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Development and evaluation of a new PCR assay for detection of *Pseudomonas aeruginosa* D genotype

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ABSTRACT

This report describes a new PCR-based assay for the detection of *Pseudomonas aeruginosa* genotype D in occupational saturation diving systems in the North Sea. This genotype has persisted in these systems for 11 years (1993–2003) and represents 18% of isolates from infections analysed during this period. The new PCR assay was based on sequences obtained after randomly amplified polymorphic DNA (RAPD)-PCR analysis of a group of isolates related to diving that had been identified previously by pulsed-field gel electrophoresis (PFGE). The primer set for the D genotype targets a gene that codes for a hypothetical class 4 protein in the *P. aeruginosa* PAO1 genome. A primer set able to detect *P. aeruginosa* at the species level was also designed, based on the 23S-5S rDNA spacer region. The two assays produced 382-bp and 192-bp amplicons, respectively. The PCR assay was evaluated by analysing 100 *P. aeruginosa* isolates related to diving, representing 28 PFGE genotypes, and 38 clinical and community *P. aeruginosa* isolates and strains from other species. The assay identified all of the genotype D isolates tested. Two additional diving-relevant genotypes (TP2 and TP27) were also identified, as well as three isolates of non-diving origin. It was concluded that the new PCR assay is a useful tool for early detection and prevention of infections with the D genotype.

Keywords Genotypes, identification, infections, PCR assay, *Pseudomonas aeruginosa*, saturation diving

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INTRODUCTION

Saturation diving is used regularly during the maintenance and inspection of offshore subsea petroleum production systems in the North Sea. The working and living environment of the divers is unique, in that they are chronically exposed to hyperbaric, warm and humid surroundings that are capable of maintaining a rich microbial flora [1–5]. Exposure to such hyperbaric environments has been associated with a risk of acquiring various conditions, including decompression sickness, arterial gas embolism, neurological symptoms and pulmonary dysfunctions [6]. However, the medical problems encountered most frequently by divers are ear, nose and throat complications [7]. Of these, acute infection of the outer ear has been a well-known and frequent problem associated with saturation diving since the introduction of the technique in the late 1960s. Outbreaks of infection involving several divers have been a frequent cause of costly interruptions in operations in both the USA and the UK [8,9].

A specific cause of these outbreaks is *Pseudomonas aeruginosa*, which has also long been known as the predominant bacterial pathogen in 'swimmer's ear' [10]. *P. aeruginosa* is a common bacterium that occurs worldwide in both fresh and seawater, in soil and on plants. The organism is characterised by its metabolic versatility and its exceptional ability to adapt to and colonise various ecological niches [11]. *P. aeruginosa* is a well-known opportunistic pathogen and is of growing importance in both hospital and community-acquired infections [12].

Field research on the presence of *P. aeruginosa* in infections and the environment has been continuous since the 1980s during the

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exploration of the Norwegian petroleum sector. Systematic analysis of isolates by biotyping and pulsed-field gel electrophoresis (PFGE) during an 18-year survey has produced evidence of the involvement of specific *P. aeruginosa* genotypes in skin infections among occupational saturation divers in the Norwegian sector [3–5]. Some *P. aeruginosa* genotypes have been detected more frequently than others in single infections and recorded outbreaks [3,4]. These genotypes have been shown to persist in saturation diving systems for years, despite the fact that new genotypes are being introduced continuously [4,5]. Genotype D, which is the most prominent of these genotypes, has been isolated from single, recurrent and clusters of infections involving several different genotypes and divers [4].

For efficient control and prevention of *P. aeruginosa* infections and a better understanding of their route(s) of transmission in operational saturation diving systems, rapid and sensitive tools for detection of the genotypes involved in infections are of crucial importance. This study describes the development of a PCR-based assay for detection of *P. aeruginosa* genotype D in occupational diving environments, as well as for the identification of *P. aeruginosa* in general. Randomly amplified polymorphic DNA (RAPD)-PCR analysis was used to verify the PFGE genotypes identified previously and formed the basis for the design of the new PCR assay.

