Detection and Identification of *Pseudomonas* spp. by Polymerase Chain Reaction-Reverse Cross-Blot Hybridization (PCR-RCBH) with 16S-23S Ribosomal RNA Intergenic Spacer Probes

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ABSTRACT

Pseudomonas spp. is a bacteria type frequently found in fish and in some instances this has caused haemorrhagic bacteraemia leading to moderate to high mortality. Four species commonly isolated from tropical fish are *P. fluorescens, P. putida, P. aeruginosa* and *P. diminuta*. A variety of methods have been used to identify *Pseudomonas* spp. including biochemistry and DNA-based methods. However, these methods are unable to differentiate between different species of *Pseudomonas*. Polymerase chain reaction (PCR) followed by reverse cross blot hybridization (RCBH) was adapted in this study to speciate *Pseudomonas*. Primers were designed for amplification of the 16S-23S rRNA spacer regions of *Pseudomonas*. The PCR products were analyzed in a reverse cross blot hybridization assay with five probes specific to the genus and four species. The specificity was tested with 7 *Pseudomonas* spp. and 11 strains of other bacteria. The method was highly specific for *Pseudomonas* spp. and identified the bacteria to species level with a detection limit of 20 cells/ml.

INTRODUCTION

Pseudomonas spp. are commonly found in natural sources of water and associated with septicaemia in aquatic animals (Roberts, 1978). These bacteria are considered opportunistic pathogens, causing disease when the host is subjected to stress. A number of aquatic animals including fish, frogs and soft-shelled turtles are reported to be susceptible to *Pseudomonas* spp. with moderate to high losses (Somsiri and Soontornvit, 2002). The etiological agents commonly found are *P. diminuta, P. fluorescens, P. putida* and *P. aeruginosa* with different degrees of virulence (unpublished data). Identification of pseudomonads has been tedious since their phenotypic properties are highly uniform among the species. However, identification is valuable in terms of taxonomy and may lead to a better understanding of this genus.

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Disease diagnosis is presently based mainly on a conventional biochemical tests which are time-consuming, requiring a lengthy culturing procedure. Therefore, a rapid and more specific method of pseudomonad diagnosis would be useful for control of the disease as well as for on-farm monitoring. More advanced approaches to identification have been developed, including polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and DNA sequencing (De Vos *et al*., 1997; Widmer *et al*., 1998; Campbell *et al*., 2000). However, identification individual species was inconclusive by these methods. Here, we describe the use of PCR amplification of the intergenic spacer regions (ISRs) followed by a reverse hybridization technique to differentiate pseudomonads at species level.

MATERIALS AND METHODS

Bacterial strains and culture conditions

All bacteria used in this study (Table 1) were grown on tryptone soya agar (TSA) (Oxoid) at 30°C. They were characterized by API 20E and API 20NE with additional tests as described by Cowan (1973).

Table 1. Specificity of PCR with primers P16sf-Bio and P23sr-Bio against DNA from *Pseudomonas* reference strains and other bacteria.

Bacteria	Source	Probe				
		pAeru1	pPuti1	pFlou 1	pDim 2	pGrou1
	ATCC 27853	+	-	-	-	+
Pseudomonas aeruginosa						
P. putida	DMST 10603	-	+	-	-	+
P. fluorescens	TISTR 358	-	-	+	-	+
P. aeruginosa	AAHRI 01024 (isolated in guppy)	+	-	-	-	+
P. putida	AAHRI 95033 (isolated in frog)	-	+	-	-	+
	AAHRI 96163 (isolated in frog)	-	-	-	+	-
P. diminuta						
P. diminuta	AAHRI 96144 (isolated in frog)	-	-	-	+	-
P. diminuta	AAHRI 01158 (isolated in frog)	-	-	-	+	-
P. diminuta	AAHRI 02022 (isolated in frog)	-	-	-	+	-
P. diminuta	AAHRI 96174 (isolated in frog)	-	-	-	+	-
Staphylococcus aureus	ATCC 25923	-	-	-	-	-
Escherichia coli	ATCC 25922	-	-	-	-	-
Proteus morganii	AAHRI 98095 (isolated in soft shell turtle)	-	-	-	-	-
Staphylococcus sp.	AAHRI 00126 (isolated in catfish)	-	-	-	-	-
	AAHRI 01013 (isolated in giant gouramy)	-	-	-	-	-
Citrobacter freundii						
	AAHRI 01018 (isolated in gold fish)	-	-	-	-	-
Aeromonas sobria						
Edwardsiella tarda	AAHRI 01041 (isolated in tilapia)	-	-	-	-	-
	AAHRI 01230 (isolated in catfish)	-	-	-	-	-
Plesiomonas shigelloides						
Vibrio cholerae	AAHRI 01260 (isolated in tilapia)	-	-	-	-	-
Aeromonas hydrophila	AAHRI 01277 (isolated in discus)	-	-	-	-	-
Streptococcus sp.	AAHRI 01285 (isolated in frog)	-	-	-	-	-

