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Comparative typing of *Pseudomonas* species isolated from the aquatic environment in Greece by SDS-PAGE and RAPD analysis

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ABSTRACT

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Aims: Three broadly used typing methods were employed in order to assess and compare the identification and classification of environmental *Pseudomonas* strains. The reproducibility, typeability and discriminatory power of the methods were also compared to evaluate their application. Finally, the potential impact on public health of the isolates is to be discussed.

Methods and Results: *Pseudomonas* strains (160) isolated from the aquatic environment in Greece and identified by a rapid identification commercially available system (API20NE), were subjected to whole-cell protein electrophoresis (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and Randomly Amplified Polymorphic DNAs (RAPD) using two 10-mer primers. In general, the obtained results were in agreement. Twenty isolates that could not be identified by the API20NE system were classified by the other methods.

Conclusions: Rapid identification systems may serve only for a first rough identification of environmental Pseudomonads. In order to acquire further information, so that conclusions about their role in the ecosystem and human health could be drawn, other phenotypic or genotypic methods have to be applied.

Significance and Impact of Study: It is important, from a public health point of view, to monitor the identities of environmental *Pseudomonas* isolates using specific methods due to their ubiquity, heterogeneity and their pathogenicity, either established or potential.

Keywords: API20NE, environmental strains, *Pseudomonas*, randomly amplified polymorphic DNAs, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, typing.

INTRODUCTION

The genus of *Pseudomonas* includes species with ecological, economic and health-related importance (Widmer *et al.* 1998). For many years, this genus has been thought to consist of more than 100 species, many of which play a significant role in the biosphere. They are involved in the carbon cycle in nature, due to their ability to degrade low molecular weight organic compounds or to produce enzymes that participate in the catabolism of various aromatic compounds (Kersters *et al.* 1996).

Because of the great heterogeneity of this genus, it has been recently divided into more genera. For example, *Pseudomonas cepacia* has become *Burkholderia cepacia* and *Pseudomonas acidovorans* has been renamed to *Comamonas acidovorans*. The current genus of *Pseudomonas* is restricted to the rRNA group-I organisms that belong to the γ-subclass of the Proteobacteria (Kersters *et al.* 1996).

Pseudomonas species are found ubiquitously in nature. They occur abundantly in water, soil and plants (Römling *et al.* 1994). Even though, they may also be found in the oligotrophic environment of distilled water or antiseptic

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fluids, due to their ability to survive and multiply in low-nutrient conditions (Moreira *et al.* 1994; Bharath *et al.* 2003).

Most members of this genus are opportunistic pathogens in humans, especially immuno-compromised, and major causes of serious nosocomial infections (Römling *et al.* 1994). The predominant member, *Pseudomonas aeruginosa*, together with *B. cepacia* has been shown to cause cystic fibrosis often with fatal consequences (Mahenthiralingam *et al.* 1996; Coenye *et al.* 2001). *Pseudomonas aeruginosa* can colonize the human eye and ear, inducing corneal ulcers and otitis, respectively (Bukanov *et al.* 1994).

From a public health point of view, it is important to monitor the absence of *Ps. aeruginosa* from bottled and recreational waters because of its pathogenicity. European Union directive 98/83/EC (Anonymous 1998) prohibits the presence of *Ps. aeruginosa* in any 250 ml sample of bottled water, as it has been implicated in food borne and waterborne diseases (Warburton *et al.* 1998). Moreover, given that *Ps. aeruginosa* carries resistance to many antibiotics and antibacterial agents, its presence in the water poses the risk of transferring this resistance to other pathogenic bacteria present in the human body (Mavridou *et al.* 1994).

The detection of *Ps. aeruginosa* in environmental samples is usually performed by membrane filtration on a selective solid medium and it is presumptively identified by the production of pyocyanin and a diffusive fluorescent pigment. The EU directive (98/83/EC) sets some additional confirmatory tests such as the ability to produce ammonia from acetamide or to produce the cytochrome oxidase. The peculiarity of environmental isolates is that they often meet low nutrient or starvation conditions, so they adopt mechanisms in order to survive, which change their phenotypic characteristics (Mavridou *et al.* 1994). The problem with the above mentioned methods is that they were developed for clinical isolates and often fail to identify environmental strains correctly, at least to the species or subspecies level (Ferreira *et al.* 1996).

Taking into account the diverse nature of the genus *Pseudomonas*, as well as the obstacles of conventional cultivation methods for identification of *Pseudomonas* species, the need for the employment of more reliable identification methods becomes apparent.

One of the simplest identification methods is the API20NE identification system with 20 biochemical tests, but it has been implicated in inadequate reliability and low efficiency (Shelly *et al.* 2000), at least for environmental isolates (Sørensen *et al.* 1992; Ferreira *et al.* 1996). To overcome this, other phenotypic and genotypic techniques have been developed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has gained ground during the latest years in differentiating and classifying microorganisms (Plikaytis *et al.* 1986). It is a sensitive and relatively simple method applicable to a wide range of microorganisms (Coenye *et al.* 2001). It has been established that computer-aided comparisons of whole-cell electrophoretic protein patterns provide a reliable way to measure genomic relatedness, since bacterial strains with 90–100% DNA relatedness display only minor differences in their protein fingerprints and strains with at least 70% DNA homology tend to have similarities in their protein profiles (Pot *et al.* 1994).

Nonetheless, SDS-PAGE remains a phenotypic method with all the consequent disadvantages; analysing the phenotype does not always reflect the genotype of the strain, especially if the strain has faced severe nutrient conditions, which has led to phenotypic differentiation. As opposed to the traditional methods developed for typing *Ps. aeruginosa*, molecular typing schemes do not rely on the expression of particular properties in a specific environment (Leitão *et al.* 1996).

Randomly amplified polymorphic DNA (RAPD) techniques have been successfully applied to genetic population analysis of a broad range of microorganisms (Olive and Bean 1999) and specifically of *Pseudomonas* species (Bukanov *et al.* 1994; Elaichouni *et al.* 1994; Haase *et al.* 1995a,b; Renders *et al.* 1996; Hernández *et al.* 1997). It has been demonstrated that RAPD is a universally applicable, rapid and highly discriminating typing method (Elaichouni *et al.* 1994). It is thought to be less laborious and time-consuming than other DNA techniques since there is no requirement for restriction endonuclease digestion, nor for DNA labelling, blotting, nucleic acid hybridization, or electrophoresis in pulsed-field gels (Bukanov *et al.* 1994). Finally, it has the further merit of requiring little knowledge of the biochemistry or molecular biology of the species being studied (Welsh and McClelland 1990).

In this study we tested *Pseudomonas* strains isolated from the aquatic environment of the Achaia Prefecture in Greece. The strains were collected from potable, bottled and seawater during a 5-year period. The objectives of this work were (i) to assess whether the application of phenotypic (SDS-PAGE) and genotypic (RAPD) techniques result in a better and more comprehensive classification of environmental isolates than the commonly used technique API20NE and (ii) to compare the results obtained by the three methods, as well as their typeability, reproducibility and discriminatory power. Moreover, applying more reliable and informative methods on the isolates contributes to our understanding of their structure and epidemiology, leading to a more successful dealing with health-related issues.