MATERIALS AND METHODS

Bacteria and samples

A group of *P. aeruginosa* isolates, related to saturation diving in the Norwegian sector of the North Sea, was selected on the basis of their PFGE genotypes [2–5]. The isolates comprised 28 genotypes with different origins, persistence and frequencies of occurrence in infections. Additional *P. aeruginosa* isolates from the North Sea, hospitals and the general community, as well as reference strains for *P. aeruginosa*, other *Pseudomonas* spp. and other bacterial genera, were also included in the study (Table 1). Sampling, cultivation and biotyping of the

Table 1. Bacterial isolates used in the study

	***Pseudomonas aeruginosa* genotypes**[a]																		
Isolates	**AD**	**AE**	**AL**	**AU**	**BM**	**D**	**E**	**G**	**N**	**P**	**TP1**	**TP2**	**TP4**	**TP5**	**TP12**	**TP27**	**Others**[b]	**NT**	**No. of isolates**
Diving																			
Infections[c]	5	1		2	2	6	4	1	1	5		1			1		4	16	49
Environment[d]	4	1	2	1		9	9	3	1		2	3	2	2	1	5	3	3	51
Hospital																			
Infections																			
Sepsis	Strains 1–9				**rDN, rEN, rDS, rDO, rEO, rEP, rEQ, rEM, rDH**														9
Ear	Strains R1-R3				**rBB, rBC, rBG**														3
Environment	Strain R4				**rCE**														1
	'Snøgg'[e]				**xCCV**														1
Environment																			
Freshwater onshore	Strains K1, K2				**kCCZ, kDaA**														2
Reference strains																			
CCUG 551 T					**ER**														
ATCC 27853					**BBU**														
PAO 1					**aCB**														
PA 103					**aCC**														
PAK					**aCA**														
388					**aCD**														
PAKS 1					**BBD**														

Non-*Pseudomonas aeruginosa*

Pseudomonas mendocina	CCUG 1781 T	*Sphingomonas paucimobilis*	CCUG 6518 T
Pseudomonas stutzeri	CCUG 11256 T	*Brevundimonas diminuta*	CCUG 1427 T
Pseudomonas alcaligenes	CCUG 1425 A T	*Acinetobacter lwoffii*	CCUG 33984 T
Pseudomonas fluorescens	CCUG 1253 T	*Acinetobacter baumannii*	Own isolate
Serratia fonticola	CCUG 14186 T	*Escherichia coli*	Own isolate
Serratia marcescens	CCUG 1647 T	*Proteus mirabilis*	Own isolate
Stenotrophomonas maltophilia	CCUG 5866 T	*Klebsiella pneumoniae*	Own isolate
Comamonas aquatica	CCUG 15845 T		

T, type strain; CCUG, culture collection of University of Gothenburg; ATCC, American type culture collection; NT, non-typeable by PFGE (five RAPD-PCR genotypes).
[a]Designation according to PFGE profiles.
[b]Single genotypes: A, AY, J, TP13, TP28, V, AAY.
[c]Skin infections: external otitis, folliculitis, skin abscess, mostly localised in the face and neck areas.
[d]Isolates from freshwater, seawater, chamber interiors and divers' personal equipment.
[e]'Snøgg', a strain from a large hospital outbreak of infection in Norway.
PFGE, pulsed-field gel electrophoresis; RAPD, random amplified polymorphic DNA.

new isolates were performed by conventional microbiological and biochemical methods [2,5].

PFGE analysis

PFGE analysis of the new isolates was performed as described previously [2,3] with rare-cutting endonuclease *Spe*I (Roche Diagnostics, Mannheim, Germany).

Preparation of genomic DNA, DNA mixtures and bacterial lysates

Overnight bacterial cultures grown on Luria broth (LB) were used for DNA extraction. Genomic DNA was isolated with a Genomic Prep Cells and Tissue DNA Isolation Kit (Amersham Biosciences, Uppsala, Sweden), used according to the manufacturer's instructions. DNA concentrations were determined with a Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech, Chalfont St Giles, UK). DNA templates were diluted to a concentration of 5 ng/μL and stored at −20°C.

