Acta clin Croat 2001; 40:85-91 Original Scientific Paper

DIFFERENCES IN THE ANTIGENIC STRUCTURE AND PRODUCTION OF HEMOLYSIN OF *PSEUDOMONAS AERUGINOSA* STRAINS ISOLATED FROM A VARIETY OF ISOLATION SITES

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SUMMARY – *Pseudomonas (P.) aeruginosa* produces a number of exoenzymes, which have been implicated as virulence factors in infections caused by this organism. One of these exoenzymes is phospholipase C or heat labile hemolysin (HE) or *P. aeruginosa.* The aim of the study was to establish differences in HE production of 100 *P. aeruginosa* strains in dependence of their antigenic structure and type of specimens from which they were isolated. Isolates were serotyped by agglutination with commercial antisera according to the International Antigenic Typing System. A microtiter HE assay was used to quantitate the hemolytic activity of the strains investigated. The most commonly detected O-serogroup was O11, found in 34% of strains, followed by O6 (16%), O3 (9%), O4 (8%), O2 (7%) and O1 (6%) serogroups. Serogrups O7, O10, O13, O14, O15 and O17 were not detected. Serogroup O11 was found in 11 out of 16 strains isolated from bronchial secretion, and in 2 of 15 strains isolated from urine ($p<0.01$). HE production was detected in 88% of all isolates. The O1, O2, O3 and O16 strains mostly produced a large quantity of HE, while the epidemic O11 strains produced a small amount of HE. Urine and pharyngeal isolates produced the highest levels of HE.

Key words: *Pseudomonas aeruginosa, pathogenicity; Pseudomonas aeruginosa, classification; Hemolysins*

Introduction

Pseudomonas (P.) aeruginosa has emerged as an important nosocomial pathogen during the past three decades. It causes between 10% and 20% of infections in most hospitals[1]. Moreover, the incidence and relative frequency of hospital-acquired *P. aeruginosa* infection have increased during the last decades. This is partly due to the increased number of patients particularly prone to such infections: immunocompromised patients, and patients with malignancy, cystic fibrosis, burns or traumatic wounds[2,3]. Infection with *P. aeruginosa* is associated with severe morbidity. Despite antibiotics, the lethality directly or indirectly related to this bacterium is one of the highest among the nosocomial pathogens[4].

The virulence of *P. aeruginosa* is multifactorial and is the product of many interacting variables, involving both the bacterium and the host. Damage to the host tissue may occur directly through the action of bacterial products or indirectly through the induction of an exaggerated host response to these products. In addition to cell-associated factors, *P. aeruginosa* liberates a number of exoenzymes, including exotoxin A, proteolytic enzymes, phospholipase C, and exoenzyme S, each of which has been implicated as a pathogenic determinant in infections caused by this organism[1,3]. Exotoxin A has been shown to be a pathogenic determinant in experimental infections of the eye and burn wounds, and in chronic lung infections caused by *P. aeruginosa*[5,6]. The activity of proteases, such as alka-

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Received February 13, 2001, accepted April 20, 2001

line protease and elastase, has been well established in most *P. aeruginosa* strains[1,7]. Recent studies have shown that elastase contributes to the pathogenesis in acute and chronic lung infections[8,9,] and together with exotoxin A may contribute to the development of fatal septicemia[10]. Exoenzyme S, an adenosine-disphosphate-ribosyl (ADPR) transferase enzyme, like exotoxin A, is an important virulence factor in burn infections caused by *P. aeruginosa*[11].

The role of phospholipase C in human infections has not yet been fully clarified. Phospholipase C is a lecithinase, which liberates phosphorylcholine from lecithin, and is also known as a heat labile hemolysin[12.] Together with a heat stabile glycolipid, it acts synergistically to break down lipids and lecithin. Like *Pseudomonas* proteases, hemolysins can contribute to tissue invasion through their necrotizing effects. It has also been suggested that phospholipase C contributes to the pathogenesis of *Pseudomonas* pneumonia through the degradation of lung surfactants[12].

