Typing of *Pseudomonas aeruginosa* strains isolated from Greek water samples by three typing methods: serotyping, Random Amplified Polymorphic DNA (RAPD) and Pulsed Field Gel Electrophoresis (PFGE)

O. Pappa, G. Mandilara, A. Vatopoulos and A. Mavridou

ABSTRACT

The study of various types of *Pseudomonas aeruginosa* strains isolated from water environments is of paramount importance from a public health point of view, due to their ubiquity and pathogenicity. Molecular (Random Amplified Polymorphic DNA and Pulsed Field Gel Electrophoresis) and phenotypical (serotyping) typing methods were applied to environmental *P. aeruginosa* strains. The typeability and discriminatory power of the methods were studied and compared. The two molecular methods managed to type a number of *P. aeruginosa* strains which were non-serotypeable due to their rough phenotypes. According to our results, the combination of phenotypic and genotypic methods increased the reliability of the results, yielding several different clones that seem to circulate in Greek water environments.

Key words | environmental strains, *Pseudomonas*, Pulsed Field Gel Electrophoresis, typing, wastewater

O. Pappa (corresponding author)
Central Public Health Laboratory,
Hellenic Centre for Diseases Control and Prevention– 34,
Alexandrou Fleming, Athens,
Greece
E-mail: *olpap79@yahoo.gr*

G. Mandilara
A. Vatopoulos
National School of Public Health-Department of Microbiology,
196, Alexandras Ave. 11521 Athens,
Greece

A. Mavridou
Technological Educational Institution of Athens-Department of Medical Laboratories,
Ag.Spiridonos, 12210 Aigaleo, Athens,
Greece

INTRODUCTION

Many methods have been developed for the typing of clinical *Pseudomonas aeruginosa* strains with high reliability and reproducibility. However, literature concerning typing of environmental strains other than derived from hospital water networks is very limited (Renders *et al*. 1996; Sazakli *et al*. 2005; Khan *et al*. 2007).

Serotyping is traditionally the most important phenotypic method, but the presence of rough strains, mainly in environmental samples, is considered as the main limitation of the method restricting its typeability (Bystrova & Knirel 2006; Belkum Van *et al*. 2007; Pirnay *et al*. 2009). Thus the use of molecular techniques is necessary. Searching in the literature, limitations on the applicability and resolution power of Random Amplified Polymorphic DNA (RAPD) and Pulsed Field Gel Electrophoresis (PFGE) in typing environmental strains are obvious (Amagliani *et al*. 2011; Lanini *et al*. 2011).

RAPD is considered as the method which produces the fastest typing results with least hands-on time. RAPD presented high typeability and discriminatory power when typing clinical isolates (Speert *et al*. 2002; Satpathy *et al*. 2010; Lanini *et al*. 2011). The method was also used for a first screening of *P. aeruginosa* environmental strains (Bradbury *et al*. 2009; Amagliani *et al*. 2011). Nevertheless, a thorough search in the literature reveals that two or more primers in the application of RAPD are used (Campbell *et al*. 2000; Sazakli *et al*. 2005; Trautmann *et al*. 2006; Lanini *et al*. 2011). This fact makes the method more complicated in the interpretation of the results and more time consuming, cancelling the main advantage of the method.

As for the PFGE, a standardized protocol is used in all published studies. Taking the value of the method as an important epidemiological tool, it has been used in studying the prevalence of *P. aeruginosa* in hospital water facilities and in outbreak investigations (Vallés *et al*. 2004; Lavenir *et al*. 2008; Bradbury *et al*. 2009; Huhulescu *et al*. 2010).

The aim of the present study was to determine, to compare and correlate, and to improve the typeability and the discriminatory power of three different typing methods applied to *P. aeruginosa* strains isolated from environmental samples (water and wastewater). Moreover, the study aimed to detect the distribution of the various types of *P. aeruginosa* strains in specific habitats, since there are no similar data available for the Greek region.