ATCC: American Type Culture Collection
DMST: Department of Medical Sciences Thailand
TISTR: Thailand Institute of Scientific and Technological Research
AAHRI: Aquatic Animal Health Research Institute

Bacterial DNA isolation and amplification of spacer region

Genomic DNA was isolated as previously described by Boom *et al*. (1990), with modifications. Briefly, bacteria were resuspended in 500 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Proteinase K was added at a final concentration of 0.1 mg/ml and the bacteria were incubated for a further 1 h at 65°C. The mixture was added to 900 µl lysis buffer L1 (5 M guanidinium isothiocyanate (Sigma), 1% Triton X-100, 50 mM Tris-HCl pH 6.4, 20 mM EDTA) and 20 (l diatom (10 g Colite [Acras], 500 µl HCl, 50 ml H_2O). The tube was mixed on a rotary shaker for 10 min and centrifuged (15 s) in a microfuge (fixed angle, 12,000 x g), and the supernatant was discarded. The diatom nucleic acid pellet was subsequently washed twice with 900 µl washing buffer L2 (5 M guanidinium isothiocyanate in 0.1 M Tris-HCl, pH 6.4), twice with 1 ml ethanol 70% (vol/vol) and once with 1 ml acetone. After disposal of the acetone, the pellets were dried at 56°C with open lids for 10 min. 80 µl TE buffer was added into the tubes and incubated at 56°C for 10 min. The tube was briefly vortexed again and centrifuged for 2 min at 12,000 x g, and the supernatant containing DNA was used for PCR.

Primers

Oligonucleotide primers used for amplifying the 16S-23S rRNA intergenic spacer region were selected from the conserved regions at the 3' end of the 16S rRNA and the 5' end of the 23S rRNA genes. The sequences of the primers were 5'-TGAAGTCGTAACAAGGTAGC-3' for P16sf-Bio (from position 1490 to position 1509 using *Escherichia coli* numbering) and 5'-ATCGCCTCTGACTGCCAAGG-3' for P23sr-Bio (from position 50 to position 31 using *E. coli* numbering). These primers were described by Sawada *et al*. (1997). Both primers were labeled with biotin at the 5' end.

PCR

PCR was performed in a DNA thermal cycler (OmniGene, Hybaid Ltd., UK). A typical reaction mixture (50 µl) consisted of reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 3.0 mM $MgCl_2$) 200 µM (each) deoxynucleotide triphosphate, 10 pmol of each primer, 2 U *Taq* DNA polymerase (Promega), and a 5 µl DNA sample. The reaction mixture was cycled 40 times as follows: 1 min denaturation at 94°C, 1 min annealing at 52°C and 1 min 30 s extension at 72°C. The vials were held at 25°C until the PCR product was detected by RCBH (Puttinaowarat *et al*., 2002 modified from Kox *et al*., 1995).

Sequencing methods

The PCR products were purified with phenol-chloroform and precipitated with ethanol. The DNA pellet was dissolved in 50 µl TE buffer. The fragment was ligated into pGEM T-Easy (Promega), and the recombinant plasmid was transformed into *E. coli* by standard methods (Sambrook *et al*., 1989). Cloned plasmids were prepared from positive transformants by the alkaline lysis method (Sambrook *et al*., 1989). Inserts were amplified with M13 primers using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit. The products were then analyzed by the ABI Prism 377 automatic sequencer (Applied Biosystems) following the manufacturer's instructions.

Reverse cross blot hybridization assay

Tailing of oligonucleotide probes with dTTP

The oligonucleotide probes used in the RCBH assay, outlined in Table 2, were homologous to internal sequences of the PCR products. The probes were tailed with dTTP. This facilitated hybridization of the probes by adding a spacer sequence. 200 pmol of each oligonucleotide was added to 8.8 µl of tailing solution, which contained 1.6 µl of 5X tailing buffer, 1.6 µl of 2.5 mM $CoCl_2$, 2 µl of 10 mM dTTP (Amersham Pharmacia) and 0.2 µl of 25 U TdT (Roche Diagnostics Ltd, Lewes, UK). The mixture was incubated at 37°C for 2 h and 4 µl of 0.2 M EDTA (pH 8.0) was added to stop the reaction. The volume of dTTP-tailed oligonucleotide was made up to 400 µl with nanopure water, giving a final concentration of 0.5 µM of dTTP-tailed oligonucleotide. The tailed probes were stored at -20°C until required.