DNA mixtures were prepared with 30 ng DNA of each strain involved, as follows: mixture 1, *Serratia fonticola* CCUG 14186 T, *Serratia marcescens* CCUG 1647 T, *Stenotrophomonas maltophilia* CCUG 5866 T, *Acinetobacter lwoffii* CCUG 33984 T, *Brevundimonas diminuta* CCUG 1427 T, *Comamonas aquatica* CCUG 15845 T and *Sphingomonas paucimobilis* CCUG 6518 T; mixture 2, *P. aeruginosa* ATCC 27853, *P. aeruginosa* isolates belonging to genotypes E, A, N and G (one strain of each), isolates of *Escherichia coli* and *Proteus mirabilis*; mixture 3, *P. aeruginosa* CCUG 551 T, *P. aeruginosa* ATCC 27853, *Pseudomonas mendocina* CCUG 1781 T, *Pseudomonas stutzeri* CCUG 11256 T, *Pseudomonas alcaligenes* CCUG 1425 A T, *Pseudomonas fluorescens* CCUG 1253 T and a *P. aeruginosa* isolate belonging to genotype D; mixture 4, as mixture 3, but without the *P. aeruginosa* isolate belonging to genotype D; mixture 5, similar to mixture 1, but with *P. mendocina* CCUG 1781 T and *P. stutzeri* CCUG 11256 T added, and without *B. diminuta* CCUG 1427 T.

Cultures of single *P. aeruginosa* strains grown overnight on blood agar were used for the preparation of bacterial lysates. Cells were suspended in molecular biology grade water (Eppendorf, Hamburg, Germany) and boiled for 10 min. Lysates were prepared similarly from bacterial material gathered after filtration of water samples and cultivation of the filters on *Pseudomonas* agar for 48 h.

RAPD-PCR analysis

RAPD-PCR fingerprints were generated using a Ready-To-Go RAPD Analysis Beads Kit (Amersham Biosciences) according to the manufacturer's guidelines, with slight modifications. In brief, the PCRs were performed in 25-μL volumes using 10 ng template DNA and 25 pmol RAPD analysis primer 1, supplied with the kit. Amplifications were performed in a Mastercycler gradient thermocycler (Eppendorf), with the cycling parameters recommended by the manufacturer's guidelines. A final extension at 72°C for 7 min was added. As a control for the PCR, two DNA samples supplied with the kit (*E. coli* BL21(DE3) and *E. coli* C1a) were amplified in every experiment with RAPD analysis primer 2. In addition, one or two internal control DNAs (from PFGE genotype D and/or E) were included repeatedly in the analyses.

Fragment extraction and cloning of RAPD-PCR fragments

Fragments from the RAPD-PCR profiles were extracted with a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned using a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA, USA) according to the recommendations of the suppliers. The plasmids from the selected clones were isolated with a Plasmid Miniprep Kit (Bio-Rad, Hercules, CA, USA).

Primer design

Following comparison of the sequences of the cloned fragments, Oligo Primer Analysis software (National Biosciences, Plymouth, MN, USA) was used for primer design. The two new primer sets were: (a) for *P. aeruginosa* genotype D: forward primer DspesUp (5′-GGGAAAGGGAATGCGGT-AGA-3′) and reverse primer DspesLow (5′-CCACTTGCCT-GGTTCACCTG-3′), yielding a 382-bp PCR product; and (b) for *P. aeruginosa* in general: forward primer P.a.26Up (5′-CA-AGCAATTCGGTTGGATAT-3′) and reverse primer Ps.fell-Low (5′-GGCGTTGAGCTAACCAGTAC-3′), yielding a 192-bp PCR product.

PCR amplification with the newly designed PCR primer sets

Amplification reactions were performed in 50-μL mixtures that contained 1 × *Taq* Buffer with 1.5 mM Mg^{2+}, dNTPs (50 μM each), 2 U *Taq* polymerase (Eppendorf); each primer (synthesised by Eurogentec, Ougrée, Belgium) 4 ng/μL, 15 ng template DNA and sterile molecular biology grade water. In the experiments with bacterial lysates, 3 μL of a lysate was used in the reaction. Multiplex PCR was performed using similar conditions. Amplifications were carried out in a pre-heated thermal cycler at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 10 min. In every reaction, one or two DNA samples were included as positive controls, together with one negative control.

Gel electrophoresis

Aliquots (5 μL) of the RAPD-PCR fragments were separated in ethidium bromide-stained agarose 2% w/v gels in 1 × TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM $Na_2EDTA.2H_2O$, pH 8.3) at 7 V/cm for 150 min. Specific PCR fragments (10-μL aliquots) were analysed on agarose 1.5% w/v gels in 1 × TBE buffer at 7 V/cm for 1–2 h. The 100-bp PCR molecular ruler and 100-bp molecular ruler (Bio-Rad) were used as molecular size standards.

