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ORIGINAL PAPER

Genetic Features of Clinical *Pseudomonas aeruginosa* Strains

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Abstract

The genetic features of each isolate were determined by enterobacterial repetitive intergenic consensus (ERIC) primer sequences used in PCR and by searching for six virulence genes (*alg D*, *las B*, *tox A*, *plc H*, *plc N*, *exo S*). 49 (79%) of the isolates were distributed in three ERIC PCR subgroups and showed 62% of similarity. The remaining 13 strains generated unique patterns. The first subgroup was primarily composed of isolates from faeces, these strains indicated over 70% relationship with the next subgroup, and primarily contained strains isolated from wounds and bronchial washings and the last subgroup contained strains isolated from wounds and urine. The unique strains were isolated mainly from urine. Statistical analysis indicated that variations in distribution of virulence genes in *P. aeruginosa* isolates with respect to strain origin and genomic subgroups were not significant. In the group of 49 strains, 100% gave a positive reaction to *alg D*, *las B* and *plc H* genes, 91.8% to *tox A* and *plc N* genes and 83.7% to *exo S* gene. Among the strains that generated unique (ERIC-PCR) patterns, 69.2% gave a positive reaction to *alg D* gene, 84.6% to *las B* gene, 76.9% to *tox A*, *plc N* and *plc H* genes, and 46.15% to *exo S* gene.

Key words: *Pseudomonas aeruginosa*, ERIC-PCR, virulence genes

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is responsible for a wide range of infections. It is a common hospital-acquired pathogen and is responsible for ventilator-associated pneumonia in patients with underlying immune defects (Carratala *et al*., 1998), as well as wound and catheter-associated infections. Also intestinal colonization by *P. aeruginosa* could be a reservoir for invasive infections caused by this bacterium and for its dissemination (Zaborina *et al*., 2006). In healthy individuals, it is a leading cause of contact lens keratitis (Cheng *et al*., 1999), otitis externa (swimmer's ear), and hot-tub folliculitis (Speert, 2002). Additionally, *P. aeruginosa* is the most common pathogen in lung infections affecting cystic fibrosis (CF) patients and is the leading cause of morbidity and mortality in this patient group (Govan and Deretic, 1996). The ability of *P. aeruginosa* to cause infection is further exacerbated by a high level of resistance to antibiotics, which makes *Pseudomonas* infections difficult to treat (Hancock and Speert, 2000).

P. aeruginosa produces several virulent factors to colonize the cells of its host. Many of these factors are controlled by regulatory systems involving cell-to-cell signaling (Van Delden and Iglewski, 1998). Among these are: exotoxin A, exoenzyme S, las B elastase, phospholipases C and alginate. Exotoxin A, encoded by the *tox A* gene, inhibits protein biosynthesis by transferring an ADP-ribosyl moiety to elongation factor 2 of eukaryotic cells. Exoenzyme S, encoded by the *exo S* gene, is also an ADP-ribosyltransferase that is secreted by a type-III secretion system directly into the cytosol of epithelial cells (Rumbaugh *et al*., 1999a). Las B elastase, a zinc metalloprotease encoded by the *las B* gene, attacks eukaryotic proteins such as collagen and elastin, and destroys the structural proteins of the cell (Toder *et al*., 1994). While phospholipids are hydrolyzed by two phospholipases C encoded by *plc H* and *plc N* genes (PLC-H and PLC N, respectively). Alginate, encoded by *alg D* gene, protects the bacterium from the host's immune response and from antibiotics (Ostroff *et al*., 1990; Storey *et al*., 1997).

Genetic methods have been used to explore the genetic diversity of various populations of *P. aeruginosa* strains. Various methods, such as pulsed-field gel electrophoresis, arbitrarily primed polymerase chain reaction, and ribotyping are currently available for genotyping of *P. aeruginosa* (Bennekov *et al*., 1996).

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On the other hand, the enterobacterial repetitive intergenic consensus (ERIC) sequences are known to be dispersed throughout the prokaryotic genome, and polymerase chain reaction (PCR) studies of eubacteria have revealed that inter-ERIC distances and patterns are highly specific to the individual group of the same bacterial species (Versalovic *et al*., 1991).

