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Value of Phenotyping Methods as an Initial Screening of *Pseudomonas aeruginosa* in Epidemiologic Studies

Summary: When studying the epidemiology of *Pseudomonas aeruginosa*, determination of the similarity of isolates is crucial. In the present study the distinctive capacity of four phenotyping methods (antibiotic susceptibility patterns, serotyping, phage-typing and outer membrane protein [*OMP*] profile analysis) was determined and compared to pulsed-field gel electrophoresis (PFGE) of enzyme restricted chromosomal DNA. In all, 91 isolates of *P. aeruginosa* were cultured from ten patients. Antibiotic susceptibility patterns were concordant for all isolates. Serotyping yielded five, phage-typing eight, OMP profile analysis nine and PFGE seven distinct types of *P. aeruginosa*. Compared to PFGE, the distinctive capacities were 89% (81/91) for serotyping, 87% (79/91) for phage-typing, and 90% (82/91) for OMP profile analysis. When serotyping results were different, PFGE types also were different (exclusiveness 100%). However, isolates with the same serotype may have various PFGE patterns. In contrast, isolates with similar PFGE patterns could have different phage-types or OMP types. For the study of isolates of *P. aeruginosa*, serotyping provides a good initial selection to reduce the number of isolates that need to be genotyped.

Introduction

Ventilator associated pneumonia (VAP) is an important nosocomial infection, frequently caused by *Pseudomonas aeruginosa* [1]. When studying routes of colonization with *P. aeruginosa* that lead to VAP, determination of similarity of isolates is crucial, especially since colonization may occur with different isolates, both at one particular body site and at different body sites [2]. Comparison of phenotypes, such as antibiotic susceptibility patterns, serotypes, phage-types and outer membrane protein (OMP) types, is relatively easy to perform, but lacks specificity [3]. Pulsed-field gel electrophoresis (PFGE) of enzyme restricted chromosomal DNA has a higher specificity and discriminatory power while maintaining epidemiological linkage and has, therefore, become the method of choice to compare bacterial isolates when studying nosocomial outbreaks (4–6). However, PFGE is laborious and expensive, and therefore not feasible for all laboratories. The aim of the present study was to determine the usefulness of four methods of phenotyping as an initial method of screening of *P. aeruginosa* in order to select isolates for further genotyping. This was done by determination of typeability, distinctive capacity, and exclusiveness of antibiotic susceptibility patterns, serotyping, phage-typing and OMP profile analysis as compared to PFGE of enzyme restricted chromosomal DNA.

Materials and Methods

Isolates: Ninety-one isolates of *P. aeruginosa*, derived from ten patients who consecutively developed VAP caused by this species, were analyzed: 16 from oropharyngeal swabs (nine patients), 18 from tracheal aspirates (eight patients), 17 from bronchoalveolar lavage (eight patients), 14 from protected specimen brush (eight patients), eight from gastric aspirates (four patients), and 18 from rectal swabs (four patients).

Typing methods: Antibiotic susceptibility patterns were determined by measurement of MIC according to the NCCLS guidelines. Serotyping of *P. aeruginosa* isolates was performed using antisera to the International Antigenic Typing System (IATS) serotypes (Difco Laboratories, USA). Phage-typing of *P. aeruginosa* was performed using the following phages: 7, 16, 21, 24, 31, 44, 68, 73, F7, F8, F10, 109, 119x, 352, 1214, M4, M6, Col21, Col11, Col18. Serotyping and phage-typing were performed of all isolates in a single run. Outer membrane proteins of *P. aeruginosa* were isolated as described previously [7, 8] with minor modifications. The OMP types were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Genome fingerprinting was carried out according to the method described by *Grothues* et al. [9]. PFGE was performed using a CHEF-DRII System (Bio-Rad Laboratories, Richmond, CA, USA) after restriction digestion with *Spe*I (Boehringer Mannheim, Germany). *Saccharomyces cerevisiae* was used as marker.

