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Phenotypic and Genotypic Characteristics of *Pseudomonas aeruginosa* Strains Isolated from Hospitals in the North-West Region of Poland

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Abstract

A total of 90 *Pseudomonas aeruginosa* strains isolated from 4 hospitals in the west-north region of Poland were studied by arbitrarily primed polymerase chain reaction (AP-PCR). AP-PCR results revealed the presence of 11 main groups of patterns (A-K) and 5 unique patterns among isolates. Generally, they were characterized by high resistance to antibiotics tested and significant differences in serogroups and types of growth on Cetrimide Agar medium. It was observed that clonally related strains were isolated from patients within the same ward, among different wards as well as in distant hospitals.

Key words: *Pseudomonas aeruginosa*, arbitrarily primed PCR (AP-PCR), fingerprinting, antibiotic resistance, nosocomial infections

Introduction

Pseudomonas aeruginosa is a Gram-negative, non-fermenting rod widely distributed in nature and human environment. It is responsible for one of the most serious opportunistic infections in humans. In recent years nosocomial infections caused by *P. aeruginosa* has been recognized as an acute problem in hospitals due to its antibiotic multi-resistance. *P. aeruginosa* is one of the main causes of nosocomial respiratory tract, urinary tract and surgical site of infection. It is the primary cause of ventilated-associated pneumonia in intensive tract unit (ICU), where individuals are highly susceptible to infection than patients from the other wards of the same hospital. Severity of illness, underlying disease, immunosupression and invasive devices especially mechanical ventilation are risk factors for *P. aeruginosa* infection (Ayats *et al.*, 1997; Bertrand *et al.*, 2000; Boddie *et al.*, 2003; Cheol-In Kang *et al.*, 2003; Garcia-Garmendia *et al.*, 1999; Garrouste-Orgeas *et al.*, 1996). Patients hospitalized in ICUs have three-fold greater risk of contracting nosocomial infection and mortality may increase from 13% to 47% (Nikodemski, 1997).

According to the studies conducted by the European Prevalence of Infection in Intensive Care (EPIC) which covered 10 038 patients in 17 western European countries, *P. aeruginosa* was the third most frequently isolated microorganism (28.7% of the total number of isolates) (Spencer, 1993). As reported by Peacock and Garrad (1997) 16–31% of pneumonia cases diagnosed among artificially ventilated patients were connected with *P. aeruginosa* infections (Peacock *et al.*, 1997). Pneumonia occurrences caused by *P. aeruginosa* result in 30–80% of death cases among ICU patients in Polish hospitals (Piotrowska, 1998).

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Quite recently epidemiological investigations could rely only on classical methods based on analyses of phenotypic features, including biotype, serotype and phagotype identification as well as bacteriocin and antibiotic sensitivity of tested strains. To identify a strain precisely several method had to be applied. Phenotypic tests are expensive, time and labor consuming and very often their results are ambiguous to interpret, *e.g.* in case of endemic strains (Gospodarek and Waszak, 1995; Gillespie *et al.*, 2000; Hancock *et al.*, 1983; van Belkum, 1994; Versalovic *et al.*, 1993).

Presently, genetic techniques supported by phenotypic tests enable to conduct a detailed characteristic of strains isolated from particular time and environment. The aim of such analyses is to precisely evaluate if strains isolated from infected patients are clonally related and transmitted horizontally within the ward or infections are caused by representatives of various clonal groups. It provides data about sources of infection and route of microorganism transmission and is of crucial importance for epidemiological investigations, especially those focused on tracing nosocomial infections.

Reliability of performed analyses relies both on a typing method selected as well as on interpretation of results. Rightly performed analysis should cover three basic criteria of typing bacterial strains, *i.e.* typeability, reproducibility and discrimination (Power, 1996; Versalovic *et al.*, 1993). To fulfill the above criteria, a preliminary optimization of applied typing method is usually essential. Lack of standardization leads to non-reproducibility of the results and their wrong interpretation and may also limit wider application of genetic methods in routine clinical diagnostics. Macrorestriction analysis of genomic DNA followed by pulsed field gel electrophoresis (PFGE) has become the gold standard for molecular typing, however, interpretation of PFGE results for *P. aeruginosa* is very complicated. Efficiency of the method may be limited by species features, *e.g.* interpretation of pulsed-field gel electrophoresis (PFGE) typing patterns is complicated (Fiett *et al.*, 1998; Kersulyte *et al.*, 1995). Promising results were obtained by application of arbitrarily chosen primers in random amplified polymorphic DNA (RAPD), DNA amplification fingerprinting (DAF) and arbitrary primed PCR assay (AP-PCR).