The amplification products were photographed under UV transillumination and analysed visually.

Sequencing of RAPD-PCR fragments and specific PCR products

Sequencing of RAPD-PCR fragments was performed on an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA, USA) using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and Primer T3 (Invitrogen). PCR products (382 bp) from among the genotypes that were amplified by the primer set for genotype D were also sequenced. These amplification products were purified with a PCR Purification Kit (Qiagen)

and sequenced using the specific primers DspesUp and DspesLow, and either a BigDye Terminator Cycle Sequencing Kit and an ABI Prism 310 sequencer, or a CEQ DTCS Start Kit (Beckman Coulter, Fullerton, CA, USA) and a CEQ 8800 sequencer (Beckman Coulter). Three amplification products were sequenced at least twice to check the reproducibility.

Computer sequence analysis

All the sequences obtained were aligned using the CLUSTALW program (European Bioinformatics Institute; http://www.ebi.ac.uk) and compared with the *P. aeruginosa* PAO1 genome using data from the *Pseudomonas* Genome Project (http://www.pseudomonas.com) and the published *P. aeruginosa* PAO1 genome sequence [13]. A BLAST sequence alignment search in the GenBank database was also performed [14].

RESULTS

RAPD-PCR analysis

Analysis of the isolates in this study by RAPD-PCR showed that the groupings obtained were in agreement with the PFGE data. Examples of the RAPD-PCR profiles obtained are shown in Fig. 1. The analyses revealed fragments ranging from 150 bp to 3000 bp in size, with most of the amplicons being >250 bp in size. Nineteen isolates that were non-typeable by PFGE belonged to five RAPD-PCR genotypes, one of which was dominant.

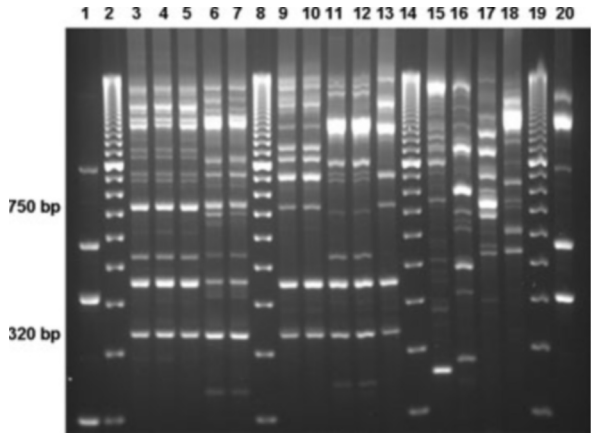

Fig. 1. Examples of RAPD-PCR analysis of *Pseudomonas aeruginosa* and other bacteria. Lanes: 2, 8, 14 and 19, 100-bp PCR molecular ruler; 1, *Escherichia coli* BL 21(DE3) control DNA; 20, *E. coli* C1a control DNA; 3, *P. aeruginosa* D1; 4, *P. aeruginosa* D2; 5, *P. aeruginosa* D10; 6, *P. aeruginosa* E1; 7, *P. aeruginosa* E4; 9, *P. aeruginosa* TP2-1; 10, *P. aeruginosa* TP2-2; 11, *P. aeruginosa* TP12-1; 12, *P. aeruginosa* TP12-2; 13, *P. aeruginosa* PAK; 15, *Acinetobacter baumannii*; 16, *E. coli*; 17, *Klebsiella pneumoniae*; 18, *Proteus mirabilis*.

Comparisons of the profiles obtained with the control DNAs analysed in every PCR run showed reproducible patterns.

Development of PCR primer sets for detection of *P. aeruginosa* genotype D and for *P. aeruginosa* in general

Two random fragments from the RAPD-PCR profiles, *c*.750 bp and 320 bp in size, were considered to be a potential source for the design of specific primers. The 750-bp fragment was found to be characteristic for only a few *P. aeruginosa* genotypes (including genotypes D and E), while the 320-bp fragment was common to all *P. aeruginosa* isolates, but absent from the profiles generated by strains belonging to other species (Fig. 1). The 750-bp and 320-bp fragments were extracted and sequenced from two genotype D isolates (D1 and D10) and two genotype E isolates (E1 and E4). The 750-bp fragment generated from an isolate of *Klebsiella* spp. was also analysed as a representative of another species.