Comparison of strains: Antibiotic susceptibility pattern, serotype, phage-type, OMP type and enzyme restricted chromosomal DNA patterns, produced by PFGE, were determined for all isolates of *P. aeruginosa*. OMP profiles and PFGE patterns were

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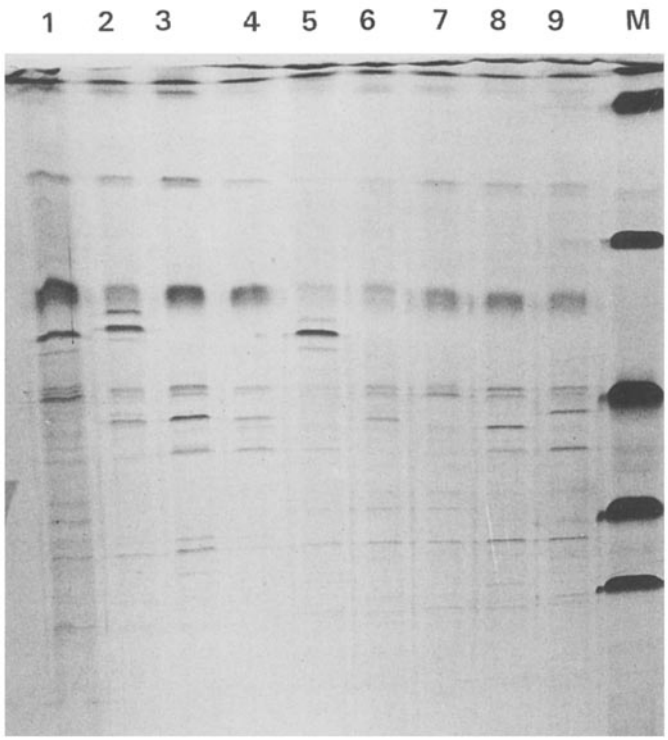


M = marker.
Figure 1: Outer membrane protein profiles of *Pseudomonas aeruginosa* strains of the ten patients developing VAP, revealing nine distinct types labelled 1 to 9.

compared visually on enlarged gel photographs to identify bands of identical relative mobility as fragments of identical size. The criteria for bacterial strain typing according to *Tenover* et al. [10] were used to interpret chromosomal DNA restriction patterns produced by PFGE.

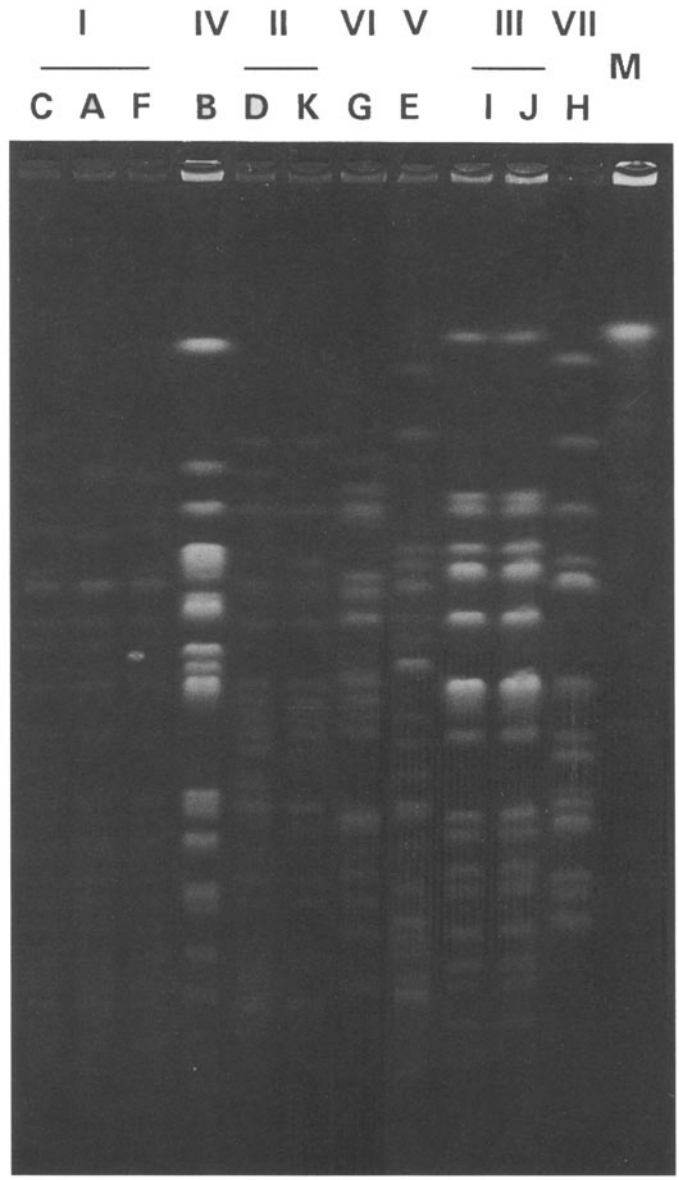
Typeability: Typeability of the various typing techniques was determined by dividing the number of typeable isolates by the total number of isolates, expressed as a percentage.

