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ORIGINAL PAPER

Development of a real-time TaqMan assay to detect *mendocina* sublineage *Pseudomonas* species in contaminated metalworking fluids

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Abstract A TaqMan quantitative real-time polymerase chain reaction (qPCR) assay was developed for the detection and enumeration of three *Pseudomonas* species belonging to the *mendocina* sublineage (*P. oleovorans*, *P. pseudoalcaligenes*, and *P. oleovorans* subsp. *lubricantis*) found in contaminated metalworking fluids (MWFs). These microbes are the primary colonizers and serve as indicator organisms of biodegradation of used MWFs. Molecular techniques such as qPCR are preferred for the detection of these microbes since they grow poorly on typical growth media such as R2A agar and *Pseudomonas* isolation agar (PIA). Traditional culturing techniques not only underestimate the actual distribution of these bacteria but are also time-consuming. The primer–probe pair developed from *gyrase B* (*gyrB*) sequences of the targeted bacteria was highly sensitive and specific for the three species. qPCR was performed with both whole cell and genomic DNA to confirm the specificity and sensitivity of the assay. The sensitivity of the assay was 10^1 colony forming units (CFU)/ml for whole cell and 13.7 fg with genomic DNA. The primer–probe pair was successful in determining concentrations from used MWF samples, indicating levels between 2.9×10^3 and 3.9×10^6 CFU/ml. In contrast, the total count of *Pseudomonas* sp. recovered on PIA was in the range of $<1.0 \times 10^1$ to 1.4×10^5 CFU/ml for the same samples. Based on these results from the qPCR assay, the designed TaqMan primer–probe pair can be efficiently used for rapid (within 2 h) determination of the distribution of these species of *Pseudomonas* in contaminated MWFs.

Keywords *Pseudomonas mendocina* sublineage · Quantitative real-time PCR · Metalworking fluids · *gyrB* · TaqMan assay

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Introduction

Metalworking fluids (MWFs) are complex mixtures of chemicals that are indispensable materials in industry. They are used as cooling and lubricating agents in different machining processes such as grinding, milling, and cutting. In addition, they help to improve the finish of work pieces and increase the tool life by preventing corrosion [13, 19]. MWFs are affected by different types of contaminations, i.e., physical, chemical, and microbial factors. They are highly vulnerable to microbial contamination; these microbes can act both as potential pathogens and deteriogens [13], as well as causing skin dermatitis and hypersensitivity pneumonitis [19, 22]. They cause biofouling by altering fluid quality and performance by changing fluid viscosity and lowering the pH of the fluid, causing corrosion and leaks in the machining system [5, 13]. The contaminated MWFs can exhibit high degrees of microbial loading, ranging from 10^4 to 10^{10} colony forming units (CFU)/ml. The organic carbon present in the chemical components of MWF supports the growth of a wide variety of microorganisms such as species of *Staphylococcus*, *Pseudomonas*, sulfate-reducing bacteria, *Acinetobacter*, *Mycobacterium immunogenum*, and the recently described *P. oleovorans* subsp. *lubricantis* [7, 9, 21].

The dominant group of bacteria found in contaminated MWFs belongs to the genus *Pseudomonas*, such as *P. fluorescens* and species belonging to the *mendocina* sublineage (*MS*) [6, 13, 18]. Based on 16S rRNA sequences, *P. mendocina*, *P. oleovorans*, *P. pseudoalcaligenes*, and *P. alcaliphila* cluster together under the *P. aeruginosa* intrageneric cluster and are considered as the *MS* [8]. They are indicator organisms because they are commonly found in used MWFs and are capable of growing at an alkaline pH (9.0–11.0), (the range that is generally found in MWFs) [19]. They are considered as primary colonizers as this growth reduces the pH, which then favors the growth of other bacteria such as enterobacteria and mycobacteria [22]. The actual distribution of these bacteria in MWFs is difficult to study as these nonfluorescent pseudomonads typically fail to grow on media commonly used with MWFs such as *Pseudomonas* isolation agar (PIA) (due to the presence of triclosan) and are also underestimated with the conventional culturing methods using media for heterotrophic plate count (HPC) and selective-differential media [1, 4, 6, 17, 23]. This observation was further confirmed by the description of a new subspecies of *Pseudomonas oleovorans*, *P. oleovorans* subsp. *lubricantis*, that failed to grow on PIA but was recovered on Middlebrook 7H11 media, used for the recovery of rapidly growing *Mycobacterium* species [14].