The purpose of the present study was to determine the *in vitro* production of hemolysin among phenotypically different *P. aeruginosa* strains isolated from a variety of clinical isolation sites.

Material and Methods

Bacterial strains: A total of 100 *P. aeruginosa* isolates from different specimens (urine, bronchial secretion obtained by fiberoptic bronchoscopy, endotracheal aspirate, sputum, ear and throat swabs, pus and wound aspirate) were collected from the Department of Microbiology, Institute of Public Health at Osijek, Croatia, from February 1 to June 17, 1998. All patients had symptoms and signs of underlying infection of the system involved, and cultures were taken during standard diagnostic procedures.

Bacteria were characterized as *P. aeruginosa* according to their biochemical characteristics established by means of API 20 NE System (API, Analytab Products, Plainview, NY, USA). Multiple isolates from the same patient were excluded from the study. Strains were stored in deep-agar tubes at +4 °C (1.5% nutrient agar, Difco Lab., Detroit, MI, USA), and subcultured by passaging on trypticase soy agar (TSA, Difco Lab., USA) before use.

Serotyping: O-serogroups of the *P. aeruginosa* strains were determined by slide agglutination using commercially available antisera (Diagnostic Antisera, Institute of Immunology, Zagreb, Croatia) according to the International Antigenic Typing System.

Measurement of hemolytic activity: A modified microtiter hemolysin assay described previously by O'Hanley et al. was used to quantitate the hemolytic activity of bacteria[13]. In short, a single bacterial colony on trypticase soy agar for each strain was inoculated into tubes containing Luria broth (Difco Lab., Detroit, USA). After incubation for 5 h at 37 °C using a shaker (Lucham Ltd.), one milliliter of this culture served as inoculum for 60 ml of medium contained in cotton-stoppered Erlenmeyer flask incubated at 37 °C on a shaker for 24 h. Bacteria were removed from culture supernatants by centrifugation (1 200 xg). The supernatant was filtered through 0.4-μm pore-size membranes (Millex-HA Filter, Millipore, Molsheim, France) and serially diluted twofold in phosphate-buffered saline (0.067 M potassium phosphate buffer, pH 6.8, with 0.077 M NaCl). Hemolytic activity was tested by combining equal volumes (50 μl each) of an erythrocyte suspension in assay buffer (human blood type 0 erythrocytes, final concentration 2%) and culture supernatant in microtiter plates (Falcon 3077, Becton Dickinson Labware, New Jersey, USA). Plates were incubated at 37 °C for 2 h, and the hemolytic titers were expressed as the reciprocal of the highest dilution at which any hemolysis was observed.

Statistical analysis: The χ^2-test was used to test the significance of differences. A probability of 0.01 was considered significant.

Results

The distribution of the *P. aeruginosa* serotypes among 100 studied isolates is summarized in Tables 1, 2 and 4. Somatic type O11 was most frequently identified (34%), followed by O6 (16%). Other somatic types accounted for less than 10.0% each, and four strains failed to agglutinate any somatic antiserum (non-typable strains, NT). Serogroups O7, O10, O13, O14, O15, and O17 were not detected among investigated strains.

Table 1 shows the distribution of most frequent serotypes among isolates from various clinical sources. Twelve strains were isolated from endotracheal aspirates, 16 from bronchial secretion obtained by fiberoptic bronchoscopy, and seven from sputum. Ten strains were isolated from pharyngeal swabs, nine from ear swabs, 17 from wound swabs, 15 from urine and seven from different materials

Table 1. Distribution of most frequent serogroups among isolates from various clinical materials