The aim of the work was to estimate intra-species differentiation of *P. aeruginosa* strains isolated from 4 hospitals in the west-north region of Poland and to evaluate effectiveness of phenotypic methods such as biotyping, serotyping, susceptibility to chemotherapeutic agents and type of growth on Cetrimide Agar medium in the epidemiological investigation.

Experimental

Materials and Methods

Bacterial strains. A total of 90 *P. aeruginosa* strains isolated from various clinical specimens in hospitals nos. 1–4 in the west-north region of Poland during 1996–2000 were examined. Strains were isolated from 56 patients admitted to different wards of these hospitals. Some patients had *P. aeruginosa* isolated from one, two sites or from the same specimens collected at different days of hospitalization.

Phenotypic study. *P. aeruginosa* isolates were identified by the biochemical profile index procedure ID 32 GN (bioMérieux, France). Pyocin production was tested on selective Cetrimide Agar (Merck, Germany). O-type lipopolysaccharide was determined according to Habs (1957) protocol based on agglutination test with anti-O sera (Sanofi Pasteur, France). Susceptibility to antibacterial drugs was studied by the disk diffusion method according to CLSI (Clinical and Laboratory Standards Institute, formerly NCCLS-National Committee for Clinical Laboratory Standards) for following agents: carbenicillin (Cb), piperacillin-tazobactam (Tzp), ceftazidime (Caz), imipenem (Imp), meropenem (Mem), gentamicin (Gn), tobramycin (Tb), netilmicin (Net), amikacin (Ak), pefloxacin (Pef), ciprofloxacin (Cip), colistin (Ks) (Performance standards for antimicrobial susceptibility testing. 1999).

Genetic analysis. DNA was extracted using DNA®ZOL reagent (Gibco, USA) according to the manufacturer's instructions. DNA concentration was quantified spectrophotometrically at 260 nm/280 nm in GeneQuant RNA/DNA Calculator (Pharmacia Biotech, UK) and diluted if necessary to obtain concentration of 20 ng/μL. To obtain complex and stable amplicon profiles, the orthogonal array assay designed by Taguchi and Wu 1980 and modified by Cobb and Clarkson (1992) was applied.

The PCR was performed in a volume of 25 μL containing 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1 U of *Taq* DNA polymerase (Roche, USA), 3.0 mM $MgCl_2$, 2.0 mM of each nucleotide, 30 pM of each primer: CagA2 (5' ATT TAG AAG CAG GCT TTA GC 3') and CMVin2 (5' GGT AGC ACC GCG GGT TTC GAC 3') and 20 ng/μL of template DNA in termocycler Gene Amp PCR System 9600 (Perkin Elmer, USA). The thermal profile consisted of an initial denaturation step at 94°C for 2 min followed by 36 cycles of a 94°C denaturation for 30 sec, a 30°C annealing for 5 min, a 72°C elongation for 1 min, proceeded with 37 cycles of a 94°C denaturation for 30 s, a 30°C annealing for 1 min and a 72°C elongation for 2 min. At the end of amplification mixture was subjected to the final extension at 72°C for 7 min. Amplified products including a negative control and a molecular weight marker (Mass Ruller™ DNA Ladder, MBI Fermentas, Lithuania) were analyzed by electrophoresis in 6% polyacrylamide gel stained with 0.5 mg/L ethidium bromide and visualized, photographed and analyzed by GelDoc 2000 (BioRad Laboratories, USA). Dice coefficient was calculated and compared to evaluate similarity among strains by BIOGENE software (Vilber Lourmat, France).

Results

The isolation sites of 90 strains *P. aeruginosa* for the 56 patients are shown in Table I. Most of strains (63–70%) were isolated from lower respiratory tract of patients admitted to ICU, 16 (17.8%) from postoperative wounds and single from catheters (4), throat (4), bile (2) and anus (1). *P. aeruginosa* was isolated from one site or from sequential samples of various respiratory tract exudates in 52 patients, and from two different sites in 4 patients (in all from respiratory tract and wound-2, or catheter-1, or anus-1).

