routine fluid monitoring is paramount to the detection of viable and non-viable microbial and/or mycobacterial populations in a timely manner for appropriate interventions. This calls for development of rapid, less expensive and adaptable assays such as those based on microscopy for the routine monitoring applications in these environments. Here, in this study, we have developed and evaluated (i) an epifluorescence microscopy technique for specific detection and quantification of total mycobacteria in presence of other co-contaminants in MWF samples and (ii) a Live/Dead fluorescence staining-based selective assay for rapid and specific detection and quantification of viable *vs* non-viable mycobacteria and/or other bacteria in contaminated MWF.

Materials and methods

Micro-organisms and culture conditions

Two MWF isolates, *Myco. immunogenum* 700506 and *Bacillus* sp. B22, and a Pseudomonads representative species *Pseudomonas fluorescens* (ATCC 13525) used in this study were maintained and cultured as described in our earlier study (Selvaraju *et al.* 2005b).

Metalworking fluids

Three different types of MWF, pristine synthetic, pristine semi-synthetic and in-use synthetic, obtained from a US-based plant were used in this study. Each MWF type was diluted to the desired concentration and used as described earlier (Yadav *et al.* 2006).

AR staining for MWF mycobacteria

Auramine-O-rhodamine (AR) staining was tested with the pristine synthetic and semi-synthetic fluids spiked with *Mycobacterium immunogenum*, an in-use contaminated synthetic MWF that contained mixed microflora other than mycobacteria, and an in-use MWF that contained mycobacteria. *Mycobacterium immunogenum* was serially diluted in MWF to get 10^1 through 10^6 CFU ml^{-1} in pristine synthetic and semi-synthetic MWF containing pre-spiked *Ps. fluorescens* and *Bacillus* sp. at 10^6 CFU ml^{-1} each. One millilitre each of the three MWF preparations was centrifuged at 14 000 rev min^{-1} for 30 min and the pellet was resuspended in 100 μl (higher count samples: $>10^3$ CFU ml^{-1}) or 10 μl (low count samples: 10^2 and 10^1 CFU ml^{-1}) of the respective MWF supernatant. Ten microlitre of this suspension was applied directly onto the marked area (1 cm^2) on a Reich counting slide (Bellco Glass Inc., Vineland, NJ, USA) meant for quantitative microscopy. AR staining protocol based on the TB Fluorescent Stain Kit T (BD Biosciences, Sparks, MD, USA) was used as per the manufacturer's instructions. In parallel, a pure cell suspension of *Myco. immunogenum* serially diluted in physiological saline was used as control.

Comparison with real-time QPCR quantification

AR staining protocol was evaluated for its minimum quantification limit by comparing it with our previously optimized mycobacteria-specific real-time QPCR method (Khan and Yadav 2004a,b; Yadav *et al.* 2006).

Live/Dead staining protocol (viable *vs* non-viable cells)

A Live/Dead staining (L/D staining) protocol based on LIVE/DEAD *Bac*Light™ Bacterial viability kit (Molecular Probes, Eugene, OR, USA) was applied to study the viable *vs* non-viable bacterial cells in MWF. The L/D staining protocol was evaluated using pure suspensions of *Myco. immunogenum* or *Ps. fluorescens* and an in-use MWF sample which contained mixed microflora other than mycobacteria. Viable and non-viable cell suspensions in MWF were prepared and the staining performed as per the manufacturer's instructions. Pure cell suspensions of *Myco. immunogenum* and *Ps. fluorescens* in physiological saline served as controls. The selective viable counting for mycobacteria in field MWF sample was carried out by killing the other co-contaminants with PANTA plus antibiotic mixture (BD Biosciences) as described earlier (Yadav *et al.* 2006). The PANTA-treated sample was examined using the L/D staining to estimate viable mycobacteria, whereas the untreated control was stained with the mycobacteria-specific AR staining protocol. The difference yielded the non-viable mycobacteria count. The field-contaminated in-use MWF fluid was directly used to count the viable *vs* non-viable total bacterial population.

Non-specific staining of MWF background flora

Simple staining using 0.3% methylene blue solution for 3 min was used for detection and quantification of the total background flora in different MWF matrices. The staining sample suspensions were prepared as described for Epifluorescence microscopy counting and examined using light microscope (100× objective).