MATERIALS AND METHODS

Bacterial strains

Pseudomonas strains were collected from the aquatic environment. In detail, 4000 municipal tap water samples, 725 bottled waters, 681 seawater samples and 80 samples from swimming pools had been tested over a 5-year period and in 160 of them, *Pseudomonas* strains were found. The type strain *Ps. aeruginosa* ATCC 27853 was used as a standard of reference for both techniques (SDS-PAGE and RAPD). Moreover, *Pseudomonas stutzeri* ATCC 17588, *C. acidovorans* ATCC 15668, *Burkholderia pickettii* ATCC 27511, *Pseudomonas putida* ATCC 12633, *Pseudomonas mendocina* ATCC 25411, *Pseudomonas fluorescens* ATCC 13525 and *Xanthomonas maltophilia* ATCC 13637 were included in the study as indicators of the individual species.

Reference strains and isolates were stored at −70°C in Tryptone Soya Broth (Oxoid Ltd., Basingstoke, Hampshire, England) containing 15% glycerol.

Phenotypic characterization

All isolates were tested for growth and the production of a fluorescent pigment on *Pseudomonas* Agar Base (Oxoid) in which *Pseudomonas* C-N Supplement (SR102, Oxoid) was added. Gram stain and cytochrome oxidase test were performed on all isolates. All grown on *Pseudomonas* agar, gram (−) and oxidase (+) isolates, were subjected to the API20NE Test System (BioMèrieux, Marcy l'Etoile, France) for biochemical characterization.

Polyacrylamide gel electrophoresis of whole-cell proteins

All isolates were cultured on MacConkey Agar (Oxoid), for 18–22 h at 37°C and subcultured on R2A Agar (Difco Lab., Detroit, MI, USA) for 18–22 h at 37°C. The whole-cell protein extracts were prepared as follows: Cells harvested from each plate were inoculated into 1 ml of NaCl 0·9% in eppendorf tubes. The bacterial suspension was centrifuged at 12000 *g* (4°C) for 20 min. The sediment was washed twice with 1 ml NaCl 0·9%, suspended in 1 ml of 0·1%SDS and sonicated for four 30-second periods separated by a 10-second interval. The whole-cell lysate was harvested by centrifugation at 5000 *g* for 10 min at 4°C and the protein amount was determined in the supernatant by the Bradford method (Bradford 1976) and standardized accordingly. The absorbance measures were performed by a Hitachi U-2001 UV/Vis spectrophotometer. The whole-cell lysate was kept at −20°C until required.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out on a slab gel apparatus (Penguin^{TM}, Owl Scientific Inc., MA, USA) with 0·5 mm spacers, by following the procedure of Laemmli (Laemmli 1970). The separation gel (13·4% T) and stacking gel (5% T) were prepared using water of high quality (MilliQ water, resistance MΩ 18·2). After the gel was set, each 10 μg bacterial protein sample mixed with 5 μl of sample marking solution (Tris-Cl/SDS buffer, pH 6·8) and 1 μl mercaptoethanol-b was being boiled for 3 min and loaded onto the gel. Bacterial whole-cell proteins were concentrated in the stacking gel at 100 V for 20 min and separated in the separation gel at 200 V for 1 h. Wide Molecular Weight Range Marker M4038 (Sigma, St. Louis, MO, USA) was used as a molecular weight marker. Whole-cell protein lysate of *Ps. aeruginosa* ATCC 27853 was used in each gel twice as a standard for correction of inter- and intra-gel variations.

All gels were stained with Coomassie Brilliant Blue solution R-250 (Merck, Darmstadt, F.R.Germany) for 30 min under shaking, destained with a solution containing 25% methanol and 10% acetic acid, for 30 min under shaking and the bands were stabilized by 7% acetic acid solution for 30 min under shaking.

Protein electrophoretic patterns were photographed with UVP ImageStore 7500 Gel Documentation System and were analysed visually as well as with the aid of computer using LabWorks^{TM} Version 3.02 software (Ultra-Violet Products Ltd., Cambridge, UK). The intensity threshold used to exclude peaks below a certain intensity level was set to 0·5% of maximum intensity level for the image class.

Isolation of genomic DNA

The bacterial strains were cultured onto MacConkey Agar (Oxoid) for 18–22 h at 37°C. A single colony was inoculated in nutrient broth (Oxoid) and grown at 37°C for 18 h. After harvest by centrifugation (12 000 *g*, 5 min, 4°C), the pellet was resuspended in 200 μl of MilliQ water and was being boiled for 10 min. The suspension was vortexed and centrifuged at 14 000 *g* for 20 min at 4°C, after the addition of 200 μl sodium acetate 3 mol l^{-1} and 400 μl phenol : chloroform : isoamyl alcohol 25 : 24 : 1 (Sigma). Ethanol precipitation was carried out in the supernatant and the genomic DNA was kept at −20°C overnight. After washing the DNA pellet twice with ethanol 70%, it was dissolved in 100 μl of MilliQ water, and the DNA was quantified by its absorbance value at 260 nm, while its purity was determined by the ratio A260 : A280 (Sambrook *et al.* 1989). All DNA suspensions were kept at −20°C until required.

Randomly amplified polymorphic DNAs analysis

RAPD 10-mers primers (40) of arbitrary sequence (Operon Technologies Inc., Alameda, CA, USA) were screened for

the ability to produce discriminatory polymorphisms between five *Ps. aeruginosa*, five *Ps. stutzeri*, five *C. acidovorans* and five *Ps. fluorescens* strains. Under the RAPD conditions used, 32 primers were found to amplify the DNA of the isolates but two, OPA-13 (sequence 5′-CAGCACC-CAC-3′) and OPD-13 (sequence 5′-GGGGTGACGA-3′), were chosen for comparison, as these gave the clearest and most discriminating RAPD fingerprints.

Randomly Amplified Polymorphic DNAs PCR reaction mixtures (50 μl) contained 200 ng of genomic DNA, 25 pmol of primer, 2·5 U μl^{-1} Taq polymerase (Perkin Elmer), 0·2 mmol l^{-1} of each deoxynucleotide triphosphate (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), 1 × PCR buffer (Applied Biosystems) and 4 mmol l^{-1} $MgCl_2$ (Perkin Elmer). A negative control in which DNA was replaced with sterile MilliQ water was also included. Each reaction mixture was amplified with PTC-200 Peltier Thermal Cycler (MJ Research Inc, MA, USA) by using the following protocol: an initial denaturation step at 95°C for 1 min, 45 cycles of 94°C for 1 min (denaturation), 38°C for 1 min (annealing) and 74°C for 1 min (extension). Amplified DNA fragments were separated by electrophoresis on 1·5% agarose gels in 0·5X Tris-Borate-EDTA buffer (0·045 mol l^{-1} Tris, 0·045 mol l^{-1} Borate, 0·001 mol l^{-1} EDTA, pH 8·00) containing ethidium bromide. 100 bp DNA ladder (Bio-Labs Inc., New England) was used as a size marker. The amplified DNA fragment of *Ps. aeruginosa* ATCC 27853 was included in each gel twice to serve as a correcting factor for inter- and intra-gel variations. The gels were running for 3 h at 80 V, then were photographed under UV illumination with UVP ImageStore 7500 System and were analysed visually and with the aid of computer analysis (LabWorks^{TM} Version 3.02 software).