Distinctive capacity: The results of each phenotypic method were compared to the results of the PFGE of enzyme digested chromosomal DNA, which was regarded as the gold standard. Distinctive capacity was defined as the number of correctly typed isolates by the phenotypic method divided by the total number of isolates as typed by the PFGE technique, expressed as a percentage.

Exclusiveness: For the isolates of each strain type, as defined by each phenotyping technique, it was established whether they were linked to a single or to multiple PFGE types. Exclusiveness of each phenotyping technique was determined by dividing the number of isolates of one particular strain type exclusively linked to a single PFGE type by the total number of isolates, expressed as a percentage.

Results

Antimicrobial susceptibility patterns were concordant for all isolates. The MIC values were: piperacillin ≤ 8 mg/l, ticarcillin/clavulanate ≤ 8 mg/l, ceftazidime ≤ 2 mg/l, imipenem/cilastatin ≤ 1 mg/l, gentamicin ≤ 1 mg/l, ciprofloxacin ≤ 0.5 mg/l. Serotyping yielded five different patterns



M = marker (*Saccharomyces cerevisiae*).
Figure 2: Pulsed-field gel electrophoresis patterns of 11 *Pseudomonas aeruginosa* strains following digestion with *Spe*I. The lanes were labelled as strains A to K (according to phenotyping techniques) and as PFGE type I to VII.

in 87 isolates; four isolates were polyagglutinable. With phage-typing eight different patterns could be distinguished, arbitrarily labelled 1 to 8. Analysis of OMP profiles yielded nine distinct patterns, arbitrarily labelled 1 to 9 (Figure 1). Combination of the four phenotyping methods identified 11 distinct strain types, labelled A to K (Table 1). PFGE of enzyme restricted chromosomal DNA showed seven different patterns, arbitrarily labelled I to VII (Figure 2). Type I was isolated most frequently (n = 47), and was isolated from five out of ten patients. Three types (II, III, and VII) were demonstrated in one patient only. Overall typeability of 100% was achieved by

Table 1: Strains of *Pseudomonas aeruginosa* based on molecular biotyping techniques.

Serotype	Phage-type[1]	OMP type[2]	Types[3]	PFGE type[4]	No. isolates	No. patients
3	6	4	G	VI	9	2
5	1	9	A	I	33	4
5	1	5	F	I	2	1
5	3	9	C	I	12	1
6	5	6	E	V	6	2
6	8	6	I	III	5	1[5]
6	8	2	J	III	2	1[5]
7	2	8	B	IV	6	2
11	4	7	D	II	11	1
11	4	1	K	II	1	1
PA[6]	7	3	H	VII	4	I

[1] arbitrarily labelled 1 to 8; [2] arbitrarily labelled 1 to 9 (Figure 1); [3] distinct bacterial types based on serotype, phage-type and OMP type, arbitrarily labelled Ato K; [4] arbitrarily labelled I to VII (Figure 2); [5] the same patient; [6] PA= polyagglutinable.