To overcome these limitations with classical culturing techniques, molecular technology such as real-time polymerase chain reaction (PCR) has been applied for the detection of these bacteria in MWFs [12]. Quantitative real-time PCR (qPCR) is an efficient molecular technique that is culture-independent and provides a more precise detection and quantification of microbial loads present in a wide variety of samples [3, 11, 15, 26]. Earlier studies with used MWFs had developed real-time assays for the detection of rapidly growing *Mycobacterium* species, mainly *M. immunogenum*, suspected to cause hypersensitivity pneumonitis [12, 24]. Khan and Yadav [5] developed an assay for the detection of culturable and nonculturable species of *Pseudomonas* by using the fluorescent dye SYBR green, which requires an additional analysis of the dissociation curve of the PCR product for determining the specificity of the primer pairs [2]. In contrast to using whole cells, extracted genomic DNA from contaminated MWF samples has also been used in qPCR assays for the detection and quantification of mycobacteria and pseudomonads that requires additional cost (labor and reagents) and turnaround time [5, 20, 24].

The objective of this study was to develop a fast and accurate molecular method for the detection and enumeration of the three *Pseudomonas* species of interest belonging to *MS* from contaminated MWFs. A TaqMan qPCR assay targeting the *gyrB* gene was developed for the identification and enumeration of the three indicator *Pseudomonas* species. The *gyrB* gene was selected because the three species used in the present study are closely related to each other and the *gyrB* sequence provided a better molecular marker for the development of the primer–probe pair [14]. TaqMan qPCR assay was developed as it is highly specific and does not require the generation of a dissociation graph. In contrast to previous studies [5, 24], the direct use of contaminated MWF containing whole cells eliminated the DNA extraction step thus making the assay fast and more cost effective.

Materials and methods

Bacterial strains

The bacterial type strains belonging to *MS* selected for the study were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA): *P. oleovorans* subsp. *lubricantis* (ATCC BAA-1494); *P. pseudoalcaligenes* (ATCC 17440); *P. oleovorans* (ATCC 8062); *P. mendocina* (ATCC 2541); and *P. alcaliphila* (ATCC BAA-571). Although *P. mendocina* and *P. alcaliphila* are in the *MS*, they were not specifically targeted for detection in MWFs as these have not been reported to be found in MWFs. *P. aeruginosa* (ATCC 15442) and *P. fluorescens* were used to test the specificity of the probe as they belong to the rRNA group I along with the *MS* species [25]. *Klebsiella pneumoniae* (ATCC 13883) was used as it is one of the enterobacteria and has also been recovered from contaminated MWFs [23]. All strains were grown on tryptic soy agar (TSA) and tryptic soy broth (TSB) (BD, Franklin Lakes, NJ, USA) for 24 h at 37°C except for *P. fluorescens*, which was grown at 30°C.

The total HPC from used MWF samples was estimated by culturing samples on TSA by spread plating as *Pseudomonas* species of interest did not grow well on the more commonly used R2A agar. The total *Pseudomonas* levels were determined by using PIA (BD, Franklin Lakes, NJ, USA) to compare its levels recovered by culturing technique with the levels of the three bacteria of interest detected by qPCR assay.

Metalworking fluid samples

The two unused or fresh MWF samples were obtained from two different MWF manufacturing companies. A total of six used MWFs were obtained from three different machining companies located in Michigan. Generally, 5% diluted (with tap water) fresh MWFs are used in machining operations; the same dilution was utilized in the present study with unused MWFs serving as a matrix in the spiking experiment. The target organism was grown on 10 ml TSB

at 37°C for 24 h. Following incubation 1 ml of the culture was taken in a microcentrifuge tube and was centrifuged at 14,000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml unused MWF solution. The spiked MWF sample was serially diluted and used for whole cell qPCR. The corresponding CFU/ml for each dilution was confirmed by spread plating on TSA agar and direct microscopic count. The used samples were analyzed without modification.

DNA isolation from bacterial cultures

The GenElute™ Bacterial Genomic DNA Kit (Sigma–Aldrich, St. Louis, MO, USA) was used for the isolation and purification of genomic DNA from the different bacterial strains. The purity and yield of the DNA was measured by using a NanoDrop® 1000 spectrometer (Thermo Scientific, Wilmington, DE, USA).