Table I
Isolation site of 90 *Pseudomonas aeruginosa* from 56 patients hospitalized in 1996–2000

Hospital/ward	1996 number of strains patients	1997 number of strains patients	1998 number of strains patients	1999 number of strains patients	2000 number of strains patients
Nº1 ICU	1a 1 1b 1	1c 1	5b 1 3(2b,1c) 1 3(2a,1b) 1 3(2b,1d) 1 3b 1 4b 2 11b 11	4b 4	16(15b,1f) 1 3(2b,1d) 1 2b,g 1 3b 3 3d 2 1g 1
Sur1				1d 1	2d 2 2g 2
Sur2			1e 1	3d 3	2d 2
DSur				2d 2	1d 1
N°2 ICU		2b 2	1b 1 1c 1		
N°3 ICU			2(1a,1b) 1 1b 1		
N°4 ICU			1b 1		
Sur					1e 1
Total	2 2	3 3	39 24	10 10	36 17

Isolation site: a – throat, b – bronchi, c – BAL, d – wound, e – bile, f – anus, g – catheter

Genetic types were determined based on cluster analysis comparing values of Dice coefficient for AP-PCR patterns. Two main groups (I–II) at the level of 30–40% of Dice coefficient were observed. AP-PCR typing revealed 11 groups of genotypes (A-K) containing from 2 to 20 isolates of a high similarity according to Dice coefficient values (70–100%) and 5 unique isolates (L, M, N, O, P) (Fig. 1 and 2). Among them, genotypes persistently present in a particular ward for some years were detected, *e.g.* AP-PCR types A and D isolated from a ICU in the hospital no 1 from 1996 to1998, as well as strains typical of different wards of the same hospital, *e.g.* AP-PCR type C (ICU, Sur1 and DSur) and type F (ICU, Sur1, Sur2, DSur). It was

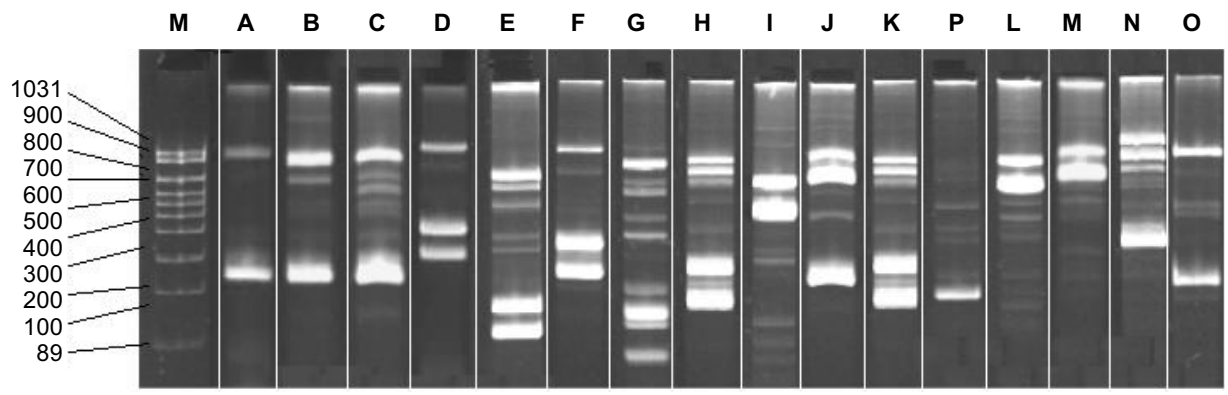

Fig. 1. AP-PCR fingerprintints as representative of 11 main groups (A – K) and 5 unique profiles (P – O) obtained from 90 isolates *Pseudomonas aeruginosa*. The lane M is a molecular weight marker (Mass Ruller ^{TM} DNA ladder, Fermantas)