Table 2: Exclusiveness of the phenotyping techniques.

	Number of isolates with an exclusive PFGE			Number of isolates with an exclusive PFGE			Number of isolates with an exclusive PFGE	
Serotype	Yes	No	Phage-type	Yes	No	OMP type	Yes	No
3	9	–	1	–	35	1	–	1
5	47	–	2	6	–	2	–	2
6	13	–[1]	3	–	12	3	4	–
7	6	–	4	12	–	4	9	–
11	12	–	5	6	–	5	–	2
PA[2]	4	–	6	9	–	6	6	5
			7	4	–	7	–	11
			8	7	–	8	6	–
						9	–	45
Total	91	–		44	47		25	66
Exclusiveness	91/91 (100%)			44/91 (48%)			25/91 (27%)	

[1] serotype 6 was associated with 2 PFGE types (III, V), however, both were exclusively seen in this serotype; [2] PA = polyagglutinable.

antibiotic susceptibility testing, phage-typing, OMP profile analysis and PFGE of enzyme restricted chromosomal DNA. Because of the four polyagglutinable isolates, typeability was 96% (87/91) for serotyping. Determination of antibiotic susceptibility patterns of the 91 isolates had no distinctive capacity, since all isolates had the same susceptibility pattern. Distinctive capacity of serotyping was 89% (81/91), and 93% (81/87) when the four polyagglutinable isolates were excluded. Distinctive capacity of phage-typing was 87% (79/91), and of OMP profile analysis 90% (82/91). Combination of two or more phenotypic typing methods did not increase the distinctive capacity. Serotyping/phage-typing 87% (79/91), serotyping/OMP profile analysis 89% (81/91), and phage-typing/OMP profile analysis 81% (74/91). The combination of serotyping, phage-typing and OMP profile analysis had a distinctive capacity of 81% (74/91).

Isolates with different serotypes always differed in their PFGE pattern (≥ 7 band differences; exclusiveness 100%; Table 2). Moreover, within a group of isolates with one and the same serotype, PFGE analysis showed ≤ 3 band differences in 85 of 91 isolates (93%). In six of 91 isolates (7%), ≥ 7 band differences were present according to PFGE analysis. In summary, isolates with a different serotype also had a different PFGE type. Isolates with the same serotype did not always have the same PFGE type.

Isolates with different phage-types were associated with different PFGE patterns (≥ 7 band differences) in 79 of 91 isolates (87%). However, in 12 of 91 isolates (13%) two different phage-types were associated with one PFGE type, exclusiveness was 48% (44/91, Table 2). Within a group of isolates with the same phage-type, PFGE analysis showed ≤ 3 band differences in 100% of isolates. In summary, isolates with a different phage-type did not always have a different PFGE type. Isolates with the same phage-type always had the same PFGE type.

Different OMP types were associated with different PFGE patterns in 86 of 91 isolates (95%). However, in five

of 91 isolates (5%) two different OMP types were associated with one PFGE type, exclusiveness was 27% (25/91, Table 2). Within a group of isolates with the same OMP types, PFGE analysis revealed ≤ 3 band differences in 86 of 91 isolates (95%). In summary, isolates with a different OMP type did not always have a different PFGE type, and isolates with the same OMP type did not always have the same PFGE type.

Discussion

The results of this study show that phenotyping may well be used as an initial method of screening for epidemiological studies of *P. aeruginosa* in an endemic setting of intensive care patients. Based on the data presented in this study, we therefore recommend serotyping for this purpose. In case of identical serotypes of strains from different patients, identity should be confirmed by a method of genotyping.