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MATERIALS AND METHODS

Sampling

Forty-eight positive for *P. aeruginosa* samples were used in the present study: 31 water samples sent to the Central Public Health Laboratory from various areas of Greece (17 mains water, four swimming pools, three wells, six boreholes, one spring water) and 17 wastewater samples (five from the influent and 12 from the effluent of the Secondary Treatment of the Central Athens Treatment Plant). The criteria for sample selection were temporal, geographical and sample type. Additionally, wastewater samples are used since they often concentrate isolates from the total population.

Colony isolation

The samples were analyzed for the detection of *Pseudomonas aeruginosa* using the standard method applied in EU countries based on the membrane filtration technique (ISO 16266:2006). Wastewater samples were handled according to ISO 6887-1:1999 and 8199:2005 concerning the preparation of decimal dilutions of samples with high background flora. The colony identification (phenotypic identification) was presumptively based on the production of pyocyanin. Pyocyanin-producing colonies are considered as confirmed *P. aeruginosa* but other fluorescing or reddish brown colonies require confirmation (i.e. ability to produce ammonia from acetamide and/or to produce the cytochrome oxidase) (ISO 16266:2006). The control strain *Pseudomonas aeruginosa* NCTC 10662 was used as a standard of reference during all the procedures (identification and typing methods).

Serotyping

Serological typing was performed using typing sera according to the International Antigenic Typing System (IATS) (Sanofi Diagnostics Pasteur). During the procedure the reference strain *Pseudomonas aeruginosa* NCTC 10662 was serotyped as serotype O:15.

DNA extraction

Isolates were grown from frozen stock on nutrient broth incubated at 37 °C for 18 h. Three different protocols were applied in order to reach the most appropriate for the isolation of pure DNA. In particular: (a) DNA extraction with proteinase K (Da Silva Filho Luiz 2001; Wilson 2001), (b) DNA extraction with CTAB (hexadecyltrimethyl ammodium bromide) and proteinase K (Wilson & Carson 2001) and (c) DNA extraction with lysozyme (Rademaker *et al.* 2000). Concentration and purity of DNA was measured with Spectrophotometer Biophotometer plus 6132, Eppendorf (260–596 nm).

RAPD-typing

Two primers, 208 5′ ACGGCCGACC 3′ and 272 5′ AGCGGGCCAA 3′ (Rademaker *et al.* 2000) were tested on 20 isolates (randomly chosen) in order to decide which one produced better results (number and intensity of bands). Amplification was performed as described previously (Rademaker *et al.* 2000). RAPD products were electrophorized in 1.5% agarose gels in 0.5× TBE, stained with ethidium bromide. A 100 bp DNA ladder (Fermentas, Gene ruler, DNA ladder, 0.5 μg/μl) was used as a size marker. Finally, the gels were seen under UV light (DOLPHIN DOC, WEALTEC Corp.)

PFGE analysis

PFGE was performed as described previously with some alterations concerning the concentration of the initial inoculums (measuring Optical Density at 600 nm) (Spencker *et al.* 2000). The isolates were grown in nutrient broth overnight at 37 °C and then were treated with lysozyme at 37 °C and with proteinase K at 50 °C, both overnight incubation. After four washing steps, DNA was digested with the rare-cutting restriction endonuclease SpeI (BcuI, 10 u/μl, Fermentas) at 37 °C overnight. The digests of the genomic DNA of *P. aeruginosa* were separated on 1% agarose gels by PFGE under the following conditions: current range 100–130 mA at 14 °C for 20 h, initial switch time 5.3 s, final switch time 34.9 s, angle 120°, field strength 6 V/cm. Banding patterns were visualized by ethidium bromide staining and photographed by DOLPHIN DOC, WEALTEC Corp.

Computational and visual analysis of RAPD profiles

RAPD profiles were analyzed statistically by Gel Compar 2, which clustered the isolates at a 70% cut-off. Profiles presenting a similarity index >70% by Gel Compar were run again side by side to confirm their identity and then they were inspected visually and compared according to criteria previously described (Renders *et al.* 1996). Thus, isolates differing by one or two bands were considered to belong in the same RAPD type.