Table 2. Oligonucleotide probes using in reverse cross blot hybridization.

Code	Specificity	Nucleotide sequences
pGrou1	*Pseudomonas* spp.	5'-CGGCGAATGTCGTCTTCACAG-3'
pAeru1		5'-GGTGTGCTGCGTGATCCG-3'
	P. aeruginosa	
pPuti1		5'-GCGGTAGATGTTGCTCCTGC-3'
	P. putida	
pFluo1		5'-GCATTCCATTGTGATGATGGTG-3'
	P. fluorescens	
pDim2		5'-GATACAAGTATACGAATAGAGCC-3'
	P. diminuta	

Hybridization assay

The hybridization assay followed the method previously described by Puttinaowarat *et al*. (2002) and modified by Kox *et al*. (1995). Basically, a nitrocellulose membrane (Optitran BA-S 83, Schleicher & Schuell) was placed in the hybridization apparatus (Schleicher and Schuell). 50 pmol of each dTTP-tailed probe was diluted in 0.5 ml of 10X saline-sodium citrate buffer (SSC: 1.5 M NaCl, 150 mM sodium citrate, pH 7.0). Each of the diluted oligonucleotide probes was added to one of the slots of the mould and incubated overnight at 28°C on a rotary shaker. The membrane was removed from the apparatus and wrapped in a piece of plastic film. The probes were fixed to the membrane by exposing them to UV light (BDH) at 312 nm until 1.5 Jcm^{-2} was reached. The membrane was washed twice with 10X SSC and then incubated in hybridization solution [5X SSC, 1% blocking agent (Roche Diagnostics Ltd.), 0.1% N-laurylsarcosine, 0.02% SDS] for 5 min. The membrane was allowed to air-dry and kept at 4°C until the next step of the process.

The Accutran cross unit was assembled with the membrane and 200 µl hybridization solution was added to each slot. The membrane was then incubated on a rotary mixer at 20°C for 5 min. 30 µl of PCR product was placed into 1.5 ml screw-cap vials and boiled at 100°C for 5 min. The vials were placed on ice immediately after boiling and 200 µl of hybridization solution was added to each vial. The hybridization solution was removed from each slot and replaced with the DNA mixture. The unit was incubated at 50°C for 1 h. The DNA mixture was discarded from each slot using a vacuum pump and the membrane was then removed from the unit. It was briefly rinsed with 0.1% SDS in 2X SSC and then incubated at 50°C for 5 min in fresh 0.1% SDS in 2X SSC. The membrane was washed briefly with 100 ml of washing buffer (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5) and then incubated in 100 ml of a blocking buffer [0.5% (w/v) blocking reagent (Roche Diagnostics Ltd.) in washing buffer] at 28°C for 30 min on a rotary shaker. The membrane was washed as described above then incubated with 10 ml of streptavidin-conjugated alkaline phosphatase (0.1 U ml^{-1}) (Roche Diagnostics Ltd.) in washing buffer for 30 min at 28°C. Unbound conjugate was removed by incubating the membrane in 100 ml washing buffer for 30 min. The membrane was equilibrated with 20 ml of a substrate buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$) for 2 min. Finally, it was incubated in 10 ml substrate solution [45 µl of 4-nitroblue tetrazolium chloride (NBT, Roche Diagnostics Ltd.), 35 µl 5-bromo-4-choloro-3-indyl phosphate (X-phosphate, Roche Diagnostics Ltd.), 10 ml substrate buffer] until the color completely developed. Rinsing the membrane with distilled water stopped the reaction.

Determination of sensitivity

DNA was extracted from reference strain *P. aeruginosa* (ATCC 27853), *P. putida* (DMST 10603), *P. fluorescens* (TISTR 358), and *P. diminuta* (AAHRI 96144) cultures diluted to 2 x 10^6, 2 x 10^5, 2 x 10^4, 2 x 10^3, 2 x 10^2, 2 x 10^1 and 2 x 10^0 cell ml-1. 5 µl of each DNA solution was added to 45 µl PCR mixture and amplified as described above. The amplified DNA was then analyzed with RCBH.