Whole cell preparation for PCR

MWF samples collected from different machining shops and freshly prepared samples were diluted 10-fold with 0.85% w/v NaCl saline before their utilization for whole cells preparation to prevent inhibition in the PCR reaction. Whole cell sample preparation was performed by placing 1 ml of sample in a microcentrifuge tube and heating it at 94°C for 10 min and immediately chilling in ice. The samples were stored in ice or at 4°C until the assay was performed.

Analysis of gyrB sequence and designing of real time TaqMan Primer-probe pair

The *gyrB* sequence obtained from *P. oleovorans* subsp. *lubricantis* [14] was analyzed along with the other closely related *Pseudomonas* species by using a Basic Local Alignment Search Tool (BLAST) search and CLUSTAL X [16] to determine sequence similarities. The primer–probe design software Allele ID 5.0 (Premier Biosoft, Palo Alto, CA, USA) was used to design the TaqMan primer–probe pair for the three *MS* species of *Pseudomonas*. The sequence of the primer–probe pair developed for the identification and estimation of the three MS *Pseudomonas* species are:

Sense primer: 5′-CGTTTCGACCGCATGATTTCC-3′
Antisense primer: 5′-CGCAGCTTGTCAATGTTGTACTC-3′
Antisense TaqMan probe: 5′-CCGATACCACAGCCGAGCGCAGT-3′

The properties of the probe were: $T_m = 65.6°C$; %GC = 65.2; self dimer (maximum ΔG) = −49.73 kcal/mol; and length = 23 bp. The probe was dual labeled with the fluorescent reporter dye (6-FAM) (IDT, Coralville, IA, USA) on the 5′ end and the quencher (BHQ-2) on the 3′ end.

Real-time PCR assays were performed by using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Each 25-µl reaction consisted of the following: 12.5 µl of TaqMan® Universal PCR Master Mix, 10 µl of genomic DNA or prepared whole cell, 1 µl each of forward and reverse primer (20 µM of working stock), and 0.5 µl (250 nM) of the probe. The PCR program used for the probe assay was as follows: cycle 1: (×1), step-1, 50.0°C for 2 min; cycle 2: (×1), step-1, 95.0°C for 10 min; cycle 3: (×40), step-1, 94°C for 15 s, step-2, 60.0°C for 60 s; and cycle 4: (×1), step-1, 25.0°C indefinite hold.

Results and discussion

Determination of the TaqMan assay specificity

The specificity of the assay was determined by using both genomic DNA and the whole cells of different *MS* species along with other bacteria (*P. aeruginosa*, *P. fluorescens*, and *K. pneumoniae*). Whole cells of the closely related members of *MS*, i.e., *P. oleovorans* subsp. *lubricantis, P. oleovorans*, *P. mendocina*, *P. pseudoalcaligenes*, and *P. alcaliphila*, were used for the determination of the specificity of the primer–probe pair. Amplification of the fluorescent signal was observed only for the three target species indicating that the assay was highly specific for these organisms. A similar result was also observed for the genomic DNA samples (Fig. 1).

Previously developed methods for qPCR assay in MWFs involved an additional step of DNA extraction from the

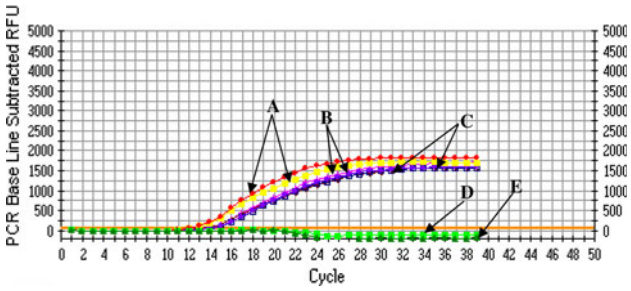

Fig. 1 Specificity assay of the TaqMan primer–probe pair for the closely related *MS Pseudomonas* species using genomic DNA targeting a specific region of the *gyrB* gene. Amplification signal was observed for *P. oleovorans* subsp. *lubricantis* (*A*), *P. pseudoalcaligenes* (*B*), and *P. oleovorans* (*C*). No amplification signal was detected for *P. alcaliphila* (*D*) and *P. mendocina* (*E*). Each species were tested in duplicate