Computational and visual analysis of PFGE profiles

PFGE profiles were analyzed statistically by Gel Compar 2, which clustered the isolates at a 79% cut-off. For PFGE, the visual inspection was performed according to Tenover's criteria for the interpretation of PFGE profiles (Tenover *et al.* 1995). The final clustering of the isolates was determined according to the findings of both methods.

Typeability and discriminatory power

To evaluate the three typing methods, two basic performance criteria were used, typeability and discriminatory power. The typeability is expressed as the percentage of the typeable isolates over the total isolates (T = Typeable isolates/total isolates × 100%) while, the discriminatory power is expressed by Simpson's index:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j (x_j - 1) \quad (1)$$

where N is the total number of strains in the sample population, S is the total number of types described and x is the number of strains belonging to the jth type. The discriminatory power should ideally be 1.0 but in practice it should be at least in the order of 0.95 for a good typing method (Belkum Van *et al.* 2007).

RESULTS AND DISCUSSION

Phenotypic identification

One hundred and twelve presumptive *P. aeruginosa* colonies (pyocyanin-producing, other fluorescing or reddish brown) were initially collected by selecting one colony from 16 positive water and seven wastewater samples and two to four different colonies from 15 positive water and 10 wastewater samples. According to ISO 16266:2006, confirmation procedures, 70 colonies in total (20 from mains water, seven from swimming pools, two from well water, 10 from boreholes, two from spring water and 29 from wastewater samples) were confirmed as *P. aeruginosa*.

Serological typing

Twenty seven out of the 70 confirmed isolates (38.6%) were proved to be typeable and were allocated into 10 different serotypes. Ten out of the 27 serotypeable isolates (37%) were typed as serotype O:11 (14.5% of the total isolates), followed by nine more serotypes in lower frequencies. Thirty one of the isolates (44.3%) were characterized as non-typeable while 12 isolates (17.1%) presented the rough phenotype (Table 1). The typeability of the method was very low (38.6%). The peculiarity of environmental *P. aeruginosa* isolates is that they often meet low nutrient or starvation conditions, so they adopt mechanisms in order to survive, which change their phenotypic characteristics (smooth to rough isolates) (Mavridou *et al.* 1994). Cross-reaction between the H- and the O-antigens results in auto-agglutination (rough phenotype = clump formation during serotyping procedure), a very common phenomenon and actually the main problem when applying this typing method (Bystrova & Knirel 2006). For clinical isolates the typeability of the method is high, as smooth phenotype, which is more pathogenic, is much more frequent than the rough one (Hernandez & Ferrus 1997; Tassios *et al.* 1998). Nevertheless in cystic fibrosis patients, *P. aeruginosa* isolation relies on the rough phenotype under oxygen stress (Lyczaka *et al.* 2000; Parsek & Fuqua 2004). Additionally, in environments such as wastewater, wells, boreholes, water tanks and so forth, rough strains are prevalent because they survive under stressed conditions (chlorine, toxic substances, oxygen stress, temperature, etc.). These findings are similar to the results of other studies (Makin & Beveridge 1996; Sabra *et al.* 2003) which use serotyping as the basic phenotypical method in typing *P. aeruginosa*, unlike the presence of high percentage of rough and non-typeable isolates.

The discriminatory power of the method was weak (0.82). This is explained from the fact that 37% of the serotypeable isolates was allocated to serotype O:11 and from the presence of the rough phenomenon at a 17.1% of the total isolates. Nevertheless, the method managed to cluster the 27 serotypeable isolates into 10 out of 16 serotypes. Three out of the 10 serotypes, O:11, O:1 and O:10, seem to be the predominant ones, which according to the literature are the main serotypes in clinical isolates too (Shehabi & Masoud 2005; Winstanley *et al.* 2005; Trautmann *et al.* 2006; Pirnay *et al.* 2009).