Determination of specificity and identification of Pseudomonas spp. by PCR-RCBH

DNA was extracted from a variety of both non-pseudomonad and reference strains of pseudomonads cultures diluted to 2 x 10^8 cell ml^{-1} (as outlined in Table 1). DNA of each sample was amplified by PCR and examined by RCBH.

RESULTS

PCR amplification and cloning

Amplification of genomic DNA from all four species, *Pseudomonas aeruginosa* (ATCC 27853), *P. putida* (DMST 10603), *P. fluorescens* (TISTR 358), and *P. diminuta* (AAHRI 96144, and AAHRI 01158), using PCR primers P16sf-Bio and P23sr-Bio, yielded a product of about 650 bp. However, amplification of *P. putida* also yielded two other products of 700 bp and 350 bp. The 650 bp fragment of each isolate was cloned using the pGEM T-Easy system (Promega) and the sequences were then analyzed.

Nucleotide sequence analysis

The sequences were aligned using http://searhlauncher.bcm.tmc.edu/cgi_bin/multi-align/multi-align.pl and compared with 16S-23S rRNA spacer regions of other prokaryotes available in the GenBank database (http://ncbi.nlm.nih.gov) including *Streptococcus pyogenes* (AF489597), *Staphylococcus aureus* (U11780), *Mycobacterium bovis* (AJ315569), *Escherichia coli* (J01702), and *Bacillus subtilis* (J01551). The 16S-23S rRNA spacer sequences of *P. diminuta* and the fluorescent pseudomonad group were found to be identical. Probes specific to the group and each species were finally designed (Table 2).