In the present study we aimed to determine the genetic features of clinical *P. aeruginosa* isolates by ERIC – based PCR (ERIC-PCR) and by assessing variations in the prevalence of six virulence genes (*alg D*, *las B*, *tox A*, *plc H*, *plc N*, *exo S*).

Experimental

Materials and Methods

Source of isolates and identification. A total of 62 strains of *P. aeruginosa*, were originally isolated from a variety of clinical specimens: faeces (26), urine (11), blood (1), bronchial washings (9), sputum (1), wound swab (9), throat swab (2), ulceration swab (1), swab from skin round tracheotomy (1) and from ear (1). The bacteria were obtained from 62 patients from different wards of the municipal hospital, main hospital and outpatients' department in Siedlce (Poland), between December 2005 and March 2006. The strains were identified as *P. aeruginosa* according to biochemical patterns in the Api 20NE system (bio Mérieux). The reference strain NCTC 6749 was also examined. Stock cultures were stored in TSB (tripticase soy broth, Difco) containing 20% glycerol at –80ºC.

DNA extraction. Isolates were grown in trypticase-soya broth at 37°C for 24 h and DNA was extracted by using the Genomic DNA Pre Plus (A&A Biotechnology, Gdańsk, Poland).

Detection of virulence genes by PCR. The prevalence of virulence genes encoding alginate (*alg D*), Las B elastase (*las B*), haemolytic phospholipase C (*plc H*), non-haemolytic phospholipase C (*plc N*), exoenzyme S (*exo S*) and exotoxin A (*tox A*) was determined by PCR. The genes were amplified with primers selected on the basis of the published PAO1 sequence (Stover *et al*., 2000). The PCR mixture contained PCR buffer (10 mM Tris/HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 200 µM of each dNTP (Boehringer), 12.5 pmol of each primer, DMSO at a final concentration of 4%, 1 U Ampli *Tag* DNA polymerase (Perkin Elmer) and 25 ng DNA template. The DNA was amplified in PTC-100 Programmable Thermo Controller (MJ Research) using the following protocol: 94°C for 3 min, 30 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1 min 30 s, and 72°C for 5 min. Each gene was amplified separately. PCR products were separated in a 1% agarose gel for 1 h at 100 V, stained with ethidium bromide and detected by UV transillumination. Amplified genes were identified on the basis of fragment size (Table I).

Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR). ERIC primer sequences were used in PCR to detect differences in the number and distribution of these bacterial repetitive sequences in the bacterial genome. ERIC PCR was carried out using the primer sequences ERIC-1R, 5'-CACTTAGGGGTCC TCGAATGTA-3' and ERIC-2, 5'-AAGTAAGTGACT GGGGTGAGCG-3' to amplify the regions in the bacterial genome placed between the ERIC sequences. Dice coefficient was calculated and compared to evaluate similarity among strains through the use of BIOGENE software. The method was described in the previous study (Wolska and Szweda, 2008).

Statistical methods. The distribution of virulence genes with respect to genomic groups or strain origin was compared using chi-square (λ^2) test.

Table I
Primers used for PCR amplification of virulence factors

Gene	Primer sequence (5'-3')	Product (bp)	Number of pair G+C
alg D	alg DF – CGTCTGCCGCGAGATCGGCT	313	14
	alg DR – GACCTCGACGGTCTTGCGGA		13
las B	las BF – GGAATGAACGAAGCGTTCTCCGAC	284	13
	las BR – TTGGCGTCGACGAACACCTCG		13
tox A	tox AF – CTGCGCGGGTCTATGTGCC	270	13
	tox AR – GATGCTGGACGGGTCGAG		12
plc H	plc HF – GCACGTGGTCATCCTGATGC	608	14
	plc HR – TCCGTAGGCGTCGACGTAC		12
plc N	plc NF – TCCGTTATCGCAACCAGCCCTACG	481	14
	plc NR – TCGCTGTCGAGCAGGTCGAAC		13
exo S	exo SF – CGTCGTGTTCAAGCAGATGGTGCTG	444	14
	exo SR – CCGAACCGCTTCACCAGGC		13