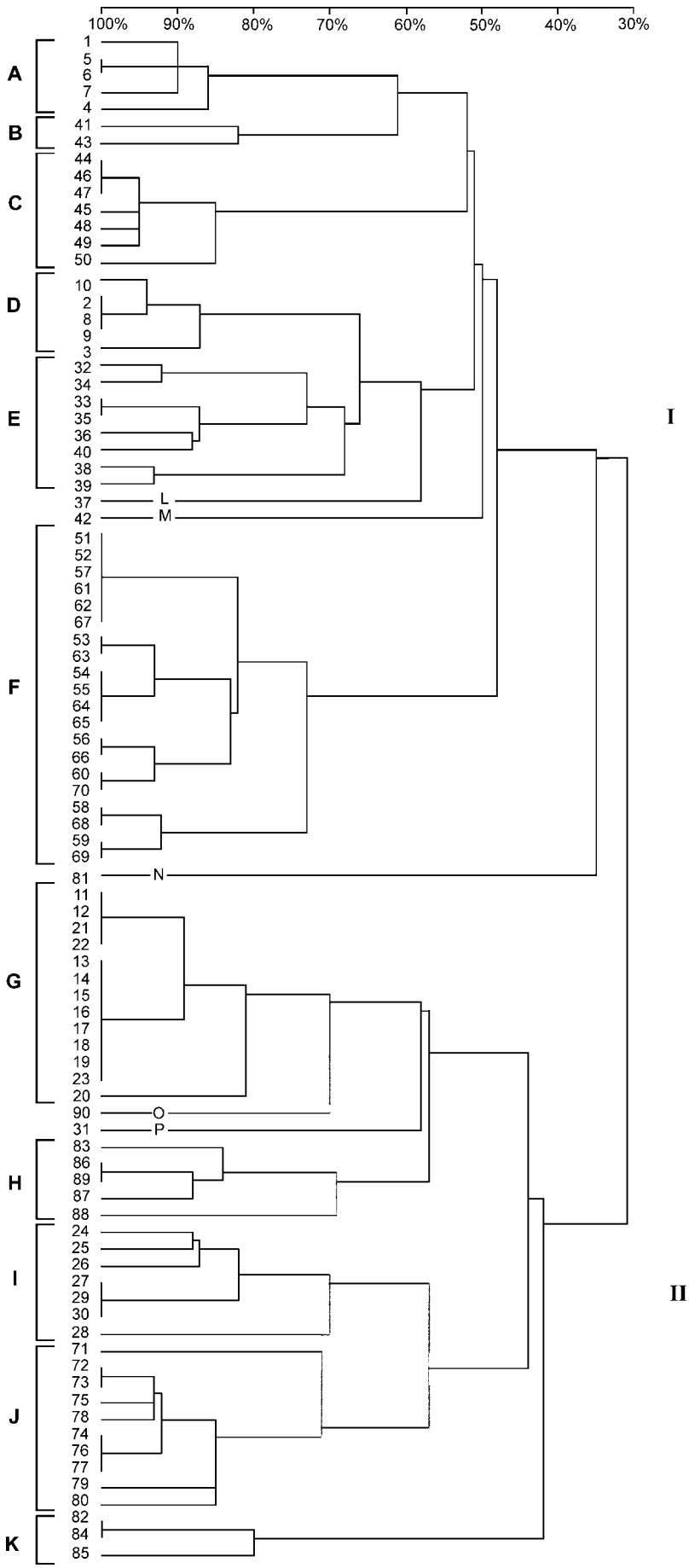

Fig. 2. Dendrogram demonstrating the genetic relationship among 90 isolates *Pseudomonas aeruginosa*. The scale corresponds to the percentage of similarity. A – K AP-PCR types, P – O-uniqe types.

Table II
AP-PCR types of *Pseudomonas aeruginosa* isolated in the north-west region of Poland

Hospital/ward		AP-PCR type (number of strains)				
		1996	1997	1998	1999	2000
N°1	ICU	A (1), D (1)	D (1)	A (4), D (3), E (5), G (12), I (7), L (1)	B (2), E (1), M (1)	C (4), F (15), J (8), O (1)
	Sur1				C (1)	F (3), J (1)
	Sur2			G (1)	E (2), N (1)	F (1), J (1)
	DSur				C (2)	F (1)
N°2	ICU		H (1),K (1)	K (2)		
N°3	ICU			H (3)		
N°4	ICU			H (1)		
	Sur					P(1)

ICU – Intensive Care Unit, Sur – General Surgery, Sur1 – General and Vascular Surgery, Sur2 – General and Transplantation Surgery, DSur – Maxillofacial and Dental Surgery

observed that some AP-PCR types occurred simultaneously for a year or longer in different hospitals, *e.g.* AP-PCR types H and K detected in 1997 (hospital no 2) and 1998 (hospitals nos 2–4). Details are presented in Table II. Most often the same clones were isolated from two to ten patients, but the different clones in sequential samples from respiratory tract or from two sites of the same patient were found also.