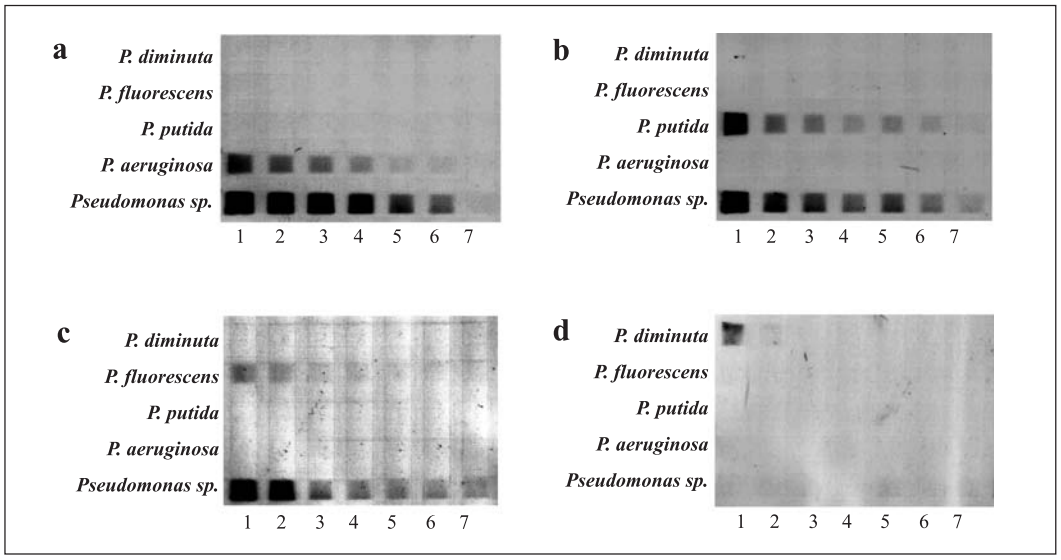

Figure 1. (a) Sensitivity of pAeru1 probe in RCBH with PCR products of *P. aeruginosa* (ATCC 27853). Lane: (1) 2 x 10^6 cell ml^{-1}, (2) 2 x 10^5 cell ml^{-1}, (3) 2 x 10^4 cell ml^{-1}, (4) 2 x 10^3 cell ml^{-1}, (5) 2 x 10^2 cell ml^{-1}, (6) 2 x 10^1 cell ml^{-1}, (7) 2 x 10^0 cell ml^{-1}. (b) Sensitivity of pPuti1 probe in RCBH with PCR products of *P. putida* (DMST 10603). Lane: (1) 2 x 10^6 cell ml^{-1}, (2) 2 x 10^5 cell ml^{-1}, (3) 2 x 10^4 cell ml^{-1}, (4) 2 x 10^3 cell ml^{-1}, (5) 2 x 10^2 cell ml^{-1}, (6) 2 x 10^1 cell ml^{-1}, (7) 2 x 10^0 cell ml^{-1} (c) Sensitivity of pFluo1 probe in RCBH with PCR products of *P. fluorescens* (TISTR 358). Lane: (1) 2 x 10^6 cell ml^{-1}, (2) 2 x 10^5 cell ml^{-1}, (3) 2 x 10^4 cell ml^{-1}, (4) 2 x 10^3 cell ml^{-1}, (5) 2 x 10^2 cell ml^{-1}, (6) 2 x 10^1 cell ml^{-1}, (7) 2 x 10^0 cell ml^{-1} (d) Sensitivity of pDim2 probe in RCBH with PCR products of *P. diminuta* (AAHRI 96144). Lane: (1) 2 x 10^6 cell ml^{-1}, (2) 2 x 10^5 cell ml^{-1}, (3) 2 x 10^4 cell ml^{-1}, (4) 2 x 10^3 cell ml^{-1}, (5) 2 x 10^2 cell ml^{-1}, (6) 2 x 10^1 cell ml^{-1}, (7) 2 x 10^0 cell ml^{-1}.

Sensitivity

The sensitivity of the primers P16sf-Bio and P23sr-Bio (for amplification of 16S-23S rDNA intergenic spacer) was determined by the RCBH assay. As illustrated in Fig. 1, the detection limits of probes pGrou1, pAeru1, pPuti1, pFlou1 and pDim2 (which correspond to *Pseudomonas* spp., *P. aeruginosa*, *P. putida*, *P. fluorescens* and *P. diminuta*, respectively) had respective detection limits of 2-20 cell ml^{-1}, 20 cell ml^{-1}, 20 cell ml^{-1}, 2,000 cell ml^{-1} and 200,000 cell ml^{-1}.

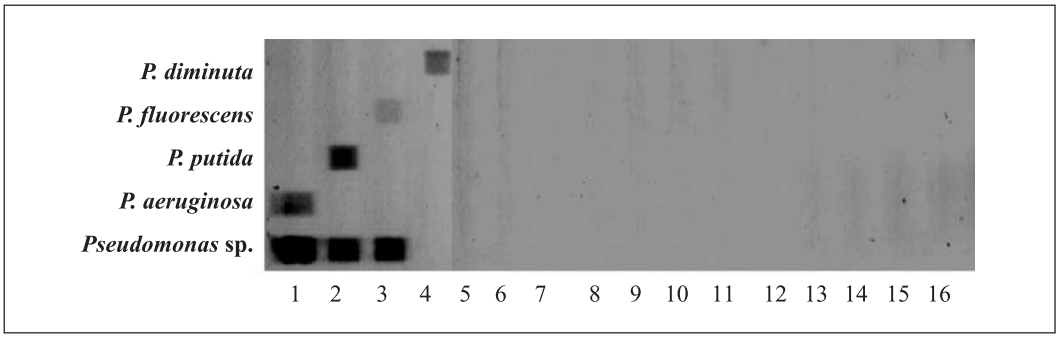

Figure 2. PCR-RCBH of other bacteria and reference strain *Pseudomonas*. Lanes: (1) *P. aeruginosa* (ATCC 27853), (2) *P. putida* (DMST 10603), (3) *P. fluorescens* (TISTR 358), (4) *P. diminuta* (AAHRI 96144), (5) *Staphylococcus aureus* (ATCC 25923), (6) *Escherichia coli* (ATCC 25922), (7) *Proteus morganii* (AAHRI 98095), (8) *Staphylococcus* sp. (AAHRI 00126), (9) *Citrobacter freundii* (AAHRI 01013), (10) *Aeromonas sobria* (AAHRI 01018), (11) *Edwardsiella tarda* (AAHRI 01041), (12) *Plesiomonas shigelloides* (AAHRI 01230), (13) *Vibrio cholerae* (AAHRI 01260), (14) *Aeromonas hydrophila* (AAHRI 01277), (15) *Streptococcus* sp. (AAHRI 01285), (16) TE buffer.