DNA extraction

RAPD-polymerase chain reaction (RAPD-PCR) requires particular amounts and purity of DNA for optimum performance. Thus, it was necessary to test more than one DNA-extraction protocol (data not shown). The protocol

Table 1 | Correlation of serotypes with RAPD- and PFGE-profiles

Serotypes	RAPD-types						PFGE-types						
[a]N = 70	R6 (10%)	R4 (8.6%)	R5 (7.1%)	R1 (7.1%)	OTHERS (37.1%)	N.T. (30%)	VIII (10%)	III (8.6%)	XI (7.1%)	VII (5.7%)	XIII (5.7%)	OTHERS (47.1%)	N.T. (15.7%)
O:11 (14.5%)	3 (2mn, 1ww)	5 (4sp, b)				2 (mn, sp)		5 (sp)				4 (ww, 3mn)	1 (b)
O:10 (6%)			1 (mn)		3 (ww)		3 (ww)					1 (mn)	
O:1 (7%)		1 (mn)	2 (mn, w)		1 (ww)	1 (mn)				2 (2mn)		3 (w, mn, ww)	
OTHERS (11.5%)	1 (mn)		1 (ww)	2 (ww)	2 (ww)	2 (ww)						7 (5ww, 2b)	1 (mn)
ROUGH (17.1%)	1 (mn)				8 (6ww, b, mn)	3 (mn, sw, ww)	4 (3ww, mn)					6 (3ww, 2 m, sw)	2 (ww, b)
N.T. (44.3%)	2 (sp, mn)		1 (mn)	3 (b, 2ww)	12 (3b, 3mn, w, wt, 4ww)	13 (2mn, 2b, wt, sw, sp, 6ww)		1 (sp)	5 (2mn, w, 2wt)	2 (ww)	4 (3b, ww)	12 (6ww, 3b, 2mn, wt)	7 (3ww, sp, 3mn)

mn: mains water, sp: swimming pools, b: boreholes, w: wells, wt: water tank, sw: spring water.
Wastewater: ww, N.T.: Non-typeable.
[a]The percentages of all types are in total number of isolates $N = 70$.

using lysozyme was selected because it produced high purity DNA. Concentration and purity of DNA were found to be ≥ 0.020 $(\mu g/ml)^{-1}$ cm^{-1} (260 nm) and ~1.8 (ratio of absorbance 260/280 nm) respectively.

RAPD-PCR

The purity of the DNA template and the concentration of primer and Mg^{+2} were optimized to obtain high intra-assay reproducibility in RAPD. Furthermore, DNA of the reference strain R29 and of one, randomly chosen isolate, was included in each RAPD assay (three in total). Strain N26 and reference strain R29 produced eight bands from 250 to 1,100 bp and 12 bands from 350 to 1,400 bp, respectively (Figure 1). Primer 208 seemed to produce more intense and higher numbers of bands than primer 272 (data not shown) in all tested isolates (20 isolates randomly chosen) producing DNA fingerprints of two to 17 bands with a molecular size ranging from 200 to 3,000 bp. Primer 208 was chosen for the RAPD-assay.

Forty-nine of the 70 (typeability = 70%) isolates identified as *P. aeruginosa* were successfully typed, while the remaining 21 (30%) were characterized as non-typeable (absence or less than four bands) (Table 1). Interestingly, 75% of the isolates with the rough phenotype and 58% of the non-serotypeable isolates have been successfully typed by the genotypic method. Gel Compar 2 clustered the isolates at a 70% cutoff value, which means that isolates differing by one or more bands were considered to belong to different RAPD-types. Thus, the analysis of the 49 typeable isolates resulted in 18 genotypes. The discriminatory power of the method was calculated to be 0.927 (Table 2) which is considered satisfactory (Belkum Van *et al.* 2007). Although, computer-assistant analysis is a

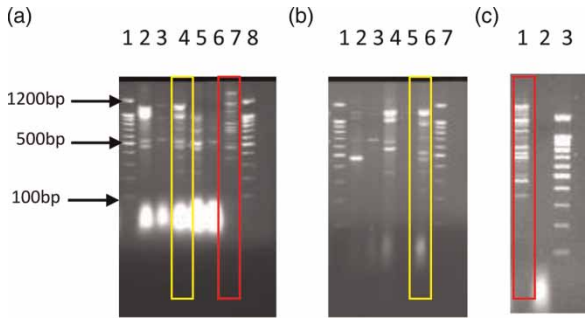

Figure 1 | Reference strain R29 (line 7 & 1 images) and the randomly chosen strain N26 (line 4 & 6, a & b images) produced identical profiles in the RAPD assay. The above gels are from 2 different RAPD-assays, while the two strains come from the same cultures but from separate DNA samples. A 100 bp DNA ladder was loaded at lines 1 & 8 (a image), 1 & 7 (b image) and at line 3 (c images).