Strains classified to the same genotypic type were not phenotypically similar, *i.e.* they did not display the same susceptibility to antimicrobial agents and the same type of growth on selective medium as well as they did not belong to the same serotype. Within the same type up to several resistance profiles, serotypes and types of growth on selective medium were presented. Detailed data on comparison of phenotypic and genotypic strain features are presented in Table III.

Irrespectively of the hospital and/or ward, most of *P. aeruginosa* isolates showed very differentiated resistance to antimicrobial agents tested. Different resistance patters in various arrangements were observed, from sensitivity to all tested antibiotics, through single resistance to carbenicillin or/and pefloxacin to multidrug resistance for almost all tested drugs. All strains were susceptible to colistin. Strains isolated in 1996 and 1997 and 4 from 5 unique types (L, M, N, P) were generally less resistant to chemotherapeutic agents than isolated since the end of 1998. Nevertheless, no correlation was found between susceptibility to antibiotics and serotype or the type of growth on selective medium.

The total of 90 *P. aeruginosa* strains were tested on selective cetrimide agar. A green-yellow type of growth appeared most frequently (59 strains – 65.5%) whereas a yellow type was the most rarely found (1.1%). Details are presented in Table III.

From all tested strains 81 isolates (89.9% of) reacted with applied sera. 11 serotypes were distinguished. Most strains (70–77.7%) typed by monovalent sera. Two serotypes: FO:11 (46–51.1%) and EO:16 (17–18.9%) were observed most frequently. Individual strains reacted with following sera EO:15 (2), AO:1 (1), AO:4 (2), AO:6 (1), CO:10 (1). Some strains were typed only by polyvalent sera: PMA (6), PME (3) and PMF (2) whereas 9 (10.0%) of strains was non-typed at all. In general, 21 of strains (23.3%) gave ambiguous typing results. A great variety of serotypes were observed among isolates from hospitals no 1 and 2. Only all strains isolated from the hospital no 3 belonged exclusively to EO:16 serotype whereas isolates from the hospital no. 4 were identified as FO:11.

Discussion

The hospital environment remarkably promotes selection and quick distribution of resistant strains (Dzierżanowska, 1997; Giedrys-Kalemba, 2000; Hanberger *et al.*, 2004; Jaworski *et al.*, 1993; Łopaciuk, 1996; Sader *et al.*, 2004). One of the essential steps leading to a reduction of nosocomial infections is a constant monitoring of etiological agents and resistance of intrahospital strains. It is of crucial importance to carry out epidemiological surveys including a detailed characteristic and relationship among strains isolated in particular environment and time, as well as to become aware of risk factors, sources and ways of infection distribution (Czekajło-Kołodziej, 2001; Łopaciuk, 1996). To obtain reliable results,

Table III
AP-PCR types *Pseudomonas aeruginosa* isolated in the north – west region of Poland and their phenotypic differentiation