Microscopy

All epifluorescence microscopy counts were performed on a Nikon eclipse TE 300 Microscope System using Texas red filter cube (excitation wavelength 530–590 nm; dichoric mirror wavelength 595 nm, barrier filter wavelength 610–690 nm) using 100× (UV-F) objective. On the other hand, a bright field microscopy was used to detect methylene blue stained cells. Randomly selected 10 microscopic fields were counted in the marked one square cm area on the Reich counting slide and the number of bacteria per ml of the original MWF sample was calculated based on the following formula: $N_b \times M_f \times 1/V \times 1/C$, 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).

Selective agar plating

Simulated pristine or in-use MWF matrices containing *Myco. immunogenum* and the background test organisms were prepared and treated with PANTA plus antibiotic mixture as described earlier (Yadav *et al.* 2006) to selectively inactivate the co-contaminants. The treated suspension was centrifuged (14 000 rev min^{-1} for 30 min) and an aliquot (100 μl) of the pellet suspension was spread plated on M7H10 agar as described earlier (Yadav *et al.* 2006). A pure *Myco. immunogenum* suspension was treated and plated out in parallel to estimate the effect of antibiotic mixture on the viability of mycobacteria, if any.

Statistics

Statistical analysis was based on log values of bacterial populations by two-tailed *t*-test for the significance of the difference between the means of two samples. A *P*-value of <0.05 was considered to be significant.

Results

Detection and quantification of MWF mycobacteria

The AR staining selectively stained the *Myco. Immunogenum* cells as distinct red to bright orange, while the Gram-positive and Gram-negative bacterial co-contaminants remained unstained. In contrast, the simple staining (methylene blue) stained the background microflora blue in all simulated MWF samples without staining *Myco. immunogenum*. The results revealed that the minimum detection limit (MDL) for the mycobacterial sub-population using AR staining was 10 cells ml^{-1} for each matrix (Fig. 1) regardless of the pure *vs* mixed nature of the suspension. A pre-concentration of the low-count samples before staining helped to achieve this MDL value. Selective agar plating (M7H10 agar supplemented with PANTA plus) showed that the MDL for the agar plating method was in the range of 10 CFU ml^{-1} (synthetic) to 1000 CFU ml^{-1} (semi-synthetic) as compared to an MDL of 10 cells ml^{-1} for all matrices with fluorescence staining. Applicability of the AR staining protocol was validated by comparing it with real-time PCR quantification and agar plating. The results showed that there was no statistical difference between the microscopy counting (3.97×10^5 cells ml^{-1}) and QPCR estimation (0.66×10^5 cells ml^{-1}; Fig. 2; $P = 0.220$) for the purpose of mycobacterial quantitative assessment. However, ≥3-log difference in mycobacterial cell number was observed between the microscopy count (3.97×10^5 cells ml^{-1}) and the agar plate count (3.0×10^2 CFU ml^{-1}), because of presence of non-culturable cells (Fig. 2; $P = 0.0019$).

Quantification of background microflora in MWF

In in-use synthetic fluid, a statistically significant difference between microscopy quantification (1.93×10^7 cells ml^{-1}) as compared with Tryptic soy agar plating (5.0×10^4 CFU ml^{-1}) was presumably due to non-culturable fraction ($P = 0.003$). In parallel, microscopy count (using simple staining) in another in-use MWF sample showed a total microbial population of 2.82×10^5 cells ml^{-1} against a cultural count of 3.1×10^2 CFU ml^{-1}, indicating the presence of even higher number of non-culturable cells ($P = 0.00077$; Fig. 5).

Differential quantification of viable *vs* non-viable bacterial sub-populations

The L/D staining for detection/quantification of viable *vs* non-viable *Myco. immunogenum* and *Ps. fluorescens* spiked individually in pristine synthetic MWF allowed direct differentiation of the viable *vs* non-viable cells (as distinct bright green and red stained cells, respectively). The viable count (*Myco. immunogenum*: 5.6×10^7; *Ps. fluorescens*: 4.5×10^7 cells ml^{-1}) and non-viable count (*Myco. immunogenum*: 3.8×10^7; *Ps. fluorescens*: 4.3×10^7 cells ml^{-1}) coincided with the spiked number of cells ($P = 0.511$ (*Myco. immunogenum*); $P = 0.395$ (*Ps. fluorescens*); Fig. 3). Similarly, the mixed microflora of in-use MWF sample showed a culture-based viable count of 0.8×10^7 CFU ml^{-1} as compared with the microscopy-based viable count of 1.1×10^7 cells ml^{-1} ($P = 0.737$; Fig. 3). In addition, microscopy counting revealed a sizeable non-viable population (4.0×10^6 cells ml^{-1}) in the sample (Fig. 3). This indicated a successful application potential of the L/D staining protocol to MWF, to differentiate the viable and non-viable microflora.