Table 2 | Typeability and discriminatory power of the typing methods and of the combination of serotyping with RAPD- and PFGE-typing

N = 70	Serotyping	RAPD-typing	PFGE-typing	Serotyping/RAPD	Serotyping/PFGE
Typeability	27	49	59		
No. of types	10	14	22	36	36
Discriminatory power (D)/Combined D	0.82	0.927	0.95	0.95	0.97

very useful tool, especially for the analysis of large numbers of isolates, a few discrepancies were obtained when the same isolates were visually inspected and correlated with epidemiological data such as the common source or the type of the sample. According to criteria previously described (Renders *et al.* 1996), isolates differing by one or two bands were considered to belong to the same RAPD-type in contrast to Gel Compar 2 which clusters such isolates into different types. Thus, the final clustering of the isolates performed according to both analysis methods resulted in 14 RAPD-types (R1-R14). Twenty-five point five per cent of the isolates (13/49) were represented by two types (R6 and R4). Twelve more types were produced represented by one to five isolates. Twenty-two out of 27 serotypeable isolates were allocated into seven RAPD-types. A random distribution of the serotypeable isolates into the genotypes was observed. The isolates of the predominant serotypes (O:11, O:1, O:10) were subdivided into six RAPD-types. It seems that RAPD is capable of discriminating strains belonging to the same serotype into different genotypes, as individual genotypes include more than four different serotypes (Table 1). This result is also enhanced by the combination of the results of the two methods, where the 70 isolates of *P. aeruginosa* were allocated into 36 types and the discriminatory power of the combination was increased to 0.95 (Table 2). Our results agree with Trautmann *et al.* (2006), who observed no correlation between the results of the two typing methods. Nine of the 12 rough isolates were allocated into six different RAPD-types, while three isolates remained ungrouped. Eighteen of the 31 non-serotyped isolates were distributed into 10 RAPD-types, while the rest of the 13 isolates remained unclustered. Twenty-one isolates were characterized as non-typeable (Table 1).

PFGE analysis

The DNA digest with SpeI generated profiles of 11–22 bands. Fifty-nine of the 70 isolates (typeability = 84.2%) were successfully typed, eight isolates (11.5%) were characterized as non-typeable (lack of bands), while three isolates were excluded from the analysis because of the small number of bands (Table 1). Interestingly, 83% of the isolates with the rough phenotype and 77.5% of the non-serotypeable isolates have been successfully typed by the genotypic method. For the control of the reproducibility of the method, DNA of the reference strain R29 was included in two out of 10 PFGE assays and produced identical patterns (data not shown). Although, the method was applied to environmental isolates, the typeability was considered quite high. The application of PFGE in typing environmental strains of *P. aeruginosa* is still at a preliminary stage. The initial protocols have been established for clinical isolates, where, high typeability and discriminatory power was achieved (Spencker *et al.* 2000; Blanc *et al.* 2004; Lanini *et al.* 2011). The literature about PFGE-typing of environmental isolates is very limited and the method was applied in a very low number of isolates derived mainly from nosocomial environments, where the typeability and discriminatory power of the method could not be evaluated (Vallés *et al.* 2004; Lavenir *et al.* 2008; Bradbury *et al.* 2009; Huhulescu *et al.* 2010).