AP-PCR type	Resistance pattern	Date of isolation	Serotype											Type of growth on Cetrimide Agar
			FO:11	EO:16	EO:15	AO:1	AO:4	AO:6	CO:10	PMA	PME	PMF	NT	
A	CbTzpPef	1996	1											green-yellow (4)
	CbGnTbPef	1997		1			1			1	1			green (1)
B	CbTzpGnTbAkPefCip	1999	2											green-yellow (2)
C	Cb	1999								2				celadan (3)
	CbGnTbNetAkPefCip	1999		1										green-yellow (2)
	TzpGnTbNetPefCip	2000	1											green (1)
	CbTzpIpmMemGnTbNetAkPefCip	2000	1											blue-green (1)
	CbTzpGnTbNetAkPefCip	2000	2											
D	CbTzpCazGnTbNetPef	1996											1	green-yellow (5)
	MemGnTbNetAkPefCip	1997											1	
	CbMemGnTbPef	1998		3										
E	CbTzpMemGnTbAk	1998	1											green-yellow (5)
	CbTzpIpmGnTb	1998	1											blue-green (2)
	CbTzpCazIpmMemGnTbAk	1998	1											green (1)
	CbTzpGnTbPefCip	1998	1											
	CbTzpImpGeTbPrfCip	1998	1											
	CbTzpGnTbAkPefCip	1999	2											
	sensitive to all	1999								1				
F	CbTzpIpmGnTbNetAkPefCip	1999	1											green-yellow (9)
	CbPef	1999	1											green (9)
	Cb	1999	1			1				1				yellow (1)
	CbMemPef	1999						1						celadan (1)
	CbTzpMemGnTbNetAkPef	1999	2											
	CbTzpCazIpmMemGnTbNetAkPefCip	1999	1											
	Caz	1999	1											
	CazTzpTbNetAkPefCip	1999	1											
	CbIpmMemGn	1999			1									
	CbTzbCazMemGnTbAkNetPef	1999	1											
	CbCazMemGnTbAkPefCip	1999									1			
	CbTzpMemGnTbNetAkPefCip	1999	1										1	
	CbTzpIpmMemGeTbNetAkPef	1999	2											
	CbImp	1999			1									
	CbTzpImpMemGrTbNetAkPefCip	1999										1		

Table III continued

AP-PCR type	Resistance pattern	Date of isolation	Serotype											Type of growth on Cetrimide Agar
			FO:11	EO:16	EO:15	AO:1	AO:4	AO:6	CO:10	PMA	PME	PMF	NT	
G	CbIpmTbPefCip	1998		2										green-yellow (12) blue-green (1)
	CbTzpIpmMemGnTbAkPefCip	1998		2										
	CbMemGnTbPef	1998		2										
	CbGnTbPefCip	1998											1	
	CbGnAkPefCip	1998	1											
	NetPefCip	1998							1					
	GnNetPefCip	1998											1	
	CbGnTbAkPefCip	1998		1										
	CbIpmMemGnTbAkPefCip	1998		1										
	Pef(I)	1998					1							
H	sensitive to all	1997								1				green-yellow (5)
	CbTzpGnTbNetPefCip	1998		2										
	CbTzpGnTbNetAkPefCip	1998		1										
	CbTzpPefCip	1998	1											
I	CbPef	1998		1										green-yellow (6) blue-yellow (1)
	CbGnTbPefCip	1998	1											
	CbCazGnTbNetAkPefCip	1998	3									1	1	
J	CbTzpIpmMemGnTbNetAkPef	2000	1											green (5) green-yellow (3) celadan (2)
	CbTzpTbNetPef	2000	1											
	CbTbNetPef	2000	1											
	CbTzpMemGnTbNetPefCip	2000	1											
	CbTzpMemGnTbNetPef	2000	1										1	
	CbTzpCazImpMemGnTbNetAkPefCip	2000	2											
	CbTzbGeTbNetPef	2000	1											
	CbTzpCazGeTbNetAkPefCip	2000											1	
K	CbPef	1997										1		green-yellow (3)
	CbTzpGnTbNetPef	1998	1											
	GnTbPef	1998									1			
L	Pef(I)	1998	1											green
M	Pef	1999	1											green-yellow
N	Pef(I)	1999	1											green-yellow
O	CbTzpCazImpMemGeTbNetAkPef	2000	1											green-yellow
P	CbPefCip	2000	1											green

Cb – carbenicillin, Tzp – piperacillin-tazobactam, Caz – ceftazidime, Imp – imipenem, Mem – meropenem, Ge – gentamicin, Tb – tobramycin, Net – netilmicin, Ak – amikacin, Pef – pefloxacin, I – intermediate

especially in case of isolates without characteristic phenotypic markers, application of molecular methods seems to be inevitable.