Selective quantification of viable mycobacteria in an MWF sample was possible by using the PANTA plus antibiotic mixture. The results showed no statistical difference between viable mycobacterial count (2.1×10^2 cells ml^{-1}) and direct cultural count (3.0×10^2 CFU ml^{-1}; Fig. 4; $P = 0.473$). Additionally, it was possible to deduce the non-viable fraction of mycobacterial population (3.96×10^5 cells ml^{-1}) from the total mycobacterial population (3.97×10^5 cells ml^{-1}) as determined using

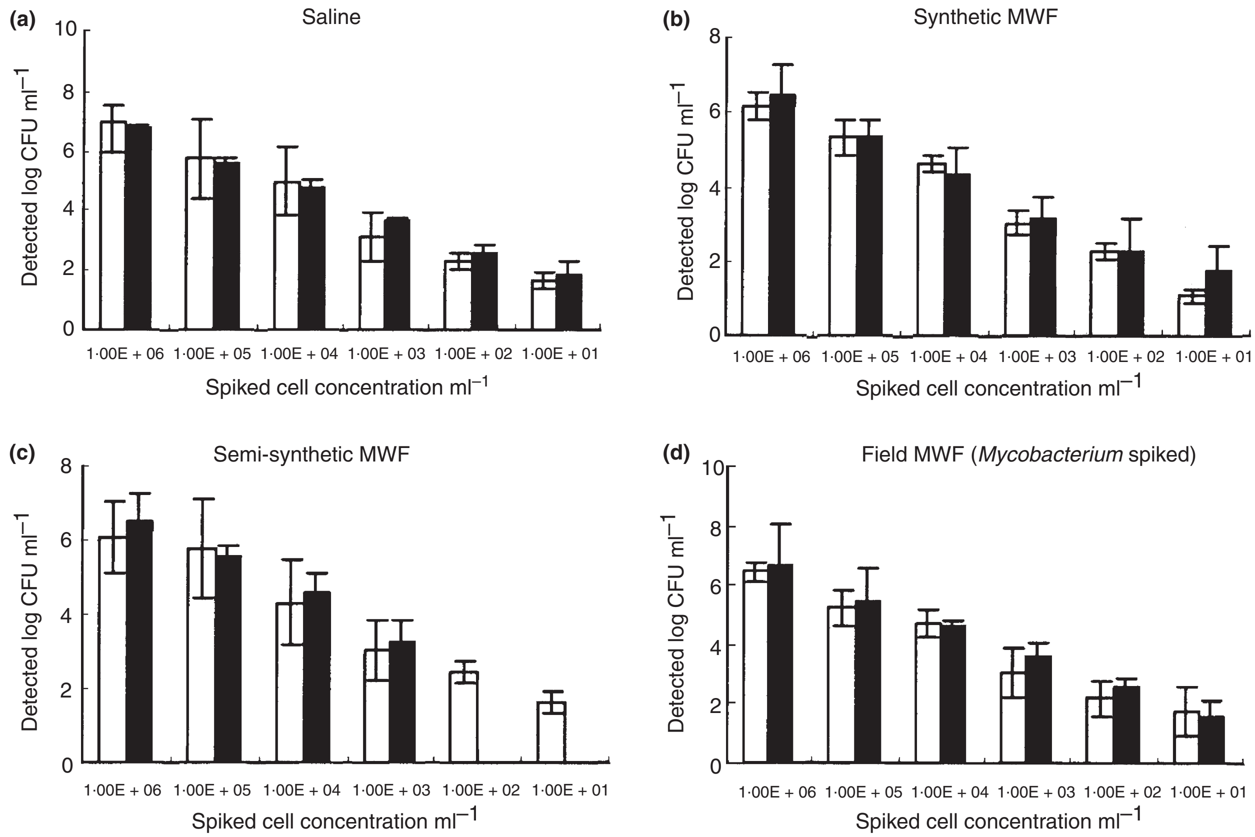

Figure 1 Applicability of the auramine-*O*-rhodamine staining for microscopic detection and quantification of *Mycobacterium Immunogenum*. The mycobacterial counts were compared in different sample matrices: saline (a), pristine synthetic metalworking fluid (MWF) (b), pristine semi-synthetic MWF (c), and in-use synthetic MWF (d). Minimum quantification limit for the microscopy protocol was compared with that of the culturing method. (□) Microscopy, (■) Plate count.