Gel Compar 2 clustered the isolates at a 79% cutoff value, which means that isolates differing by one or more bands were considered to belong to different PFGE-types. Thus, the analysis of the 59 typeable isolates resulted in 28 genotypes. Visual inspection and correlation with epidemiological data (common source, the type of the sample) were very useful in order to come up with decisions. Visual observation of the profiles and interpretation of the results were applied according to Tenover's (1995) criteria. Therefore, three isolates (non-serotypeable) which gave a small number of bands were excluded from the analysis. Thus, the final clustering of the isolates was performed according to both analysis methods resulting in 22 PFGE-types (I–XXII). The discriminatory power of the method was calculated to be 0.95 (Table 2), which is considered ideal (Belkum Van *et al.* 2007).

Eighteen out of 59 isolates (30.5%) were represented by three types (VIII, III and XI) (Figure 2). Nineteen more types were produced represented by one to four isolates. Twenty-two out of 27 serotypeable isolates were allocated

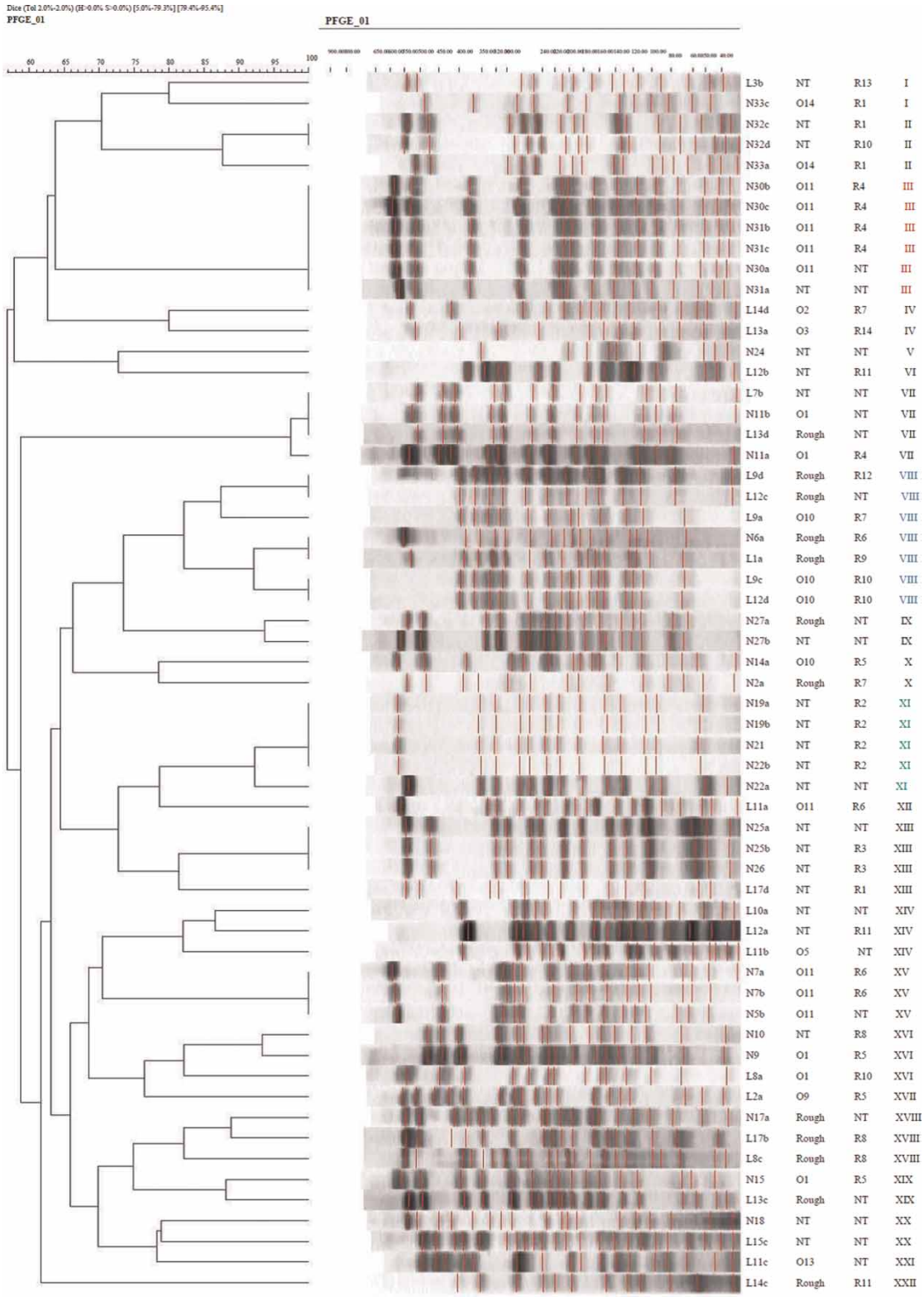

Figure 2 | Digitized PFGE patterns and dendrogram for 59 *P. aeruginosa* isolates. The dendrogram was constructed by cluster analysis by Gel compare 2 software with ≥79% cut-off.

into 14 PFGE-types. Serotypeable isolates were subdivided into various genotypes, as individual genotypes include more than four or five different serotypes.

The isolates of the predominant serotypes (O:11, O:1, O:10) were subdivided into eight PFGE-types (Table 1, Figure 2). The combination of the results of the two methods (serotyping with PFGE) increased the discriminatory power of the combination to 0.97 (Table 2) and allocated the 70 isolates of *P. aeruginosa* into 36 types. Ten out of the 12 rough isolates were allocated into five PFGE-types, while two isolates remained ungrouped. Twenty-four out of the 28 non-serotyped isolates were distributed into 13 PFGE-types, while four remained unclustered. Eight isolates were characterized as non-typeable (Table 1).

The combination of phenotypical and genotypical methods is necessary during an epidemiological study (Belkum Van *et al*. 2007). In this study, serotyping is used as the standard phenotypic typing method. Nevertheless, as our results showed, discriminatory power was weak (0.82); high numbers of non-typeable (44.3%) and rough isolates (17.1%) decreased the typeability of the method. These data show clearly that the use of molecular typing methods is necessary. According to our study, even the use of one primer (208), in every RAPD-assay managed to type non-serotypeable