Numerical analysis

The normalization of each protein or DNA profile was accomplished by using the equation proposed by Plikaytis (Plikaytis *et al.* 1986), and with the use of the profile of *Ps. aeruginosa* type strain, as a reference. Similarity coefficients were calculated across the entire absorbance profile between all pairs of normalized patterns by the Pearson product moment correlation coefficient (r), and clustering was achieved by the unweighted-pair group method with arithmetic average algorithm (UPGMA).

Discriminant analysis

The clustering results of each method were subjected to discriminant analysis. The classification efficiency was assessed by 'leave-one-out classification', i.e. by classifying each strain while leaving it out from the model calculations.

The computations were performed using the SPSS Version 11.5 software (SPSS Inc., IL, USA).

Discriminatory power

The discriminatory power of all three methods was calculated by application of Simpson numerical index of diversity (Hunter and Gaston 1988).

RESULTS

API20NE

A total of 160 pseudomonads isolated from seawater, bottled water and tap water over a 5-year period, were tested with the API20NE identification system for their phenotypic characteristics. 61 strains (38%) were found to be *Ps. aeruginosa* and 30 isolates (19%) were identified as *Ps. stutzeri*. The origin and classification of the isolates, as derived from the API20NE system, is presented in detail in Table 1. Twenty isolates were identified by the API20NE system as *Pseudomonas* spp. and were considered as unidentified to the species level. The discriminatory power of API20NE system was calculated to be 0·771.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Pseudomonas aeruginosa type strain ATCC 27853, which was used as a reference strain, gave average similarities with a mean and SD of (98·5 ± 2·0%) when analysed in the 25 different gels included in the study.

154 environmental isolates (the rest six isolates could not be recovered) and eight type strains when subjected to SDS-PAGE produced protein profiles with 12 to 41 bands, their molecular weight ranging from 13 to 245 kDa.

The numerical analysis of the whole-cell SDS-PAGE profiles of 162 strains resulted in the formation of 20 distinct clusters (correlation coefficient $r = 0{\cdot}550$) while three strains did not belong to any cluster (Fig. 1). The predominant cluster A comprised 49 strains (30% of total bacterial population) sharing 65% similarity. This one, together with cluster B ($r = 0{\cdot}700$) contained all strains identified as *Ps. aeruginosa* by API20NE system, including the type strain ATCC 27853.

Clusters D (13 strains, $r = 0{\cdot}771$), E (six strains, $r = 0{\cdot}685$) and F (six strains, $r = 0{\cdot}630$) contained the majority of isolates (81%), which were identified as *Ps. stutzeri* by the API20NE system. Nine strains produced cluster O at 0·549 similarity, six of which were considered as unidentified by the API20NE system. Clusters R (six

Table 1 Compilation of API20NE, SDS-PAGE and RAPD typing results of the isolates

Strain no	Source	API20NE	SDS-PAGE	RAPD primer OPA-13	RAPD primer OPD-13
1, 4, 6, 8, 14	Bottled	*Pseudomonas aeruginosa*	A	I	1
39, 49	Potable	*Ps. aeruginosa*	A	I	1
2, 3, 5, 9, 11, 12, 13, 15, 16, 17, 19, 46	Bottled	*Ps. aeruginosa*	A	I	2
7	Bottled	*Ps. aeruginosa*	A	I	3
10	Bottled	*Ps. aeruginosa*	A	I	4
18	Bottled	*Ps. aeruginosa*	B	II	5
20, 21, 139	Bottled	*Ps. aeruginosa*	B	II	6
22	Bottled	*Ps. aeruginosa*	A	I	7
23, 35, 36, 37, 38, 53, 54, 55	Seawater	*Ps. aeruginosa*	A	I	7
24, 25, 26, 27	Potable	*Ps. aeruginosa*	A	I	8
28	Potable	*Ps. aeruginosa*	B	I	1
29	Bottled	*Ps. aeruginosa*	A	I	6
30	Distilled water	*Ps. aeruginosa*	B	I	8
31	Swimming pool	*Ps. aeruginosa*	B	I	9
32, 33, 34	Swimming pool	*Ps. aeruginosa*	A	I	9
40	Seawater	*Ps. aeruginosa*	A	III	9
41	Seawater	*Ps. aeruginosa*	A	I	9
42	Bottled	*Ps. aeruginosa*	A	I	10
43	Bottled	*Ps. aeruginosa*	B	I	2
44, 45	Bottled	*Ps. aeruginosa*	A	I	5
47	Potable	*Ps. aeruginosa*	B	IV	11
48	Potable	*Ps. aeruginosa*	A	IV	11
50, 52	Seawater	*Ps. aeruginosa*	A	I	12
51	Seawater	*Ps. aeruginosa*	A	III	7
56	Seawater	*Pseudomonas stutzeri*	Unclustered	I	13
57	Seawater	*Ps. stutzeri*	C	V	14
58, 64	Seawater	*Ps. stutzeri*	E	V	15
59	Seawater	*Ps. stutzeri*	G	VI	16
60	Seawater	*Ps. stutzeri*	F	VII	10
61	Seawater	*Ps. stutzeri*	C	IV	10
62	Seawater	*Ps. stutzeri*	F	VI	17
63	Seawater	*Ps. stutzeri*	F	V	17
65	Seawater	*Ps. stutzeri*	E	IV	13
66	Seawater	*Ps. stutzeri*	E	VI	18
67	Seawater	*Ps. stutzeri*	F	IX	5
68	Seawater	*Ps. stutzeri*	E	X	19
69	Potable	*Ps. stutzeri*	E	VIII	18
70, 71	Seawater	*Ps. stutzeri*	D	VIII	20
72	Bottled	*Ps. stutzeri*	G	VIII	21
73, 74	Bottled	*Ps. stutzeri*	D	VIII	21
75, 77, 80, 81	Bottled	*Ps. stutzeri*	D	IX	21
76	Bottled	*Ps. stutzeri*	D	XI	22
78, 79	Bottled	*Ps. stutzeri*	D	VIII	23
82	Swimming pool	*Ps. stutzeri*	D	IX	24
83	Potable	*Ps. stutzeri*	D	III	19
84, 85	Seawater	*Comamonas acidovorans*	H	XII	16
86	Seawater	*C. acidovorans*	H	Unclustered	25
87, 88	Bottled	*C. acidovorans*	H	XIII	26
89	Bottled	*Ps. aeruginosa*	A	I	Unclustered
90	Seawater	*Ps. stutzeri*	F	I	13
91	Potable	*Ps. aeruginosa*	A	I	7
92	Seawater	*Ps. stutzeri*	C	VII	Unclustered