Specimen	Serogroup							
	O11[a]	O6	O3	O4	O2	O1	Others	Total (N, %)
Endotracheal aspirate	3	3	2	0	0	0	4	12
Bronchial secretion	11	1	1	0	0	0	3	16
Sputum	2	1	2	0	0	0	2	7
Pharyngeal swab	0	2	3	2	2	0	1	10
Ear swab	1	3	0	1	1	3	0	9
Wound aspirate	8	1	1	2	3	0	2	17
Urine	2	3	0	1	1	3	5	15
Biomaterials[b]	4	1	0	1	0	0	1	7
Others	3	1	0	1	0	0	2	7
Total (N, %)	34	16	9	8	7	6	20	100

[a] Differences statistically significant (χ^2=17.11; p<0.01)
[b] Cultivation of bacteria from different biomaterials (catheters, tubes, etc.) was performed by use of standardized microbiological techniques

(pus, exudates, etc.). Seven strains were cultivated from different biomaterials (chateters, tubes, etc.) during the standard diagnostic procedure. A statistically significant difference was observed in the distribution of strains in which O11 serogroup was detected (χ^2=17.11, DF=4, p<0.01). The difference occurred due to a higher than expected frequency of O11 serotype among strains isolated from bronchial secretion, wound aspirates and biomaterial cultures, and a lower than expected frequency of O11 strains among other respiratory tract isolates and isolates from urine. Serogroup O6 was most frequently detected among strains isolated from urine, ear swabs, and endotracheal aspirates. The O1, O2, O3 and O4 serotypes were mostly detected among isolates from ear and pharyngeal swabs and wound aspirates.

Table 2 shows the distribution of most frequent serogroups in *P. aeruginosa* isolates depending on strain origin. Twenty-six strains were recovered from outpatients, whereas 74 strains were recovered from patients at different clinical wards of the Osijek University Hospital. The difference in the distribution of O11 serogroup was statistically significant. The O11 strains were mostly isolated from patients at the pulmonology and surgery wards, and from intensive care unit patients (χ^2=15.84, DF=5, p<0.01). The O6 serotype was rarely detected among strains isolated from these clinical wards, but was frequently detected among strains from the pediatric ward. Antigenic variability was greatest among the strains isolated from outpatients.

The ability of hemolysin (HE) production of investigated strains was compared by hemolysis titer (Table 3). Hemolysin production was recorded in 88% of all isolates, but 39% of strains produced low amounts of HE. The differences between strains isolated from different materials were statistically significant (χ^2=25.02, DF=6, P<0.01). Strains isolated from bronchial secretion mostly produced low amounts of HE (hemolysis titer 2-4) or did not produce HE at all, while strains isolated from urine, pharyngeal and ear swabs mostly produced high amounts of HE (hemolysis titer ≥8). The highest HE production was detected in a strain isolated from urine (hemolysis titer 128).

As regards serogroup specificity, it was observed that epidemic O11 strains mostly produced low amounts of HE, while O1, O12 and O16 strains, and O2, O3 and O4 strains mostly produced high HE amounts (Table 4). The observed differences were statistically significant (χ^2 = 25.35, DF=4, p<0.01 after the fusion of "0" and "2-4" columns).

Discussion

In this study, the most frequently detected serogroups were O11 (34%) and O6 (16%), followed by O3 (9%), O4 (8%), O2 (7%), and O1 (6%) serogroups. Limitations to serotyping include poor discriminating power, polyagglutination for some strains, failure of serotyping in lipopoly-

Table 2. Distribution of most frequent serogroups in Pseudomonas aeruginosa isolates according to strain origin

Strain origin	Serogroup									
	O11[b]	O6[c]	O3	O4	O2	O1	012	016	Others	Total (N, %)
Outpatients	2	5	2	2	4	6	1	0	4	26
OUH - ICU[a]	6	0	1	1	0	0	1	2	1	12
OUH - Surgery	6	2	1	3	3	0	0	0	1	16
OUH - Pulmonology	11	1	2	0	0	0	0	0	5	19
OUH - Pediatrics	3	5	2	2	0	0	0	2	0	14
Others	6	3	1	0	0	0	2	0	1	13
Total (N, %)	34	16	9	8	7	6	4	4	12	100