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Results

We used PCR to assess the prevalence of six virulence genes. PCR detected *alg D*, *las B*, *tox A*, *plc H*, *plc N* and *exo S* in 58 (93.55%), 60 (96.8%), 55 (88.7%), 59 (95.2%), 55 (88.7%) and 47 (75.8%) isolates, respectively. 40 (64.5%) isolates gave positive PCR results for all studied genes. In this group, 17 (65.4%) isolates were obtained from faeces, 5 (45.45%) isolates were obtained from urine, 7 (77.8%) from wound, 8 (88.9%) from bronchial washings and individual strains were isolated from sputum, skin and blood. The remaining strains (22–35.5%) gave a negative result for one or more genes. All studied genes were not detected in two strains; the first strain (number 2) was isolated from urine and the second one (42) was obtained from throat swab. The strain isolated from faeces (59) gave a negative PCR reaction to *alg D*, *tox A*, *plc N* and *exo S* genes. The next strain isolated from the same source (53) gave a negative PCR reaction to *alg D*, *plc N* and *exo S* genes, and the last strain of this origin (8) gave a negative PCR reaction to *plc N* and *exo S* genes. The other strains gave a negative result to one of the three following genes: *exo S*, *tox A* and *plc N*. *Exo S* gene was not presented in strains isolated from urine (4 isolates), faeces (1), throat swab (1), wound (2), bronchial washings (1) and ear (1). *Tox A* gene and *plc N* were not detected in 2 and 3 strains isolated from faeces. The variations in distribution of virulence genes in *P. aeruginosa* isolates with respect to origin were not significant by the use the chi-square analysis ($\lambda^2 = 4.01$) (Table II).

Nearly all strains, with the exception of 2, 42 and 59 which gave negative reactions to nearly all studied virulence genes, showed 50% of similarity according to Dice coefficient values (Fig. 1). 41 (66.1% of all) isolates revealed a high similarity, over 70%. Dendrogram analysis enabled the division of strains into groups (50–62% of similarity), then subgroups (64–80%), genotypes (80–86%) and lastly subtypes (86–100%). 13 (21%) strains generated unique ERIC-PCR patterns. 49 (79%) isolates and reference strain of *P. aeruginosa* creating group 1 showed 62% of similarity. The chi-square analysis indicated that variations in distribution of *P. aeruginosa* isolates in genomic subgroups with respect to ecological origin were significant ($\lambda^2 = 80.186$) (Table III). The most numerous clonal subgroup of 1, which was characterized by nearly 80% of similarity, consisted of 2 genotypes. This subgroup contained 29 isolates, among which, 24 (92.3%) were isolated from faeces and the individual strains in this group were isolated from wound, skin, bronchial washings, urine and throat swab. The mentioned subgroup demonstrated over 70% of similarity with the next clonal subgroup, which included 12 isolates situated in 2 types. Among

Table II
Distribution of virulence genes in *Pseudomonas aeruginosa* isolates with respect to origin

Origin (No) of strains	Virulence genes					
	alg D	*las B*	*tox A*	*plc H*	*plc N*	*exo S*
Faeces (26)	24 (92.3%)	26 (100%)	23 (88.5%)	25 (96.1%)	21 (80.8%)	22 (84.6%)
Urine (11)	10 (90.9%)	10 (90.9%)	9 (81.8%)	10 (90.9%)	10 (90.9%)	6 (54.5%)
Wound (9)	9 (100%)	9 (100%)	9 (100%)	9 (100%)	9 (100%)	7 (77.8%)
Bronchial washings (9)	9 (100%)	9 (100%)	9 (100%)	9 (100%)	9 (100%)	8 (88.9%)
Throat (2)	1	1	1	1	1	–
Ulceration (1)	1	1	–	1	1	1
Ear (1)	1	1	1	1	1	–
Blood (1)	1	1	1	1	1	1
Skin (1)	1	1	1	1	1	1
Sputum (1)	1	1	1	1	1	1
Reference (1)	1	1	1	1	1	1