Table 1 *Continued*

Strain no	Source	API20NE	SDS-PAGE	RAPD primer OPA-13	RAPD primer OPD-13
93	Seawater	*Burkholderia pseudomallei*	B	III	7
94	Bottled	*Ralstonia pickettii*	J	XI	27
95	Seawater	*R. pickettii*	J	XI	12
96	Swimming pool	*R. pickettii*	J	XI	9
97	Bottled	*R. pickettii*	J	XIV	28
98, 99	Seawater	*S. paucimobilis*	K	X	3
100	Bottled	*Pseudomonas alcaligenes*	L	Unclustered	28
101	Seawater	*Ps. alcaligenes*	L	XV	29
102	Potable	*Ps. alcaligenes*	L	XVI	29
103	Potable	*Ps. alcaligenes*	L	XIV	28
104	Potable	*Ps. alcaligenes*	L	XV	14
105, 108	Bottled	*Stenotrophomonos maltophilia*	M	XIV	4
106	Potable	*S. maltophilia*	M	V	29
107	Bottled	*S. maltophilia*	M	VI	29
109	Bottled	*S. maltophilia*	M	XX	26
110	Potable	*S. maltophilia*	Q	XXI	30
111	Swimming pool	*Pseudomonas putida*	N	VIII	24
112	Swimming pool	*Ps. putida*	N	V	25
113	Bottled	*Burkholderia diminuta*	N	V	31
114	Bottled	*B. diminuta*	N	V	Unclustered
115, 116	Bottled	*Aeromonas hydrophila/caviae*	O	XVIII	32
117	Swimming pool	*Aer. hydrophila/caviae*	O	XVIII	3
118	Swimming pool	*Aer. hydrophila/caviae*	N	Unclustered	26
119, 129, 130	Bottled	*Pseudomonas fluorescens*	I	III	27
120	Swimming pool	*Ps. fluorescens*	I	III	33
121, 122	Potable	*Ps. fluorescens*	I	III	27
123	Bottled	*Ps. fluorescens*	I	XVI	34
124	Seawater	*Pseudomonas mendocina*	P	XXII	24
125	Seawater	*Ps. mendocina*	P	Unclustered	9
126	Seawater	*Burkholderia cepacia*	Unclustered	I	7
127	Seawater	*B. cepacia*	P	VI	Unclustered
128	Swimming pool	*B. cepacia*	P	I	7
131	Seawater	*R. pickettii*	–	Unclustered	16
132	Bottled	*Ps. aeruginosa*	–	Unclustered	26
133	Potable	*Ps. aeruginosa*	–	I	6
134	Bottled	*B. diminuta*	–	V	Unclustered
135	Seawater	*Ps. Aeruginosa*	–	III	12
136, 137	Potable	*S. maltophilia*	M	XVII	–
138	Seawater	*Ps. fluorescens*	–	Unclustered	33
140	Seawater	Unidentified	S	XX	23
141	Swimming pool	Unidentified	T	Unclustered	14
142	Bottled	Unidentified	T	XIX	34
143	Bottled	Unidentified	S	XVIII	23
144	Bottled	Unidentified	S	XV	25
145	Bottled	Unidentified	S	XVIII	34
146, 154, 157	Bottled	Unidentified	O	XVIII	32
147, 148, 149	Potable	Unidentified	R	XXI	34
151	Bottled	Unidentified	R	XXI	34
150	Bottled	Unidentified	O	XVIII	22
152	Potable	Unidentified	O	V	Unclustered
153	Potable	Unidentified	O	Unclustered	Unclustered
155	Potable	Unidentified	R	XIX	22

Table 1 *Continued*

Strain no	Source	API20NE	SDS-PAGE	RAPD primer OPA-13	RAPD primer OPD-13
156	Potable	Unidentified	R	XIX	34
158	Potable	Unidentified	Q	XXI	30
159	Swimming pool	Unidentified	N	XVII	31
160	Swimming pool	*Ps. alcaligenes*	L	–	29
ATCC 12633	Type strain	*Ps. putida*	N	XXII	Unclustered
ATCC 13525	Type strain	*Ps. fluorescens*	Unclustered	XXI	33
ATCC 13637	Type strain	*S. maltophilia*	M	V	–
ATCC 15668	Type strain	*C. acidovorans*	H	Unclustered	Unclustered
ATCC 17588	Type strain	*Ps. stutzeri*	F	VIII	17
ATCC 25411	Type strain	*Ps. mendocina*	P	XVI	3
ATCC 27511	Type strain	*R. pickettii*	J	XI	Unclustered
ATCC 27853	Type strain	*Ps. aeruginosa*	B	I	1

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RAPD, randomly amplified polymorphic DNAs.
SDS-PAGE profiles are indicated by capital letters. Latin numbers are indicative of RAPD typing results with primer OPA-13 while arabic numbers are indicative of RAPD typing results with primer OPD-13.

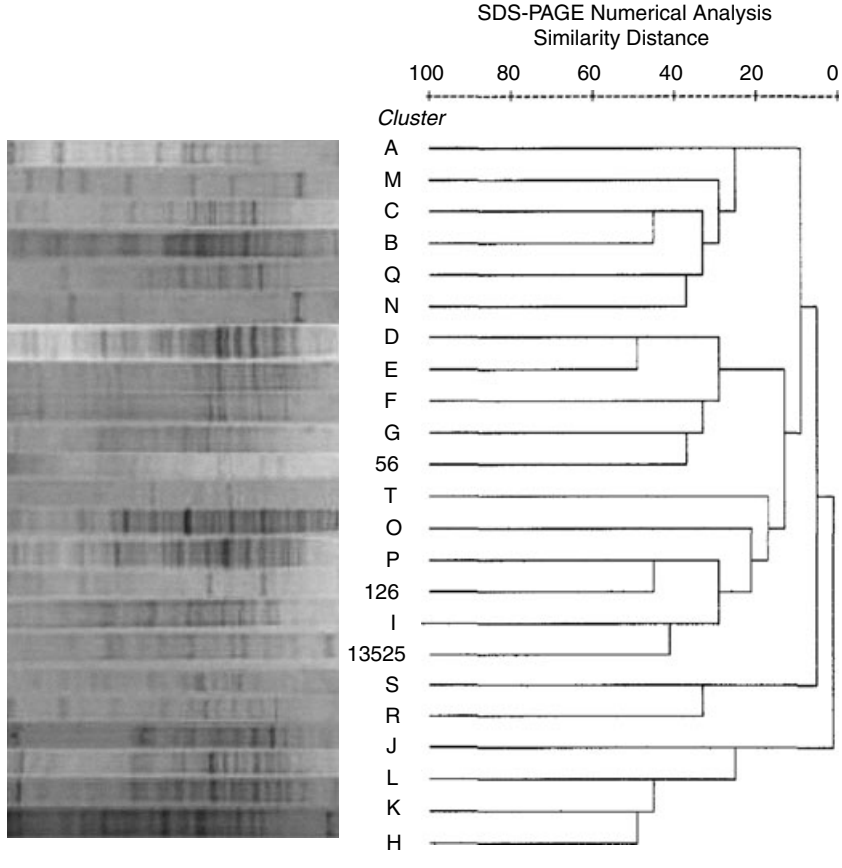

Fig. 1 Clustering analysis of SDS-PAGE patterns of the strains. The dendrogram was generated by the UPGMA method. The numbers on the horizontal axis indicate the percentage similarities as determined by the Pearson product-moment correlation coefficient (r). The vertical axis shows the defined groups in capital letters while the unclustered strains are indicated by the strain number

strains, $r = 0{\cdot}640$), S (four strains, $r = 0{\cdot}751$) and T (two strains, $r = 0{\cdot}926$) contained strains that could not be identified by the biochemical tests. Three strains remained unclustered; *Ps. fluorescens* type strain between them.