[a] OUH=Osijek University Hospital; ICU=intensive care unit
[b] Differences statistically significant (χ^2=15.84; p<0.01)
[c] Differences statistically significant (χ^2=7.17; p<0.01)

Table 3. Production of hemolysin of Pseudomonas aeruginosa strains isolated from different clinical specimens

Specimen	Titer of hemolysis[a]			
	0	2-4	≥8	Total (N, %)
Endotracheal aspirate	2	6	4	12
Bronchial secretion	3	10	3	16
Sputum	2	3	2	7
Pharyngeal swab	0	1	9	10
Ear swab	0	3	6	9
Wound aspirate	4	5	8	17
Urine	0	2	13	15
Biomaterials[b]	1	4	2	7
Others	0	5	2	7
Total (N, %)	12	39	49	100

[a] Differences statistically significant (χ^2=25.02; p<0.01)
[b] Cultivation of bacteria from different biomaterials (catheters, tubes, etc.) was performed by use of standardized microbiological techniques

Table 4. Comparison of serogroup specificity and hemolysin production ability of investigated strains

Serogroup	Titer of hemolysis[b]			
	0	2-4	≥8	Total (N, %)
O11	3	25	6	34
O6	4	2	10	16
O3	2	0	7	9
O4	2	2	4	8
O2	0	2	5	7
O1	0	1	5	6
O16	0	1	3	4
O12	0	0	4	4
O5	0	2	1	3
NT[a]	1	3	0	4
Others	0	1	4	5
Total (N, %)	12	39	49	100

[a] NT=non-typable strain
[b] Differences statistically significant (χ^2=25.35; p<0.01)

saccharide-defective strains, and autoagglutination in cystic fibrosis strains[3,14]. However, serotyping of *P. aeruginosa* remains useful and epidemiologically significant[3]. It has been shown that the most frequent serotypes involved in nosocomial infections are O6, O11, O1 and O3, and that serotype O12 is characterized by an exceptional multiresistance pattern[15-20]. Serogroup O11 was recognized as the major cause of epidemics both in the USA and Europe[17,18]. Importance of serogroup O11 in hospital environments was also confirmed in the present study, but

serogroup O12 was detected only four times, and none of these isolates were multiresistant (data not presented). As the serotype determination and antibiotic susceptibility pattern are insufficient for precise characterization of epidemiological circulation of *P. aeruginosa* strains, further studies are needed to establish whether epidemic O11 and O6 strains consist of homogeneous genotypes, or whether subtypes or clones could be identified by use of genotyping methods.

Evidence has also been reported indicating the prevalence of *P. aeruginosa* serotypes O4, O6, O11 and O12 in hospital-acquired urinary tract infections (UTIs)[18,21]. In addition, by combining multiple typing techniques, consistent epidemiological correlation has been established, leading to the hypothesis of a possible clonal origin for some uropathogenic strains belonging to serotypes O4 and O12[19]. It appears, therefore, that as with uropathogenic *Escherichia (E.) coli,* only a few serogroups of *P. aeruginosa* cause a major proportion of UTIs. In the present study, out of 15 strains isolated from urine of patients with established UTI ($\geq 10^5$ bacteria/ml urine), serogroups O12, O6 and O1 were detected in three strains each, O11 serogroup in two, and O2, O4, O5 and O16 in one *P. aeruginosa* strain. Comparing with other clinical specimens, strains isolated from urine produced the highest levels of hemolysin. This enzyme was also produced at very high levels in 100% of *P. aeruginosa* strains from UTIs studied by Woods et al.[22], but not in all strains studied by Visca et al.[21], who observed a high degree of heterogeneity among isolates. In this study, elevated levels of hemolysin and other exoenzymes were characteristic of serotypes of minor numerical importance, while low levels of hemolysin were produced by strains belonging to the predominant serotypes[21]. Similarly, we noticed that epidemic O11 strains produced low levels of hemolysin, and strains in which O1, O2, O3, O12 and O16 serotypes were detected mostly produced high levels of hemolysin.