Table III
Distribution of the 63 *Pseudomonas aeruginosa* isolates in genomic subgroups with respect to origin

Genomic subgroups (No of strains)	Faecies (n = 26)	Urine (n = 11)	Wound (n = 9)	Bronchial washings (n = 9)	Throat (n = 2)	Ulceration (n = 1)	Ear (n = 1)	Blood (n = 1)	Skin (n = 1)	Sputum (n = 1)	Reference (n = 1)
1 (29)	24 (92.3%)	1 (9.1%)	1 (11.1%)	1 (11.1%)	1 (50%)	–	–	–	1	–	–
2 (12)	–	1 (8.3%)	5 (55.5%)	4 (44.4%)	–	1	1	–	–	–	–
3 (9)	–	3 (27.3%)	3 (33.3%)	1 (11.1%)	–	–	–	–	–	1	1
The unique patterns (13)	2 (7.7%)	6 (54.5%)	–	3 (33.3%)	1 (50%)	–	–	1	–	–	–

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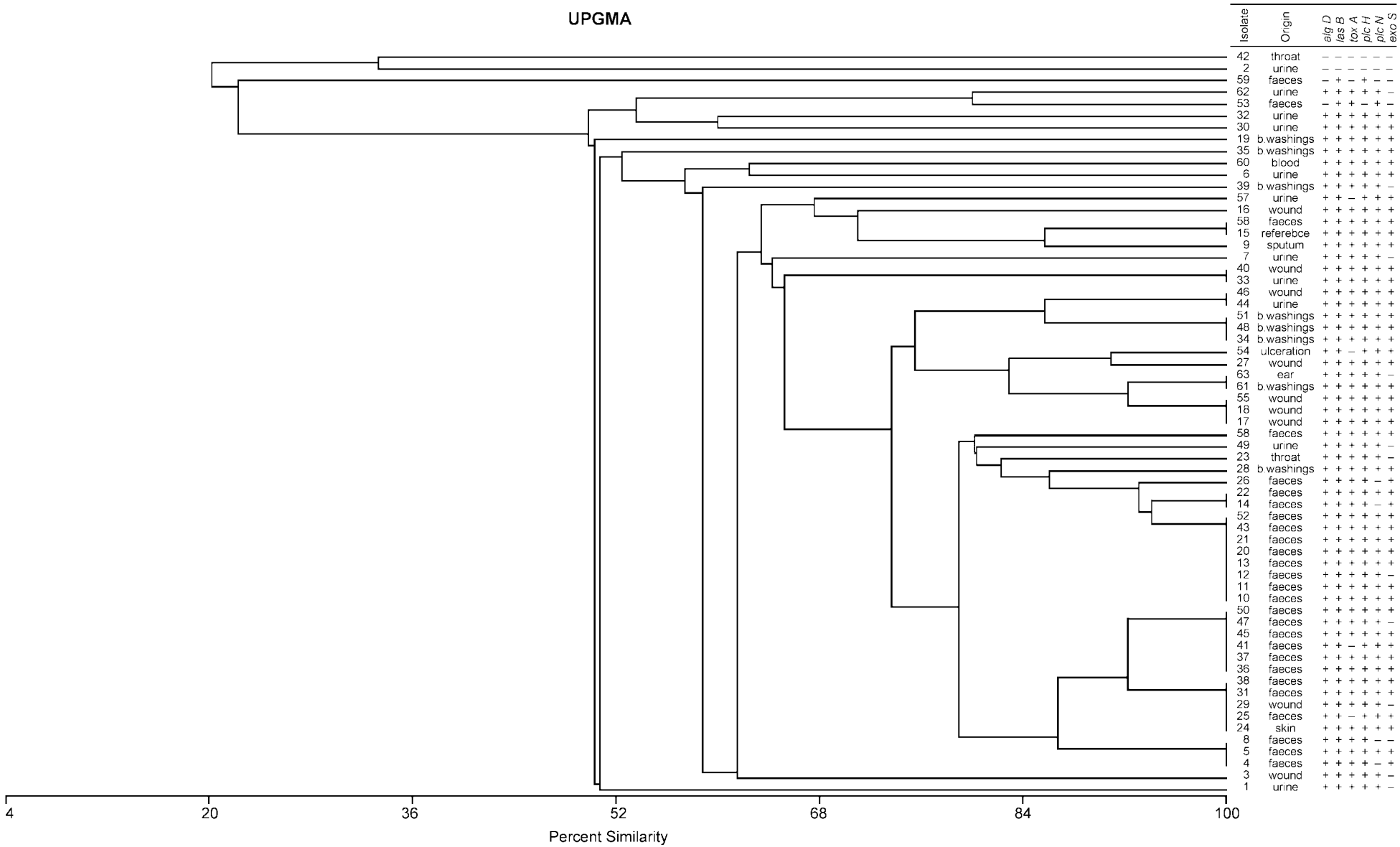