Sequencing and alignment of the 750-bp fragments demonstrated that the sequences from D1 and D10 were almost identical for a 415-bp region (only five mismatches), and were different from the sequences of isolates E1 and E4 and the *Klebsiella* spp. The D1 and D10 sequences formed the basis for the design of a primer set for detection of *P. aeruginosa* genotype D. Comparison of the sequences of the D-specific PCR products with the *P. aeruginosa* PAO1 genome showed that the new primer set targets a sequence that is partly homologous to a gene in the PAO1 genome encoding a hypothetical class 4 protein (106 bp of 382 bp).

Sequencing and alignment of the sequences obtained from the 320-bp fragments of the four *P. aeruginosa* isolates showed only five mismatches in a 239-bp region. On the basis of this alignment, a primer set capable of detecting the species *P. aeruginosa* was designed. Comparison with the *P. aeruginosa* PAO1 genome indicated that the species-specific primer set amplifies a sequence that is complementary to the ends of 23S and 5S rDNA and the 23S-5S intergenic spacer.

The BLAST search for primer target sequence homologies with the available sequence data in the GenBank database showed a perfect match (20 bp/20 bp) for DspesLow- and P.a.26Up-primer sequences only with sequences from *P.*

aeruginosa PAO1. No perfect matching sequence was found for primer DspesUp, while primer Ps.fellLow matched perfectly with *P. aeruginosa* sequences and a genomic sequence from an uncultured bacterium 463 (accession number AY458641). When combined, the species-specific primers for *P. aeruginosa* recognised only *P. aeruginosa* PAO1 from among the available sequences in the GenBank database. None of the sequences in this database could be recognised simultaneously with both primers for *P. aeruginosa* genotype D.

Evaluation of the sensitivity, specificity and applicability of the new PCR assay

The two primer sets were tested on all the isolates listed in Table 1. The analyses were performed on single isolates and on mixtures of isolates. Extracted DNA samples and bacterial lysates were used as templates for the PCR assays.

Extracted DNA samples and lysates from single bacterial isolates. In total, 27 (19.6%) of 138 isolates gave a 382-bp product with the primer set for *P. aeruginosa* genotype D. Of 28 PFGE genotypes related to saturation diving, three were detected with the PCR. The assay recognised all 15 of the genotype D isolates, as well as all the isolates belonging to the TP2 and TP27 genotypes (four and five, respectively). The PCR was also positive with three isolates of non-diving origin: one from a hospital environment (R4) and two (designated as 3 and 9) from hospitalised patients with sepsis. Fig. 2(A) shows examples of amplification with the D genotype primer set. A weak band with a molecular size comparable to the genotype-specific PCR product was observed for one *E. coli* isolate (lane 16), but extended electrophoresis showed significant differences in size and intensity.

The primer set specific for *P. aeruginosa* recognised all 123 of the *P. aeruginosa* isolates included in this study, but none of the isolates belonging to other genera and species. Fig. 2(B) shows examples of PCR amplification using the primer set specific for *P. aeruginosa*.

The results of the parallel experiments for PCR amplification of 16 *P. aeruginosa* isolates using extracted DNA and bacterial lysates of the same isolates as templates were in complete agreement.