The discriminatory power of SDS-PAGE method was estimated at 0·883. The discriminant analysis of 'leave-one-out classification' yielded 93·7% of the cases correctly classified.

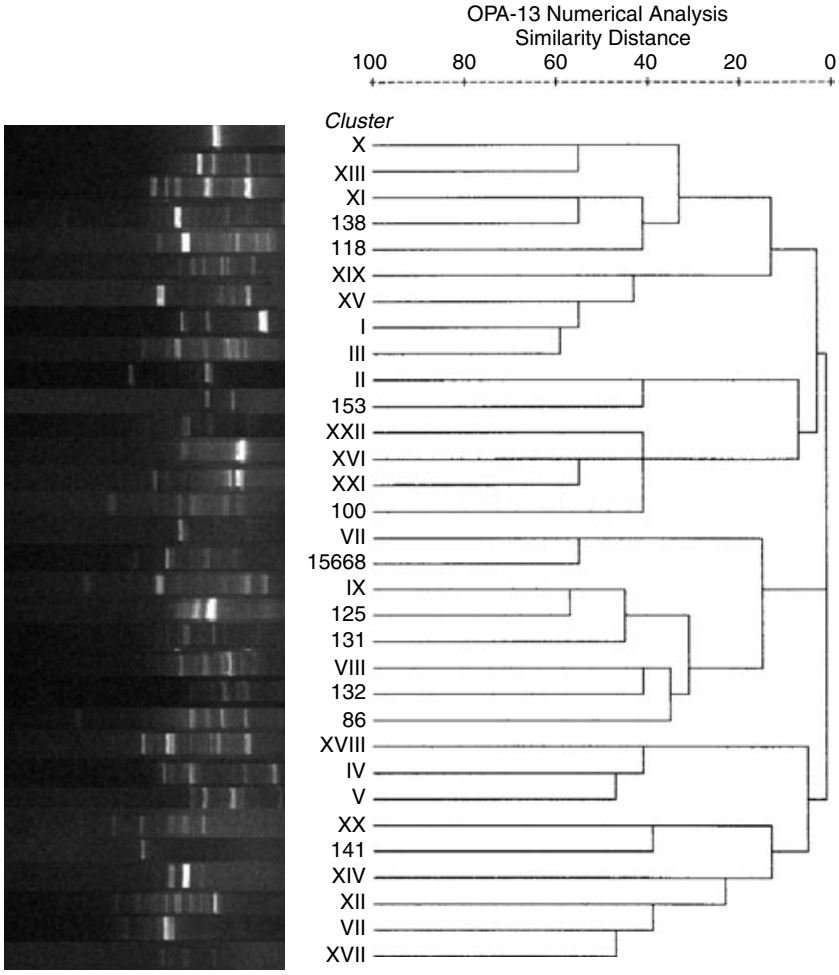

Fig. 2 Clustering analysis of RAPD patterns obtained with primer OPA-13. The dendrogram was generated by the UPGMA method. The numbers on the horizontal axis indicate the percentage similarities as determined by the Pearson product-moment correlation coefficient (r). The vertical axis shows the defined groups in latin numbers while the unclustered strains are indicated by the strain number

Randomly amplified polymorphic DNAs assay

Typeability and reproducibility. The typeability of the OPA-13 primer was estimated at 99·4%, since 167 out of 168 environmental isolates subjected to RAPD assay, yielded amplification products, while with OPD-13, 165 out of 168 isolates generated amplification products (typeability 98·2%).

To check the reproducibility of the method, DNA of 30 randomly chosen strains was tested on two different subcultures. Identical RAPD banding patterns were observed. Moreover, *Ps. aeruginosa* type strain ATCC 27853 was included in each RAPD assay and gave identical patterns. In detail, the patterns of ATCC 27853 in 20 different gels with OPA-13 were identical ($r = 1{\cdot}00$, CV $= 0{\cdot}02$) while with OPD-13, the reference strain gave average similarities with a mean and standard deviation of (97·5 ± 2·2)% when analysed on the 20 different gels used in the study.

Each primer amplified a DNA fingerprint ranging from two to 12 bands for OPA-13 and from two to 16 bands for OPD-13, over a size range of 200–2500 bp.

OPA-13. Strains (167:159 environmental isolates and eight type strains) when subjected to RAPD analysis using OPA-13 primer, resulted in the formation of 22 clusters at similarity level $r = 0{\cdot}579$, while 10 strains remained unclustered (Fig. 2). The discriminatory power of OPA-13 was at the same level as SDS-PAGE, since it was estimated at 0·868. The largest cluster (cluster I) contained the 34% (56 strains) of the population under study. These strains shared 0·632 similarity, the 52 of which were identified as *Ps. aeruginosa* by the API20NE system. Six other clusters (III, V, VIII, IX, XXI and XVIII) were defined at similarity levels varying from 0·579 to 0·712 and contained from 6 to 11 strains each. 110 strains (66% of the total population) formed these seven clusters while the remaining 57 strains were distributed into 15 clusters, each containing from two to five strains. The unidentified by the API strains were mainly included in clusters XXI (seven strains, $r = 0{\cdot}650$) and XVIII (nine strains, $r = 0{\cdot}712$). Two unidentified isolates remained ungrouped with OPA-13.