Fig. 1. Dendrogram showing genetic relationships between 62 *P. aeruginosa* isolates.
The results were obtained by ERIC-PCR analysis using two primers.

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Table IV
Distribution of virulence genes in *Pseudomonas aeruginosa* isolates with respect to genomic subgroups

Genomic subgroups (No of strains)	Virulence genes					
	alg D	*las B*	*tox A*	*plc H*	*plc N*	*exo S*
1 (29)	29 (100%)	29 (100%)	27 (93.1%)	29 (100%)	25 (86.2%)	24 (82.7%)
2 (12)	12 (100%)	12 (100%)	11 (91.7%)	12 (100%)	12 (100%)	11 (91.7%)
3 (9)	9 (100%)	9 (100%)	8 (88.9%)	9 (100%)	9 (100%)	7 (77.8%)
The unique patterns (13)	9 (69.2%)	11 (84.6%)	10 (76.9%)	10 (76.9%)	10 (76.9%)	6 (46.15)

these 12 isolates, 5 (55.6%) strains were obtained from wound, 4 (44.4%) from bronchial washings, 1 (9.1%) from urine and 1 from ulceration. Among 41 strains forming subgroups 1 and 2, 29 (70.7%) gave a positive reaction to all six genes. 11 (26.8%) strains gave a positive reaction to five genes and only 1 (2.4%) strain gave a positive reaction to four genes. All isolates of these subgroups gave positive reactions to *alg D*, *las B* and *plc H* genes. The prevalence of *tox A* gene in isolates of subgroup 1 was similar to isolates of subgroup 2. And the prevalence of *exo S* gene was higher in the isolates of group 2 than in group 1. The last 9 strains forming group 1, showed 62% of similarity with subgroups 1 and 2. Among which, there were isolates from urine (3 (27.3%) strains), wound (3 (33.3%)), sputum (1), bronchial washings (1 (11.1%)) and the reference strain of *P. aeruginosa* NCTC 6749. These strains gave a positive reaction to nearly all virulence genes with the exception of two strains isolated from urine. One gave a negative result for *exo S* gene and the second one gave a negative result for *tox A* gene. The remaining strains which were collected (13–21%) generated unique (ERIC-PCR) patterns, showing the genetic diversity of these bacteria. They were isolated from urine (6 (54.5%)), bronchial washings (3 (33.3%)), blood (1), faeces (2 (7.7%) and throat swab (1). Among these strains, 6 (46.15%) gave positive reactions to all virulence genes, but 7 (53.85%) gave negative results to one or more (from 3 to 6) virulence gene. The statistical analysis showed that variations in distribution of virulence genes in *P. aeruginosa* isolates with respect to genomic subgroups were not significant ($\lambda^2 = 4.01$) (Table IV).