The importance of hemolysin production in *P. aeruginosa* pathogenesis is not clear. It has been suggested that hemolysin plays a role in the colonization of mucous membranes, implicating a local rather than systemic role for this enzyme in *P. aeruginosa* pathogenesis[22,23]. Saiman et al.[24] studied the adherence ability of *P. aeruginosa* strains to the respiratory tract epithelial cells and showed that phospholipase C and proteases increased the adherence ability of the bacteria by modification of the glycoconjugates on the surface of epithelial cells. On the contrary, Woods et al.[25] have suggested a systemic role of this enzyme, based on the results of the neutropenic mouse model of infection. They observed that the presence of high levels of exoenzyme S in association with phospholipase C correlated with a low number of bacteria required to elicit the 50% lethal dose[25].

It is possible that the low levels of hemolysin production observed in the present study in epidemic O11 strains isolated from bronchial secretion of patients hospitalized on the pulmonology ward were the result of colonization duration. Burke et al.[26] showed the duration of colonization to correlate with bacterial phenotype. They confirmed that *P. aeruginosa* from chronically colonized patients tended to be less motile, and produced lower levels of elastase, protease, exotoxin A and hemolysin. Similarly, Woods et al.[27] observed a significant decrease in the levels of endotoxin A, exoenzyme S and phospholipase C by using a chronic pulmonary infection model.

Conclusions

1. The present study confirmed previous observations of the epidemiological predominance of O11 serogroup. Multiresistant O12 strains, also epidemic in Europe, were not detected, and moreover, O12 serogroup was of minor numerical importance.

2. The data presented indicate that the hemolysin levels varied significantly, depending on the site of isolation and serotype of the strain.

3. As in uropathogenic *E. coli* infections, hemolysin production seems to be a virulence factor in *P. aeruginosa* UTIs.

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SAŽETAK

RAZLIKE U ANTIGENOJ STRUKTURI I STVARANJU HEMOLIZINA MEĐU SOJEVIMA *PSEUDOMONAS AERUGINOSA* IZOLIRANIM IZ RAZLIČITIH MATERIJALA

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Pseudomonas (P.) aeruginosa stvara brojne egzoenzime koji se drže činiteljima virulencije u infekcijama koje izaziva ovaj mikroorganizam. Jedan od njih je fosfolipaza C ili termolabilni hemolizin *P. aeruginosa.* Svrha ovoga istraživanja bila je utvrditi razlike u stvaranju hemolizina 100 sojeva *P. aeruginosa* u ovisnosti o antigenoj strukturi soja i vrsti materijala iz kojega je soj izoliran. Izolati su serotipizirani pomoću aglutinacije s komercijalnim antiserumima prema Internacionalnoj shemi za antigeno tipiziranje. Metoda mikrotitracije rabljena je za kvantificiranje hemolitičke aktivnosti istraživanih sojeva. Najčešće je utvrđena serogrupa O11, tj. u 34% sojeva, potom serogrupe O6 (16%), O3 (9%), O4 (8%), O2 (7%) i O1 (6%). Serogrupe O7, O10, O13, O14, O15 i O17 nisu utvrđene među istraživanim sojevima. Od 16 sojeva izoliranih iz sekreta bronha, serogrupa O11 nađena je u 11 sojeva, dok je od 15 sojeva izoliranih iz mokraće ista serogrupa nađena u dva soja ($p<0,01$). Stvaranje hemolizina utvrđeno je u 88% svih izolata. Sojevi O1, O2, O3 i O16 stvarali su veliku količinu hemolizina, dok su epidemični sojevi O11 stvarali malu količinu hemolizina. Izolati iz mokraće i ždrijela stvarali su najviše hemolizina.

Ključne riječi: *Pseudomonas aeruginosa, patogeničnost; Pseudomonas aeruginosa, klasifikacija; Hemolizini*