isolates, produced reliable and more easily interpreted results and increased the typeability compared to serotyping (70% of the isolates) with satisfactory discriminatory power (0.927). This study was the first to apply PFGE in a high number of environmental isolates deriving from the Greek area and managed to achieve even higher typeability (84.2% of the isolates), the highest discriminatory power (0.95) producing the most reliable results of the three methods applied (Table 2).

The combination of PFGE with serotyping increased even more the discriminatory power (0.97), producing reliable results, but the method is much more time consuming and thus not very useful when an outbreak is on the way. On the other hand, the use of only one primer made the RAPD method more simple and rapid (even in 1 day) and when combined with serotyping, discriminatory power was increased (0.95); the use of non-specific primer and the restrictions in the PCR conditions (e.g. annealing temperature) are the major disadvantages of the method. Nevertheless, it is very useful for a first fast screening in an outbreak investigation.

The importance of typing *P. aeruginosa* isolates deriving from water and wastewater samples is crucial for public health reasons; *P. aeruginosa* in bottled water can be considered a risk to profoundly immunocompromised patients. According to European regulations (C. D. 98/83/EC), *P. aeruginosa* should be absent in potable water. Moreover the choice of pool and spa waters for medical use is increasing. A number of recent studies emphasize the high prevalence of *Pseudomonas aeruginosa* in hospital water facilities resulting in outbreaks (Blanc *et al*. 2004; Bradbury *et al*. 2009; Lanini *et al*. 2011). Epidemiological investigation to determine the source of an outbreak requires fast and reliable methods. In the present study, 12% of the positive water samples (mains water) and water samples intended for bottling (boreholes and spring water) yielded more than one serotype and genotype. Therefore, the optimization of RAPD and PFGE in typing *P. aeruginosa*, is very important.

CONCLUSIONS

According to the present study, PFGE produced the most reliable results with high discriminatory power and reproducibility and, when combined with the epidemiological data, its reliability increased. Combination of PFGE-typing and serotyping revealed several different clones circulating in Greek water environments. These conclusions, which constitute the first step of this work, will be used for further epidemiological studies on environmental and clinical *P. aeruginosa* isolates in the Greek area.

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