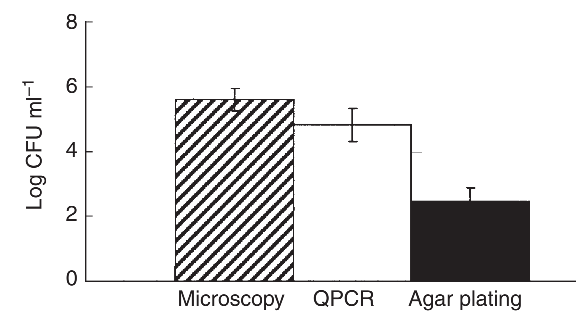

Figure 2 Comparison of the auramine-*O*-rhodamine staining-based microscopy, real-time QPCR and selective agar plating for quantification of mycobacteria in in-use metalworking fluid.

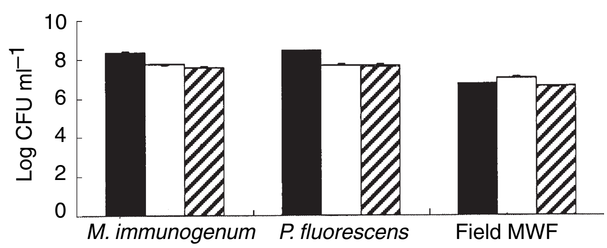

Figure 3 Differential epifluorescence-based quantification of viable *vs* non-viable cells in pure suspensions (water) and mixed microflora (metalworking fluid) using the optimized Live/Dead staining protocol and its comparison with the cultural method. (■) Plate count, (□) viable count, (▨) non-viable cells.

AR staining (Fig. 4). Of the total microbial load (2.82×10^5 cells ml^{-1}) in in-use sample, as determined by simple staining, the viable and non-viable subpopulations as determined by the L/D staining were 0.69×10^5 and 1.86×10^5 cells ml^{-1}, respectively (Fig. 5). However, the culturable count was 3.0×10^2 and 1.0×10^1 CFU ml^{-1} on M7H10 agar and Tryptic soy agar, respectively (Fig. 5). Comparison of the culturable count with the microscopy-based viable count (culturable + non-culturable) revealed a viable-but-non-culturable (VBNC) population of 6.86×10^4 cells ml^{-1} in the field MWF sample (Fig. 5).

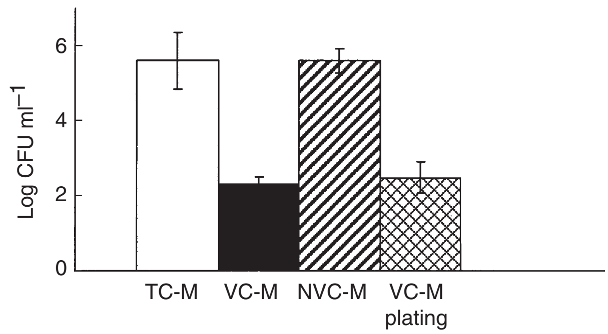

Figure 4 Mycobacterial sub-populations (viable and non-viable) in field in-use metalworking fluid as determined by the optimized fluorescence microscopy methods *vs* the culturing method. The following counts were determined: Mycobacterial total count (TC-M based on auramine-*O*-rhodamine staining), Mycobacterial viable count (VC-M based on Live/Dead (L/D) staining on the PANTA antibiotic mixture-pretreated sample), Mycobacterial non-viable count (NVC-M based on L/D staining on the PANTA antibiotic mixture-pretreated sample), and Mycobacterial viable count (VC-M plating) based on culturing of the PANTA antibiotic mixture-pretreated sample on M7H10. The PANTA pretreatment conditions are as described under Materials and methods section.