Discussion

The relationship between isolates was calculated by numerical analysis of genetic features determined by ERIC PCR. 79% of isolates were distributed in three ERIC PCR subgroups and showed 62% of similarity. The remaining strains generated unique patterns. The first subgroup was primarily composed of isolates from faeces that were strictly related with each other. These strains indicated over 70% relationship with the next subgroup, and primarily contained strains isolated from wound and bronchial washings. Above all, the last subgroup contained strains isolated from wound and urine. The unique strains were isolated mainly from urine. Virulence genes were detected more frequently in strains isolated from bronchial washings and wound rather than in strains isolated from urine and faeces. However, the statistical analysis indicated that variations in distribution of virulence genes in *P. aeruginosa* isolates with respect to strain origin and genomic subgroups were not significant. This result is in agreement with the studies of Hamood *et al*. (1996) and Rumaugh *et al*. (1999b). They suggest that elastase, phospholipase C, exotoxin A and exoenzyme S are produced by *P. aeruginosa* isolates from the different sites of infection. The studies also demonstrated that the production of higher levels of elastase and phospholipase C is important in all types of infections. In some bacterial infections, complex systems of coordinate regulation control the expression of virulence genes (Mecalanos, 1992). Storey *et al*. (1997) found a positive correlation between *alg D* transcript accumulation and both *las B* and *las A* transcript accumulation levels. This indicates a common regulatory element in a cascade of regulators or a common environmental cue that triggers transcription. Numerous authors (Joly *et al*., 2005; Rietsch *et al*., 2005; Yahr *et al*., 1995) have documented the presence of a type III secretion system in *P. aeruginosa* that appears to play a major role in the virulence of this organism. Endimiani *et al*. (2006) showed that 100% of *P. aeruginosa* isolates from bloodstream infections were positive for the following genes: *exo T*, *exo U*, *las B*, *plc H*, *plc N*, *tox A* and *nan 2*. *Exo S*, *exo Y* and *nan 1* genes were detected in 78.9%, 73.7% and 57.9% of isolates. Our data demonstrated that the studied genes: *alg D*, *las B*, *tox A*, *plc H*, *plc N* and *exo S* were present in 93.55%, 96.8%, 88.7%, 95.2%, 88.7% and 75.8% *P. aeruginosa* isolates, respectively. Feltman *et al*. (2001) and Ferguson *et al*. (2001) indicated that over 90% of clinical *P. aeruginosa* strains contain *exo T* and *exo Y* genes, but clinical isolates from urine frequently have the *exo Y* gene present at a relatively lower level-about 70%. Similarly, in our study *exo S* gene was rarely detected in *P. aeruginosa* strains isolated from urine. According

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to Lin *et al*. (2006), *exo U* gene of *P. aeruginosa* is the major contributor to cytotoxicity against mammalian cells. Others (Rumbaugh *et al*., 1999a) revealed that *P. aeruginosa* isolates recovered from patients suffering from urinary tract infections produced significant levels of exotoxin A. Over 80% of studied isolates from urine contained the *tox A* gene. In all probability, this toxin can play a significant role as a virulence factor of *P. aeruginosa* within catheter-associated urinary tract infections (Goldsworthy, 2008). Lanotte *et al*. (2004) demonstrated that 80% of all pulmonary isolates harboured *exo S*, indicating that this gene plays a major role in pulmonary infections. In our study also, strains isolated from bronchial washings showed the prevalence of *exo S*. *Exo S* gene was frequently associated with multi-drug resistant (MDR) strains which suggests that *P. aeruginosa* isolates showing MDR trails may be more virulent than susceptible strains (Endimiani *et al*., 2006). Results of Zaborina *et al*. (2006) showed that the multi-drug resistant *P. aeruginosa* clinical strains that disrupted the barrier function of cultured intestinal epithelial cells are characterized by the *exo U* gene. The presence of virulent strains of *P. aeruginosa* within the intestinal tract could be the major source of systemic sepsis and death among immuno-compromised patients.

The above presented data has been founded in Poland. So far nobody has applied the ERIC-PCR method in discriminating clinical *P. aeruginosa* strains and evaluated the occurrence of such numerous groups of virulence genes.

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