To differentiate precisely among particular *P. aeruginosa* strains isolated from 4 hospitals in the west-north region of Poland, AP-PCR typing was carried out. However, due to the large number of strains and their different origin, the classification of strains/genetic patterns was conducted at the level of 70% and more of Dice coefficient (Struelens *et al.*, 1993). Dendrogram analysis enabled to divide strains into main groups (40–50% of similarity), then subgroups (55–65%), genotypes (70–75%) and subtypes (76–100%). AP-PCR typing revealed presence of 16 AP-PCR types of *P. aeruginosa*.

A high number of AP-PCR types pointed to marked intrahospital differentiation of *P. aeruginosa* strains that are widely distributed in nature, especially in humid environments. It indicated various sources of strains and their constant exchange, also with the same patients. Such strains were generally highly resistant to antibiotics what confirmed the development of secondary resistance and their intrahospital selection. At the same time strains of unique fingerprints, frequently expressing higher susceptibility to chemotherapeutic agents, were isolated. It gave evidence of the temporary incidence of new endogenous strains entering the hospital environment.

Some of the genetic types expressing the same/similar of AP-PCR pattern were numerically dominant within the ward(s) for some months/years. It might prove horizontal transmission of clones or clonally-related groups and epidemic/endemic character of registered infections (Fiett *et al.*, 1998). The incidence of the same genetic types of *P. aeruginosa* in different hospitals drew attention to a possibility of a long-distance strain transmission. It might be linked to the movement of patients, visitors, medical and paramedical staff.

Based on dates of strains isolation and their resistance to antibiotics, it is highly probable that selection of highly resistant isolates takes place in ICUs, where *P. aeruginosa* is one of the most frequent and severe cause of infections, especially in patients with mechanical ventilator. In the absence of epidemic clones, secondary resistance development during combined antibacterial therapy appeared to be the main factor contributing to the prevalence of resistance in ICU, what was observed in sequential samples from the same patient. On the other side, the significance of patients relocated from different wards to ICUs and colonized with ward-specific microflora should not be underestimated.

Phenotypic methods are based on a presence or absence of expressed and strain characteristic features. Instability of such features in various environmental conditions is the main disadvantage of phenotypic assays that frequently precludes a precise strain characteristic. Types of *P. aeruginosa* growth on Cetrimide Agar medium differed significantly within strains belonging to one genotype as well as among strains of different genotypes. The green-yellow type was observed most frequently (65.6%) whereas the yellow type was the rarest (1.1%). Also serotyping based on the somatic antigen evaluation according to Habs protocol did not reveal correlation between genotypes and serotypes. Both FO:11 (51.1%) and EO:16 (18.9%) were the most widespread serotypes. The FO:11 serotype was isolated from almost all wards in hospitals covered by our studies. Intriguingly, a FO:12 serotype (the dominant type among multi-resistant *P. aeruginosa* strains in the hospitals all over the world) was not detected (Bingen, *et al.*, 1996; Cavallo *et al.*, 2000). Serotyping is not considered as the method of high discriminatory power. The occurrence of antigenic variations within strains causes that some strains do not respond to commercial sera (10.0% of strains analyzed in our studies). A susceptibility to antibiotics is not also the most practical tool for unambiguous epidemiological evaluation of strains. However, it may serve as the preliminary criterion indicating the incidence of a potential intrahospital strain and signaling the necessity of conducting further investigations.

Results of AP-PCR typing did not reveal consistence between strain fingerprints and their phenotypic features. A majority of *P. aeruginosa* strains presented a high differentiation of phenotypic patterns within a genotype. It confirmed lack of correlation between molecular and conventional typing, *e.g.*: types of growth on Cetrimide Agar, serotype or susceptibility/resistance pattern to antimicrobial agents. Similar results also with the other genera of microorganisms were proved (Bouza *et al.*, 1999; Fierobe *et al.*, 2001; Dinesh *et al.* 2003; Giedrys-Kalemba *et al.*, 2001). The lack of correlation between *P. aeruginosa* AP-PCR types and their phenotypic features indicates that phenotypic analysis should not be exclusive method of evaluating of strain relationship and conducting epidemiological investigations of nosocomial infections. It is necessary to carry out analysis at the molecular level (Cavallo *et al.*, 2000; Gomez *et al.*, 2000). However, phenotypic studies are a valuable tool supplementing genotyping as they enable tracing of phenotypic feature expression influenced by different environmental conditions (Biendo *et al.*, 1999).

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