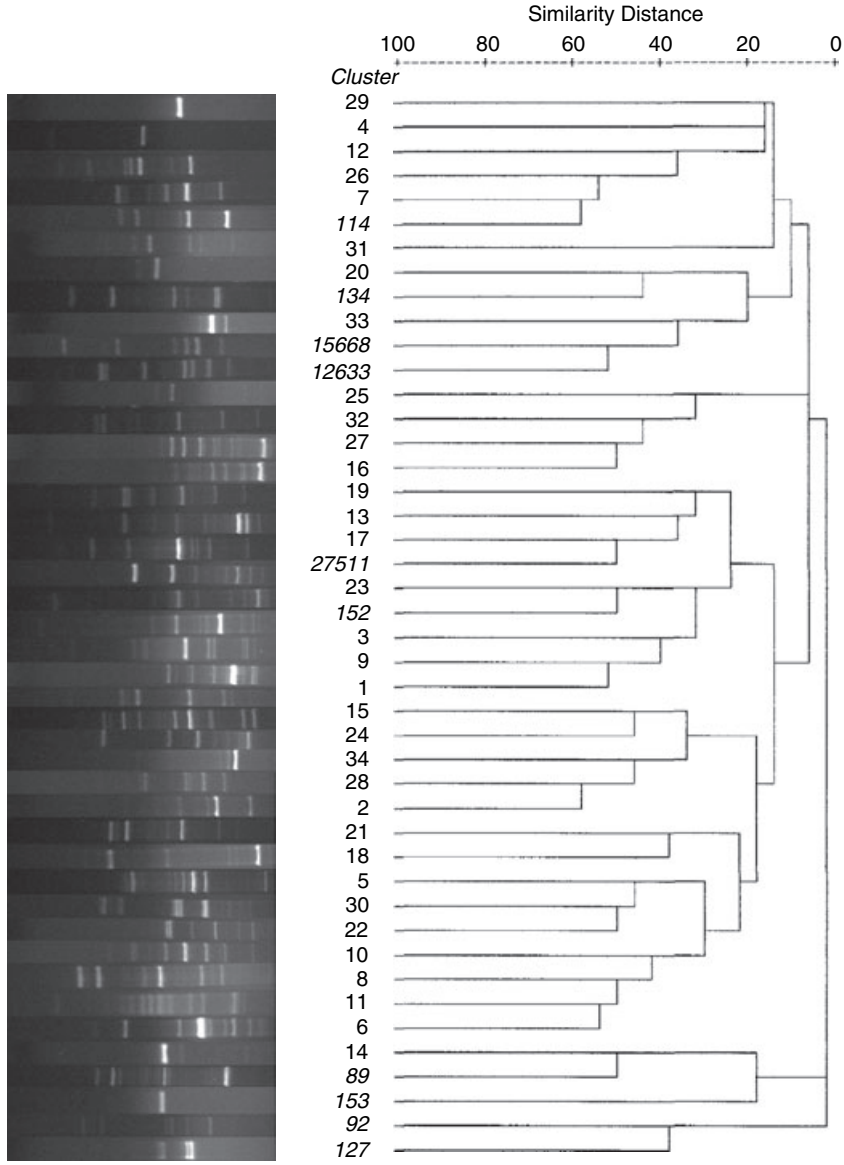

Fig. 3 Clustering analysis of RAPD patterns obtained with primer OPD-13. The dendrogram was generated by the UPGMA method. The numbers on the horizontal axis indicate the percentage similarities as determined by the Pearson product-moment correlation coefficient (*r*). The vertical axis shows the defined groups in arabic numbers while the unclustered strains are indicated by the strain number in italics

A percentage of 88·5 of cross-validated cases were correctly classified as it was estimated by the discriminant analysis.

OPD-13. The numerical analysis of the RAPD profiles obtained with primer OPD-13 yielded 34 clusters at the 58·2% similarity level while 10 strains remained ungrouped. 35% (58 strains) of the study population formed six major clusters at or above 0·600 similarity level. As it is exhibited in Table 1, there is general agreement between OPD-13, OPA-13 and SDS-PAGE classification results. The unidentified by the API strains, clustered mainly into groups 38 and 40. Two unidentified isolates remained ungrouped with OPD-13. The defined groups of OPD-13 are depicted in Fig. 3. The discriminatory power of the primer OPD-13 was the higher found in this study, since the index of discrimination achieved the value of 96·9%. The discriminant analysis of 'leave-one-out' classification yielded 82·6% of the cases classified correctly.

The similarity levels among isolates of the groups defined by each method are exhibited in Table 2.

Comparison of the three methods. Out of 160 strains isolated, the API20NE identification system discerned 14 species, while SDS-PAGE produced 20 groups. In RAPD assay, with primer OPA-13 the strains were distributed to 22 groups, while with OPD-13 34 clusters were defined.

In general, there was agreement between API and PAGE. *Pseudomonas aeruginosa* strains formed protein clusters A and B. Clusters C–G comprised *Ps. stutzeri* isolates, while *C.*

Table 2 Groups formed by each method and similarity levels among isolates

SDS-PAGE			RAPD (primer OPA-13)			RAPD (primer OPD-13)					
Cluster	Strains (*n*)	Similarity level	Cluster	Strains (*n*)	Similarity level	Cluster	Strains (*n*)	Similarity level	Cluster	Strains (*n*)	Similarity level
A	49	0·650	I	56	0·632	1	9	0·598	23	4	0·630
B	11	0·700	II	4	0·971	2	13	0.637	24	3	0·613
C	3	0·723	III	11	0·611	3	5	0.675	25	3	0·664
D	13	0·771	IV	4	0·626	4	3	0.758	26	5	0·584
E	6	0·685	V	11	0·596	5	4	0.635	27	6	0·688
F	6	0·630	VI	5	0·694	6	5	0.827	28	3	0·690
G	2	0·741	VII	2	0·750	7	14	0·616	29	5	0·616
H	6	0·995	VIII	10	0·579	8	5	0·693	30	2	0·706
I	7	0·643	IX	6	0·660	9	8	0·645	31	2	0·623
J	5	0·674	X	3	0·764	10	3	0·776	32	5	0·582
K	2	1·000	XI	5	0·652	11	2	1·000	33	3	0·674
L	6	0·588	XII	2	0·799	12	4	0·753	34	8	0·631
M	8	0·880	XIII	2	0·927	13	3	0·629			
N	7	0·572	XIV	4	0·591	14	3	0·666			
O	9	0·550	XV	3	0·663	15	2	0·688			
P	5	0·618	XVI	3	0·693	16	4	0·584			
Q	6	0·695	XVII	3	0·690	17	3	0·666			
R	4	0·640	XVIII	9	0·712	18	2	0·663			
S	2	0·751	XIX	3	0·662	19	2	0·588			
T	2	0·926	XX	2	0·588	20	2	0·630			
			XXI	7	0·650	21	7	0·755			
			XXII	2	0·635	22	3	0·614			

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RAPD, randomly amplified polymorphic DNAs.

acidovorans strains defined cluster H and *Ps. fluorescens* cluster I. *B. pickettii* gave cluster J, *Stenotrophomonos paucimobilis* strains formed cluster K and *Pseudomonas alcaligenes* joined at cluster L. Cluster M contained *S. maltophilia* isolates and cluster O the *Aeromonas hydrophila/caviae* strains. Only in two situations, SDS-PAGE could not discern between what appeared to be *Ps. putida/Burkholderia diminuta* strains and *Ps. mendocina/B. cepacia* strains.

As far as RAPD assay is concerned, *Ps. aeruginosa* strains defined four clusters when amplified with OPA-13 and eight major clusters when OPD-13 was used. Isolates regarded as *Ps. stutzeri* with API, were distributed in 10 clusters with OPA-13 and in 13 clusters in the case of OPD-13. All *Ps. fluorescens* strains defined two clusters with OPD-13 and one cluster with OPA-13 except for three isolates. Four out of six *Ralstonia pickettii* isolates defined one cluster with OPA-13 while OPD-13 was unable to correlate them.