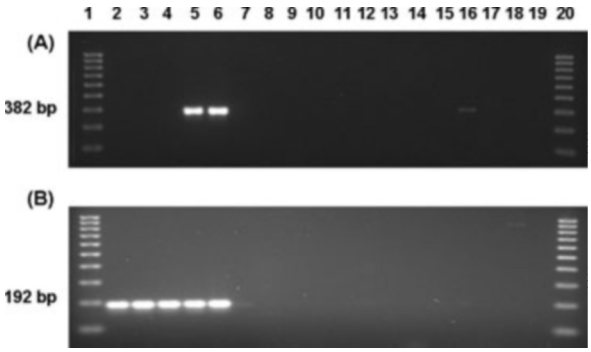

Fig. 2. Examples of PCR amplification of *Pseudomonas aeruginosa* and other bacteria with the two primer sets: (A) PCR products obtained with the primer set specific for *P. aeruginosa* genotype D (382-bp amplicon); (B) PCR products obtained with the primer set specific for *P. aeruginosa* (192-bp amplicon). Lanes: 1 and 20, 100-bp molecular ruler; 2, *P. aeruginosa* ATCC 27853; 3, *P. aeruginosa* CCUG 551 T; 4, *P. aeruginosa* PAO1; 5, *P. aeruginosa* D1; 6, *P. aeruginosa* TP2-1; 7, *Pseudomonas mendocina* CCUG 1781 T; 8, *Pseudomonas stutzeri* CCUG 11256 T; 9, *Pseudomonas fluorescens* CCUG 1253 T; 10, *Pseudomonas alcaligenes* CCUG 1425 A T; 11, *Sphingomonas paucimobilis* CCUG 6518 T; 12, *Serratia marcescens* CCUG 1647 T; 13, *Stenotrophomonas maltophilia* CCUG 5866 T; 14, *Acinetobacter lwoffii* CCUG 33984 T; 15, *Comamonas aquatica* CCUG 15845 T; 16, *Escherichia coli*; 17, *Serratia fonticola* CCUG 14186 T; 18, *Proteus mirabilis*; 19, negative control (no DNA added).

Composed DNA mixtures and mixtures with unknown composition of bacteria. All five mixtures of DNA were used as templates in PCR assays with the two new primer sets. The results demonstrated 100% recognition of the presence of isolates belonging to *P. aeruginosa* genotype D and *P. aeruginosa*, respectively.

Multiplex PCR for simultaneous detection of *P. aeruginosa* and genotype D was used with the lysates of mixtures of bacterial species originating from seven saturation-related freshwater samples. The results (Fig. 3) demonstrate that three of the samples were positive for both primer sets, while two samples were positive only for *P. aeruginosa*. A very weak amplification product was obtained for *P. aeruginosa* in sample 2, which may have been caused by a low concentration of *P. aeruginosa* in the sample.

Classical isolation and genotyping (PFGE) of single colonies from the same samples were also performed. The presence of *P. aeruginosa* was detected in samples 1, 2, 5, 6 and 7, and the TP2 genotype was detected in samples 5 and 7. None of the expected genotypes was detected in sample 6.

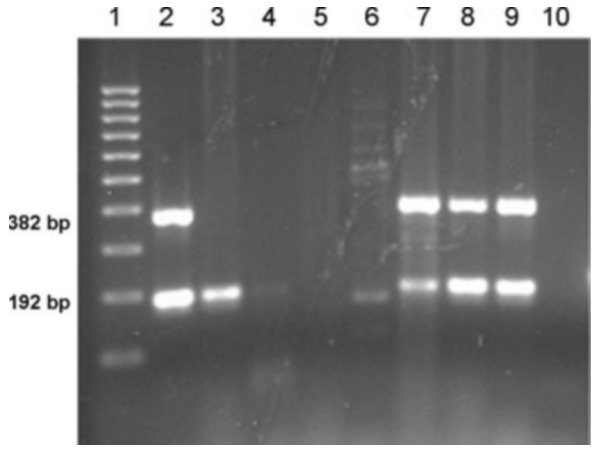

Fig. 3. Amplicons generated from lysates of bacterial mixtures originating from freshwater samples from the diving environment following multiplex PCR with primer sets for *Pseudomonas aeruginosa* and *P. aeruginosa* genotype D. Lanes: 1, 100-bp molecular ruler; 2, *P. aeruginosa* D5 – extracted DNA (positive control); 3, sample 1; 4, sample 2; 5, sample 3; 6, sample 4; 7, sample 5; 8, sample 6; 9, sample 7; 10, negative control (no DNA added).

Analysis of the 382-bp sequences obtained using the primer set for genotype D

Sequence alignment of the 382-bp PCR products from 20 of the isolates revealed only minor differences. All four sequences from strains with a TP2 genotype had one deletion (at position 84 from the beginning of the forward primer) in comparison with the other sequences. The amplification products from the isolates with a D or TP27 genotype were identical to each other and to the sequence from one of the isolates from patients with sepsis (strain 9). Similarly, the sequences from the other two hospital-related isolates (strains 3 and R4) revealed only minor differences compared with each other and with the rest of the sequences analysed.