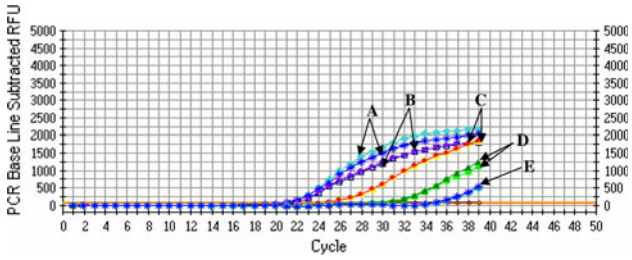

Fig. 2 Sensitivity assay of the TaqMan primer–probe pair targeting a specific region of the *gyrB* gene using varying concentrations of whole cells of *P. oleovorans* subsp. *lubricantis* (*A–E* 10^5–10^1 CFU/ml, respectively). The assay was capable of detecting as low as 10^1 CFU/ml. Each dilution was tested in duplicate

samples [5, 20, 24]. There is also a probability of differences in the yield of DNA depending on the extraction kit used [10]. The advantage of directly using contaminated MWFs in the real-time assay is the elimination of the DNA extraction step. A DNA extraction procedure usually requires 1–2 h depending on the extraction kit used, whereas the preparation of the whole cells requires only 15 min.

Real-time assay with *P. oleovorans* subsp. *lubricantis* cells spiked into MWF and development of standard curves

The assay was performed by using different concentrations of cells (10^1–10^5 CFU/ml). Amplification signals were obtained for all the concentrations studied, indicating the sensitivity of the assay (Fig. 2). Similar results were obtained with the different concentrations of genomic DNA (data not shown). The assay was able to detect as low as 13.7 fg of genomic DNA in a sample. The sensitivity of the assay indicated that it could be used for the detection and estimation of these bacteria in used MWFs with widely varying degrees of contamination. The sensitivity and the specificity of the whole cell assay in the present study were similar to the values obtained in earlier studies (10^1 CFU/ml) with genomic DNA [5, 20].

Standard curves were constructed by using different concentrations of both whole cells (10^1–10^5 CFU/ml) and genomic DNA (13.7 fg to 13.7 ng) of *P. oleovorans* subsp. *lubricantis*, *P. oleovorans*, and *Pseudomonas pseudoalcaligenes* for the primer–probe pair spiked in freshly prepared MWF. Plots of cycle threshold (Ct) versus nanograms of DNA and also CFU/ml were obtained from the qPCR assay and used to determine the concentration of cells present in used MWF samples collected from different industries. The standard graph obtained by using whole cells of *P. oleovorans* subsp. *lubricantis* indicated both the sensitivity and efficiency of the real-time assay, as was evident from the correlation coefficient value ($R^2 = 0.998$) (Fig. 3). Similar results were obtained for *P. oleovorans* and *P. pseudoalcaligenes*.

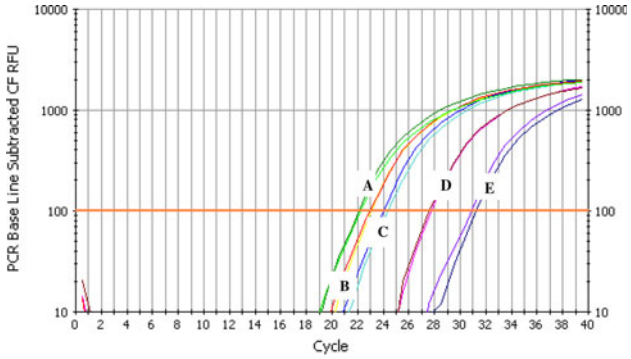

Fig. 4 Detection and enumeration of three *MS Pseudomonas* species from used metalworking fluid samples MWFs. Logarithmic curve of real-time detection and enumeration of three *Pseudomonas* species from used metalworking fluid samples (*C–E* 10^4 to 10^2 CFU/ml whole cells of *P. oleovorans* subsp. *lubricantis*, respectively; *A* and *B* represent used MWF samples). Each sample was tested in duplicate

qPCR detection and enumeration of three MS *Pseudomonas* species from used MWF samples

The TaqMan primer–probe pair was successfully used in whole cell qPCR for identification and enumeration of the three species of *Pseudomonas* in used MWF samples collected from different machining operations (Fig. 4). The concentrations were determined from the standard graph constructed by using known concentrations of *P. oleovorans* subsp. *lubricantis*. The HPC levels were in the range of $<1.0 \times 10^1$ to 3.5×10^6 CFU/ml (Table 1), whereas the