All the unidentified by the API strains were classified into groups under the SDS-PAGE assay while via the RAPD method 90% of them could be classified. In more detail, 8 strains produced the same classification result under all methods used. Twelve strains were classified into the same groups by their protein profiles and amplification products of primer OPD-13. The same results were obtained for 11 strains by their classification with SDS-PAGE and OPA-13, and with OPA-13 and OPD-13. The results are summarized in Table 3.

Table 3 Summary of the classification results of the isolates as defined by each method

Classification method	Total No. of strains	No. of groups	No. of clustered strains	Ungrouped or unidentified strains
API20NE	168	14	148 (88·0%)	20 (11·9%)
SDS-PAGE	162	20	159 (98·1%)	3 (1·9%)
RAPD with primer OPA-13	167	22	157 (94·0%)	10 (6·0%)
RAPD with primer OPD-13	165	34	155 (93·9%)	10 (6·1%)

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RAPD, randomly amplified polymorphic DNAs.

DISCUSSION

In recent years several studies have been performed in order to analyse the diversity of *Pseudomonas* genus and related species. These studies have used a large number of typing techniques, either conventional or recently developed, such as molecular typing schemes. However, more of the studies about *Pseudomonas* refer to clinical isolates while there is little information about environmental strains (Sørensen *et al.* 1992; Elomari *et al.* 1995; Morais *et al.* 1997; Widmer *et al.* 1998).

Two conventional phenotypic (API20NE and SDS-PAGE) and one molecular method (RAPD) were applied to determine the relatedness of aquatic isolates belonging to the *Pseudomonas* family.

The 20 enzymes, that API20NE identification system tests, may be inadequate for credible identification. Previous studies raise questions about the trustworthiness of such identification systems and in particular, one calculates at least a 20% probability of a false negative result (Shelly *et al.* 2000). Moreover, API20NE has been developed for the identification of pathogenic species, and as a consequence, often fails to identify environmental isolates (Ferreira *et al.* 1996). Another serious drawback of many commercial biochemical systems lies mainly in their incomplete databases (Soler *et al.* 2003), for instance, in the database of API20NE very few *Pseudomonas* species are included, while in nature a large number of different species has been reported. The above-mentioned shortcomings make the API20NE a useful tool only for a first characterization of environmental strains but not for a definite identification. This study verifies the limited usefulness of API20NE for environmental isolates, since 11·9% of the bacterial population, which remained unidentified to the species level by this system, could be classified by the other methods used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell lysates has been reported as a specific assay for strain detection of Pseudomonads in environmental samples (Sørensen *et al.* 1992). Bacterial whole-cell protein profiles are a reflection of the genetic composition of a given bacterial strain, a fact that makes SDS-PAGE a valuable tool for bacterial classification, identification and grouping (Li and Hayward 1994). The classification of the 20 strains, unidentified by the API, confirms SDS-PAGE application. Given that unique protein molecules can be encoded by DNA sequences differing as much as 30%, because of the degeneration of the genetic code (Li and Hayward 1994), the few discordant classification results between the SDS-PAGE and the RAPD assays can be explained.

Molecular typing techniques search the bacterial identities in more depth since the genome of any organism reflects its uniqueness. It has been reported that genetic analysis at the species level gives insight into the variability within a bacterial population and generates evidence on bacterial adaptation to various environmental conditions (Renders *et al.* 1996).

In this study, two primers were used for genetic typing of the isolates. The first primer (OPA-13) had lower discriminatory power but a higher percentage of correctly classified strains, in comparison to the other (OPD-13), as it was assessed by the discriminant analysis. A few discrepancies in the classification results obtained by each primer may be explained by the fact that the 10-mer primers detect polymorphisms occurring in distinct chromosomal regions and, therefore, variations in one region may not necessarily affect other regions (Nociari *et al.* 1996). Especially for species, such as *R. pickettii* or *Ps. putida*, that appeared to be particularly dispersed in their RAPD fingerprints, one possible explanation could be the amplification of DNA loci displaying a high frequency of mutation or rearrangements caused by intra- or inter-strain exchange of genetic material (Renders *et al.* 1996). Therefore, the combination of data obtained by different primers or typing procedures gives optimal insight into strain relatedness (Renders *et al.* 1996).

Furthermore, apart from the evaluation of the methods, the structure, epidemiology and potential role on public health of the isolates were under concern. There have been three to six strains, identified by the API as *Pseudomonas* spp., which, by the other methods, were clustered together with *Aer. hydrophila/caviae* strains. *Aeromonas* genus, and in particular *Aer. hydrophila*, have been previously demonstrated as opportunistic pathogens, prevalent in both chlorinated and unchlorinated water supplies (Massa *et al.* 2001) and in bottled waters (Tsai and Yu 1997). The diseases mainly linked to *Aeromonas* genus are septicemia and gastroenteritis (Massa *et al.* 2001). Recently *Aer. hydrophila* has been placed on the United States Environmental Protection Agency Contaminant Candidate List of emerging pathogens in drinking water (Borchardt *et al.* 2003). The list includes drinking water contaminants of public health concern, needing further investigation before a decision would be made about their addition in the new regulations (Borchardt *et al.* 2003).

In conclusion, rapid identification systems, such as API20NE, may be reliable only for a first screening of environmental pseudomonads but not for a definite identification, since it is possible for opportunistic pathogens not to be recognizable by the API. SDS-PAGE and RAPD provide further information about the potential roles of possible unidentified subgroups of this genus in various ecosystems and their impact on public health. The need for re-evaluation of the water quality standards currently used is another issue. There are species, wide spread in the aquatic

environment, which can serve as causative agents of diseases, especially in high risk population.

REFERENCES

Anonymous (1998) Council Directive of 3 November 1998 on the quality of water intended for human consumption. *Official Journal L330*, 05/12/1998 p. 0032–0054.

Bharath, J., Mosodeen, M., Motilal, S., Sandy, S., Sharma, S., Tessaro, T., Thomas, K., Umamaheswaran, M. *et al.* (2003) Microbial quality of domestic and imported brands of bottled water in Trinidad. *Int J Food Microbiol* **81**, 53–62.

Borchardt, M.A., Stemper, M.E. and Standridge, J.H. (2003) *Aeromonas* isolates from human diarrheic stool and groundwater compared by Pulsed-Field Gel Electrophoresis. Emerging Infectious Diseases [serial online] 2003 Feb. Available from URL: http://www.cdc.gov/ncidod/EID/vol9no2-0031.htm

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Bukanov, N., Ravi, N.V., Miller, D., Srivastava, K. and Berg, D.E. (1994) Pseudomoans aeruginosa corneal ulcer isolates distinguished using the arbitrarily primed PCR DNA fingerprinting method. *Curr Eye Res* **13**, 783–790.

Coenye, T., Vandamme, P., Govan, J.R.W and Lipuma J.J. (2001) Taxonomy and identification of the *Burkholderia cepacia* complex. *J Clin Microbiol* **39**, 3427–3436.