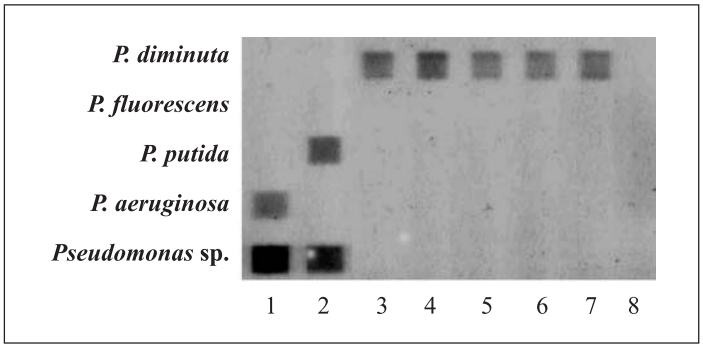

Figure 3. Characterization of *Pseudomonas* spp. isolated from fish in Thailand by PCR-RCBH. Lanes: (1) AAHRI 01024, (2) AAHRI 95033, (3) AAHRI 96163, (4) AAHRI 96144, (5) AAHRI 01158, (6) AAHRI 02022, (7) AAHRI 96174, (8) TE buffer.

Specificity

The specificity of the PCR with primers P16sf-Bio and P23sr-Bio was tested against 11 different other bacterial isolates, mainly fish pathogens. The primers amplified all bacteria listed in Table 1 with different product sizes (data not shown). The specificity of all five probes used in RCBH was also tested against the DNA of the 11 different other bacteria as well as the reference strains (Fig. 2). The reference strains reacted specifically with the corresponding probes.

Detection of amplification DNA by RCBH

The isolates from fish in Thailand gave positive reactions to four probes, pGrou1, pAeru1, pPuti1, and pDim2 (Fig. 3) which were specific for fluorescent pseudomonads, *P. aeruginosa, P. putida*, and *P. diminuta*, respectively. All isolates except *P. diminuta* tested positive using the pGrou1 probe (genus-specific in fluorescent pseudomonads).

DISCUSSION AND CONCLUSION

The rRNA-coding regions (16S rDNA) have been used extensively to analyse phylogenetic relationships at the species level or above (Woese, 1987). The 23S rDNA sequences are available for a few bacterial species and variations in these sequences have been used for typing clinical isolates (Ludwig *et al*., 1994; Anthony *et al*., 2000). However, intergenic spacer regions (ISRs), especially those located between the 16S and 23S rDNAs have been shown to be under less evolutionary pressure (Rijpens *et al*., 1996; Smart *et al*., 1996; Sawada *et al*., 1997; Berridge *et al*., 1998; Chun *et al*., 1999).

Multiple 16S-23S spacer amplicons of varying lengths, like those detected in *P. putida*, have been observed in other bacteria. For *P. putida*, amplification produced two bands other than the 650 bp fragment. Subsequent nuscleotide sequence analysis has indicated that these multiple bands were not to be caused by lack of primer specificity but were due to the existence of multiple copies of the 16S-23S spacers (data not shown). This heterogeneity among the spacers within the various copies of the rRNA operon has been reported previously in bacterial genomes and has made this region useful as a means of differentiating closely related bacterial species (Berridge *et al*., 1998).

DNA purified from the reference strains was used to determine the level of sensitivity of the method. The species-specific probes were able to identify from 20 up to 2 x 10^5 cell ml^{-1}. For diagnostic purposes, the RCBH has the advantage of not only being more sensitive than other methods, but also allowed identification of bacteria to species level. The sensitivity has been shown to vary in different bacteria, e.g. 2.8 x 10^4 CFU ml^{-1} for *Brucella* spp. (Rijpens *et al*., 1996) and 20 mycobacteria cells for *Mycobacterium* spp. (Puttinaowarat *et al*., 2002), and could be increased by using a nested PCR. However, when a nested PCR is applied in practice, one has to consider stringent measures to avoid contamination (Rijpens *et al*., 1996).

The specificity of primers in the PCR was also examined by RCBH. Primer P16sf-Bio and P23sr-Bio, amplified a gene coding for 16S-23S rRNA. The primers cross-reacted with another bacteria but this was eliminated by subsequent use of RCBH. The pDim2 probe reacted only with *P. diminuta* because the 16S-23S rRNA sequence differed from fluorescent pseudomonads likewise reported. According to Palleroni (1992) the present five groups of *Pseudomonas* described fluorescent pseudomonads in RNA group I and *P. diminuta* in RNA group IV.

Although pseudomonads do not always cause a high mortality, antibiotic treatment is commonly introduced into the farm practice and this may result in drug residue problems. This study has demonstrated that the identification of pseudomonads by PCR-RCBH is highly specific and less time-consuming than the conventional bacterial culture method. This may be useful in preventing disease outbreaks and in limiting the use of antibiotic prophylaxis.

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