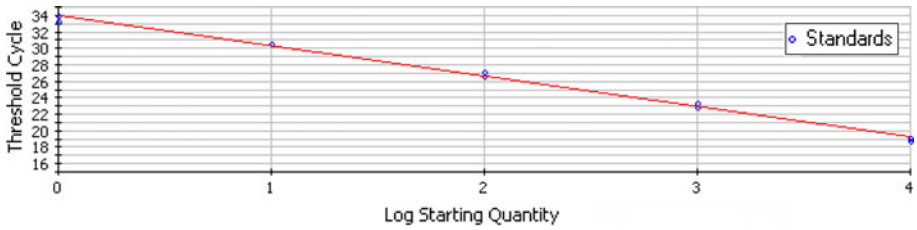

Fig. 3 Linear representation of the standard graph constructed by using different concentrations of whole cells (10^5–10^1 CFU/ml) of *P. oleovorans* subsp. *lubricantis*. The TaqMan primer probe pair targeted a region in the *gyrB* gene. Each dilution was tested in duplicate

Table 1 Quantitative real-time TaqMan PCR (qPCR) analysis of three *mendocina* sublineage *Pseudomonas* species (*P. oleovorans*, *P. pseudoalcaligenes*, and *P. oleovorans* subsp. *lubricantis*) in used metalworking fluid samples

Location[a]	Sample type[b]	Sample age (year)	Viable count[c]		qPCR quantification of *MS Pseudomonas*	
			HPC[d]	*Pseudomonas* sp.[e]	qPCR count	Ct[f]
A	1	2.5	1.0×10^5	3.3×10^4	$3.9 (\pm 0.4) \times 10^6$	22.79 ± 0.1
B	2	2.0	4.5×10^4	$<1.0 \times 10^1$	$2.1 (\pm 0.01) \times 10^6$	23.69 ± 0.08
C	3a	0.33	$<1.0 \times 10^1$	$<1.0 \times 10^1$	ND	ND
	3b	0.67	$<1.0 \times 10^1$	$<1.0 \times 10^1$	$2.9 (\pm 0.3) \times 10^3$	35.16 ± 0.2
	4a	2.0	3.5×10^6	1.4×10^5	$2.1 (\pm 0.01) \times 10^5$	28.73 ± 0.03
	4b	0.5	9.2×10^4	$<1.0 \times 10^1$	$7.8 (\pm 1.1) \times 10^4$	30.24 ± 0.2

ND not detected

[a] Used metalworking fluids (MWFs) collected from different industries in Michigan

[b] MWF samples collected from different machining systems

[c] All viable counts of bacteria are in colony forming units (CFU) per ml

[d] Tryptic soy agar (TSA) was used for the heterotrophic plate count (HPC) of bacteria

[e] *Pseudomonas* isolation agar (PIA) was used to recover culturable *Pseudomonas* species not sensitive to triclosan

[f] Mean value of cycle threshold (Ct) of two replicates of used MWF samples ($n = 2$)

numbers of *Pseudomonas* sp. recovered on PIA were between $<1.0 \times 10^1$ to 1.42×10^5 CFU/ml. However, the qPCR assay results indicated that concentrations of the three *MS Pseudomonas* species were several times higher than the total number of *Pseudomonas* sp. recovered on PIA. Similar results were observed in a previous study by Khan and Yadav [5]; when assessing used MWFs the total culturable pseudomonads recovered on PIA and by qPCR ranged from 6.0×10^2 to 2.0×10^3 CFU/ml and 5.2×10^2 to 7.0×10^5 CFU/ml, respectively. Recently, 3.4×10^1 to 1.9×10^4 CFU/ml of *M. immunogenum* were detected by qPCR assay in industrial MWF samples, which was about 38.5% more than with the traditional culturing method [12].

In contrast to the TaqMan assay, SYBR green qPCR [5] requires the generation of a dissociation or melting curve to determine the specificity of the PCR product. The fluorescent SYBR green dye intercalates nonspecifically to any double-stranded nucleic acid [2], whereas TaqMan fluorogenic probes are highly specific and bind only to the target sequence [12]. The real-time technology has reduced the recovery time of microorganisms from 48 to 2 h. The study has also indicated that the designed primer–probe pairs were successfully used for the identification of the three indicators *Pseudomonas* species commonly found in contaminated MWF samples that are responsible for both biofouling and deterioration of fresh MWFs. The primer–probe pairs were not only efficient for both genomic and whole cells assays but also worked very efficiently when used in different contaminated MWFs collected from different industries. Thus, the development of a real-time assay incorporating whole cells and TaqMan probe could be successfully applied for the rapid detection and quantification of *Pseudomonas* species from different metalworking fluid systems used in industries.

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