Elaichouni, A., Verschraegen, G., Claeys, G., Devleeschouwer, M., Godard, C. and Vaneechoutte, M. (1994) Pseudomonas aeruginosa serotype O12 outbreak studied by Arbitrary Primer PCR. *J Clin Microbiol* **32**, 666–671.

Elomari, M., Coroler, L., Izard, D. and Leclerc, H. (1995) A numerical taxonomic study of fluorescent *Pseudomonas* strains isolated from natural mineral waters. *J Appl Bacteriol* **78**, 71–81.

Ferreira, A.C., Morais, P.V., Gomes, C. and da Costa, M.S. (1996) Computer-aided comparison of protein electrophoretic patterns for grouping and identification of heterotrophic bacteria from mineral water. *J Appl Bacteriol* **80**, 479–486.

Haase, A., Melder, A., Smith-Vaughan, H., Kemp, D. and Currie, B. (1995a) RAPD analysis of isolates of *Burkholderia pseudomallei* from patients with recurrent melioidosis. *Epidemiol Infect* **115**, 115–121.

Haase, A., Smith-Vaughan, H., Melder, A., Wood, Y., Janmaat, A., Gilfedder, J., Kemp, D. and Currie, B. (1995b) Subdivision of *Burkholderia pseudomallei* ribotypes into multiple types by Random Amplified Polymorphic DNA analysis provides new insights into epidemiology. *J Clin Microbiol* **33**, 1687–1690.

Hernández, J., Ferrús, M., Hernández, M., Owen, R.J. (1997) Arbitrary primed PCR fingerprinting and serotyping of clinical *Pseudomonas aeruginosa* strains. *FEMS Immunol Med Microbiol* **17**, 37–47.

Hunter, P.R. and Gaston, M.A. (1988) Numerical index of the discriminatory ability of typing systems: an application of simpson's index of diversity. *J Clin Microbiol* **26**, 2465–2466.

Kersters, K., Ludwig, W., Vancanneyt, M., De Vos, P., Gillis, M. and Schleifer, K.H. (1996) Recent changes in the classification of the Pseudomonads: an overview. *Syst Appl Microbiol* **19**, 465–477.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* **227**, 680–685.

Leitão, J.H., Alvim, T. and Sá-Correia I. (1996) Ribotyping of *Pseudomonas aeruginosa* isolates from patients and water springs and genome fingerprinting of variants concerning mucoidy. *FEMS Immunol Med Microbiol* **13**, 287–292.

Li, X. and Hayward, A.C. (1994) Bacterial whole cell protein profiles of the RNA group II pseudomonads. *J Appl Bacteriol* **77**, 308–318.

Mahenthiralingam, E., Campbell, M.E., Foster, J., Lam, J.S. and Speert, D.P. (1996) Random Amplified Polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J Clin Microbiol* **34**, 1129–1135.

Massa, S., Altieri, C. and D'Angela, A. (2001) The occurence of *Aeromonas* spp. in natural mineral water and well water. *Int J Food Microbiol* **63**, 169–173.

Mavridou, A., Papapetropoulou, M., Boufa, P., Lambiri, M. and Papadakis, J.A. (1994) Microbiological quality of bottled water in Greece. *Lett Appl Microbiol* **19**, 213–216.

Morais, P.V., Mesquita, C., Andrade, J.L. and Da Costa, M.S. (1997) Investigation of persistent colonization by *Pseudomonas aeruginosa*-like strains in a spring water bottling plant. *Appl Environ Microbiol* **63**, 851–856.

Moreira, L., Agostino, P., Morais, P.V. and da Costa, M.S. (1994) Survival of allochthonous bacteria in still mineral water bottled in polyvinyl chloride (PVC) and glass. *J Appl Bacteriol* **77**, 334–339.

Nociari, M., Catalano, M., García, D.C., Copenhaver, S.C., Vasil, M.L. and Sordelli, D.O. (1996) Comparative usefulness of ribotyping, Exotoxin A genotyping, and *Sal*I restriction fragment length polymorphism analysis for *Pseudomonas aeruginosa* lineage assessment. *Diagn Microbiol Infect Dis* **24**, 179–190.

Olive, D.M. and Bean P. (1999) Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol* **37**, 1661–1669.

Plikaytis, B.D., Carlone, G.M. and Plikaytis, B.B. (1986) Numerical analysis of normalized whole-cell protein profiles after Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. *J Gen Microbiol* **132**, 2653–2660.

Pot, B., Vandamme, P. and Kersters, K. (1994) Analysis of electrophoretic whole-organism protein fingerprints. In *Chemical Methods in Prokaryotic Systematics* ed. Goodfellow, M. and O'Donell, A.G. pp. 493–521. New York: John Wiley and Sons Ltd.

Renders, N., Römling, U., Verbrugh, H. and Van Belkum, A. (1996) Comparative typing of *Pseudomonas aeruginosa* by random amplification of polymorphic DNA or pulsed-field gel electrophoresis of DNA macrorestriction fragments. *J Clin Microbiol* **34**, 3190–3195.

Römling, U., Wingender, J., Müller, H. and Tümmler, B. (1994) A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats. *Appl Environ Microbiol* **60**, 1734–1738.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning, a Laboratory Manual*, 2nd edn ed. pp. E.5. USA: Cold Spring Harbor Laboratory Press.

Shelly, D.B., Spilker, T., Gracely, E.J., Coenye, T., Vandamme, P. and LiPuma, J.J. (2000) Utility of commercial systems for identification of *Burkholderia cepacia* complex from cystic fibrosis sputum culture. *J Clin Microbiol* **38**, 3112–3115.

Soler, L., Marco, F., Vila, J., Chacón, M.R., Guarro, J. and Figueras, M.J. (2003) Evaluation of two miniaturized systems, MicroScan W/A and BBL Crystal E/NF, for identification of clinical isolates of *Aeromonas* spp. *J Clin Microbiol* **41**, 5732–5734.

Sørensen, J., Skouv, J., Jørgensen, A. and Nybroe, O. (1992) Rapid identification of environmental isolates of *Pseudomonas aeruginosa, P. fluorescens* and *P.putida* by SDS-PAGE analysis of whole-cell protein patterns. *FEMS Microbiol Ecol* **101**, 41–50.

Tsai, G.-J. and Yu, S.-C. (1997) Microbiological evaluation of bottled uncarbonated mineral water in Taiwan. *Int J Food Microbiol* **37**, 137–143.

Warburton, D., Harrison, B., Crawford, C., Foster, R., Fox, C., Gour, L. and Krol, P. (1998) A further review of the microbiological quality of bottled water sold in Canada: 1992–1997 survey results. *Int J Food Microbiol* **39**, 221–226.

Welsh, J. and McClelland, M. (1990) Fingerprints genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**, 7213–7218.

Widmer, F., Seidler, R.J., Gillevet, P.M., Watrud, L.S. and Di Giovanni, D. (1998) A highly selective PCR protocol for detecting 16S rRNA genes of the genus *Pseudomonas* (Sensu stricto) in environmental samples. *Appl Environ Microbiol* **64**, 2545–2553.