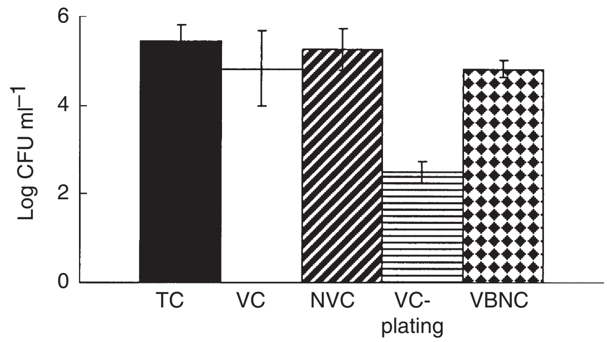

Figure 5 Bacterial sub-populations (viable, non-viable and viable-but-non-culturable) in the field MWF microflora. The different counts determined are as follows: Total bacterial count (TC-simple staining with 0.3% Methylene blue), viable count (VC-L/D staining), non-viable count (NVC-L/D staining), and culturable bacterial count (a sum of the counts from Tryptic soy agar and M7H10 agar plating). The viable-but-non-culturable (VBNC) count was derived by subtracting the viable microscopy count and viable plate count.

Discussion

Accurate assessment of mycobacteria in MWF matrices by the conventional culturing method is difficult because of the required long incubation time (4–5 days) and presence of the competing fast-growing background microflora and the non-culturable microbial subpopulation (Yadav *et al.* 2006). Instead microscopic method based on AR staining could be applied for the specific and rapid detection and enumeration of mycobacteria in these matrices. The AR staining approach has been successfully applied to clinical mycobacteria (Tenover *et al.* 1993; Somoskovi *et al.* 2001). Our results showed that the AR staining method as applied to MWF is rapid (1 h) and as sensitive as culturing (10 cells ml^{-1}) to detect or quantify MWF mycobacteria. However, the observed higher limit of detection (1000 cells ml^{-1}) for the agar plating in semi-synthetic MWF as compared to the other fluids might be due to the inhibitory effect of the components in the former even at 2% concentration.

As is known, the microscopy-based counting reveals the total population of cells as against the culture-based counting using agar plating that yields only the culturable fraction of the population. Consistent with this phenomenon, quantification of co-contaminants in in-use MWF in this study, revealed a clear difference between the microscopic count (simple staining) and cultural count (agar plating) and the difference could be attributed to the presence of non-viable or VBNC cells. Despite the above advantage, the AR staining was not applicable for differentiating the viable and non-viable cells of mycobacteria. Hence, an alternate fluorescence-based protocol using L/D staining was applied to MWF matrix for this purpose. Considering the presence of industrial debris and other impurities in used-MWF, we had to optimize the differential L/D staining protocol for the MWF matrix. The L/D staining has been used for viability analysis of bacterial species in several matrices such as seawater, drinking water, and food products (Joux *et al.* 1997; Boulos *et al.* 1999; Duffy *et al.* 2000). However, this study reports the first application of this approach to the MWF. The fluorescence microscopy-based viable count coincided with the culturable count in the freshly-spiked MWF. In addition, our study demonstrated the possibility of detection of the non-viable and VBNC sub-populations by the L/D staining in MWF matrices. In-use MWF results using this method clearly suggest that high percentage of cells are present in viable-but-non-culturable state in MWF and detection of this fraction is important as these cells do contribute to the microbial antigens and endotoxins in MWF (Bernstein *et al.* 1995). For instance, in a previous study (Mattsby-Baltzer *et al.* 1989), the culturable count of the MWF aerosol was shown to grossly underestimate the total microbial mass and endotoxins inhaled by the machine workers as the viable counts represented <10% of the total bacterial mass present.

To our knowledge, this is the first report on the specific epifluorescence microscopy-based detection and quantification of mycobacteria in MWF samples. Furthermore, a differential fluorescence-based microscopy protocol was applied to MWF to detect/quantify both viable and non-viable cells of mycobacteria and/or background co-contaminants to estimate the total microbial load without any culturing. Considering that these microscopy-based methods are rapid and not skill-intensive, an early knowledge on the mycobacterial contamination of MWF such as that based on the developed specific and rapid microscopy-based protocols could help in efficient fluid management of MWF and prevention of exposure of machine workers to the aerosolized contaminated MWF.

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