DISCUSSION

This study describes the design of a new PCR assay for detection of the most frequent *P. aeruginosa* genotype (D) encountered in infections associated with occupational saturation diving. Together with the species-specific primer set for *P. aeruginosa*, the new assay has the potential to be a powerful tool for early detection and prevention of *P. aeruginosa* infections in this specific niche.

Various strategies have been employed previously to develop primer sets and probes for rapid detection and identification of bacteria. In the present study, random genomic sequences were chosen to develop the PCR assay. Subsequent analyses showed that the random sequence used to design the primer set for *P. aeruginosa* genotype D corresponds, in part, to a sequence in the PAO1 genome coding for a hypothetical class 4 protein [13].

The assay for detection of genotype D was tested with 100 isolates related to diving, and two additional diving-related PFGE genotypes (TP2 and TP27) were recognised. The TP2 genotype has persisted in diving systems for 3 years, and there are indications of its high frequency of occurrence in infections during this period (personal unpublished data). Little is known concerning the TP27 genotype, as it has not been recorded previously. The five TP27 isolates in the present study, all of which originated from freshwater samples, were isolated on a single occasion. The absence of data for these two genotypes, as well as for the target of the primer set discussed here, makes it impossible at this stage to determine the precise reason for their occurrence, apart from noting that these genotypes are related genetically to genotype D.

To evaluate the potential of the new PCR assay, *P. aeruginosa* isolates from hospitals and other environments were also included in the study. Three hospital-related isolates (two from infections and one from the hospital environment) were recognised, indicating a potential for the assay beyond the diving niche. However, the main aim of the present study was to develop a rapid method capable of detecting the *P. aeruginosa* D genotype in diving-related microbial material containing an unknown bacterial population. As the North Sea diving environment contains an abundance of microbial species, the possibility that the primer set for the D genotype might amplify a fragment of the same size from another identified or unidentified species cannot be excluded. For this reason, parallel recognition of *P. aeruginosa* at the species level would strengthen the specificity of the new PCR assay.

Many diagnostic PCR assays and oligonucleotide probes for *P. aeruginosa* and *Pseudomonas* spp. target genes for 16S rRNA [15–18] and 16S-23S intergenic spacers [19,20], as well as other specific genes [21–27]. In the present study, the primer set amplified the intergenic spacer region between

the 23S and 5S rDNA genes. PCR assays for *P. aeruginosa* that amplify the 23S-5S rDNA spacer have not been reported previously. However, the paucity of sequence data in this region for non-*P. aeruginosa* strains makes any theoretical prediction of its specificity difficult. The *Pseudomonas* genus has a complex taxonomy, and a reassessment of the phylogenetic affiliation of the pseudomonads has been published [28]. In the present study, 123 *P. aeruginosa* isolates, four other pseudomonads and 11 representatives of other α-, β- and mostly γ-Proteobacteria were investigated. The primer set was capable of recognising all the *P. aeruginosa* isolates studied, and showed potential when it was applied to mixtures of bacterial strains. The theoretical match with available sequences in GenBank also pointed to high specificity. However, further evaluation of the specificity by testing species that are closely related phylogenetically to *P. aeruginosa* is necessary.

The time required to complete the analysis was reduced by eliminating the need to isolate single colonies from the diving-related microbial material and extract DNA. A further reduction could be achieved by the use of multiplex PCR. In this connection, the assay was tested with bacterial DNA mixtures of relevance to the diving environment to check for possible interference in the PCR. Multiplex PCR was also performed on lysates from diving-related microbial material with an unknown bacterial composition. The fact that it was not possible to isolate any of the target *P. aeruginosa* genotypes from one of the PCR-positive samples (sample 6) does not exclude the possibility that they were present in the bacterial mixture. This can be explained by a high level of biodiversity among the strains belonging to this species, and the limitations of culture methods for isolation [29].

In conclusion, a rapid detection assay was developed for the *P. aeruginosa* genotype (D) that occurs most frequently in skin infections among occupational saturation divers, as well as for rapid detection of *P. aeruginosa* in general. Although further evaluation of the specificity and applicability of the method is necessary, the results suggest that the PCR assay described in the present study may contribute to early detection of infection and the initiation of suitable treatment. Combined with other molecular typing methods, the assay could be a useful tool for the effective control of reservoirs, sources and spread of infection, thus improving prevention strategies.

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