The purpose of this study was not to determine reproducibility and discriminatory power of typing techniques, which has already been studied extensively [11–13]. Furthermore, because a number of the studied isolates are closely related, the set of isolates analyzed in the present study was unsuitable for determination of discriminatory power [14]. For epidemiological studies, genotyping with PFGE of enzyme restricted chromosomal DNA of bacterial isolates currently is the typing method of choice. However, this method is expensive and time consuming. In general, phenotyping techniques are less expensive and easier to perform. The starting point of our investigation was to determine sensitivity and specificity of four phenotyping techniques. The best phenotyping technique may be used to make a preselection out of a large series of isolates, thereby reducing the number of isolates that need to be genotyped in epidemiological studies. The results of four phenotyping techniques were compared to PFGE of enzyme restricted chromosomal DNA, which we considered to be the gold standard. Although the methods of analysis used (e.g. determination of distinctive capacity and exclusiveness) are not common in microbiological studies, we feel that they are appropriate for these purposes.

Various typing methods have been used to study the epidemiology of *P. aeruginosa*. Serotyping has been used most frequently. However, reported drawbacks of this method are the high frequency of polyagglutinable strains [15], the fact that most clinical isolates belong to a limited number of serotypes [16] and that, especially in cystic fibrosis patients, a considerable proportion of strains are non-typable [9]. The results of the present study are partly in line with this: among the 91 isolates five different serotypes could be distinguished, only four isolates were polyagglutinable. Although phage-typing has been reported to allow fairly detailed strain typing in 85% of clinical isolates, its reproducibility is poor [3, 12]. Phage-typing of a series of isolates should, therefore, always be performed in one session. Moreover, phage-typing usually can only be performed in reference laboratories where the various phages are in stock. Analysis of outer membrane proteins, although laborious and time consuming, has been used in epidemiological studies of *P. aeruginosa*, although several OMP types of *P. aeruginosa* strains showed high degrees of similarity [17]. In contrast, *Maloney* et al. demonstrated considerable variation in OMP types between strains isolated from different patients [18]. Restriction enzyme analysis with PFGE has been reported to provide the highest discriminatory power between *P. aeruginosa* isolates [12] and has, therefore, been advocated as the method of choice to investigate outbreaks of infections due to this species [4]. To reduce the number of isolates for PFGE when studying the epidemiology of *P. aeruginosa* in an endemic setting, a phenotyping technique as an initial method of screening can be advocated.

Antibiotic susceptibility testing was not discriminatory in our setting because none of the *P. aeruginosa* strains were resistant to the antibiotics tested. In the presence of resistant strains the distinctive capacity of antibiotic susceptibility testing may increase. Serotyping, phage-typing and OMP profile analysis all had a good distinctive capacity, ranging from 87% to 90%. Any combination of these methods did not improve distinctive capacity. Isolates with different serotypes were always associated with different PFGE patterns (exclusiveness = 100%). This group-specific relationship between DNA fingerprint and serotype has been described (12, 19). Moreover, within a group of isolates with the same serotype, 93% of isolates were closely related or indistinguishable. In contrast, within a group of isolates with the same PFGE patterns, different phage-types and OMP types were seen. For phage-typing this phenomenon has been reported before by *Grothues* et al. [9]. In a study by *Boukadida* et al. [20] both phenotyping and genotyping techniques were used in the analysis of an outbreak of gut colonization by *P. aeruginosa* in six immunocompromised children. They also found different phage-types within a group of isolates with similar PFGE types [20]. However, serotyping results from that study were difficult to interpret, since eight of 13 isolates were polyagglutinable [20]. Of the four phenotypic techniques tested in the present study, serotyping showed the best discriminative properties. The phenotypic technique of choice in our laboratory, therefore, is serotyping, which is also a simple, cheap and fast method, with good reproducibility [11, 13]. For example, when two patients are colonized or infected with *P. aeruginosa* with different serotypes, cross-colonization is highly unlikely. When both patients have strains with similar serotypes, PFGE is needed to confirm similarity.

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