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Virulence factors genes and drug resistance in *Pseudomonas aeruginosa* strains derived from different forms of community and healthcare associated infections*

Obecność genów kodujących czynniki wirulencji i lekooporność szczepów *Pseudomonas aeruginosa* izolowanych z różnych typów zakażeń szpitalnych i pozaszpitalnych

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: The pathogenesis of *Pseudomonas aeruginosa* (PA) infection is multifactorial and depends mainly on two types of virulence determinants: virulence factors involved in acute infections and membrane bound factors, and virulence factors involved in chronic infections. The aim of this laboratory-based study was to analyse the resistance and virulence of PA strains isolated from different types of infections in hospitalised and non-hospitalised patients in Southern Poland.

Material/Methods: Non-repetitive samples from urinary tract infections (UTI), bloodstream infections (BSI) and pneumonia (PNU) were collected. Isolates were screened for the presence of virulence factors by PCR method. Antimicrobial susceptibility testing was performed using disc diffusion method. Metallo-beta-lactamases (MBLs) were detected using an imipenem-EDTA double-disc synergy test.

Results: There were 232 specimens collected: UTI-152, PNU-69, BSI-11. Fifty-one (22%) strains were classified as multidrug resistant (MDR), 23 (10%) as extensively-drug resistant (XDR). MDR/XDR strains were more frequently isolated from pneumonia (OR = 2.28). The prevalence values for the genes of effector proteins were 82.8% for *exo*S, 89% for *exo*Y, and 24% for *exo*U. The simultaneous detection of four effector proteins was confirmed in 10.4% of the strains. *pil*B was more prevalent in isolates from the elderly (p=0.013). *las*B occurred more frequently in

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PNU (p=0.048). Three of the genes were more prevalent in isolates from patients hospitalised in ICU (*las*B (p=0.017), *apr*A (p=0.022), *phz*S (p=0.039)).

Conclusions: Understanding the contribution of selected virulence genes to the outcome of an infection may be important for the therapeutic management of patients infected with PA. Simultaneous detection of virulence factors and antimicrobial resistance might be particularly useful because both are clinically important during an infection.

Keywords: ***Pseudomonas aeruginosa*** **virulence • XDR • antimicrobial resistance • pathogenesis**

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INTRODUCTION

Pseudomonas aeruginosa (PA) is an opportunistic pathogen that is a common cause of hospital acquired infections. Infection with PA may be associated with significant morbidity and mortality. PA adapts easily to environmental changes, rapidly develops resistance, and produces a wide variety of virulence factors. The pathogenesis of PA infection is multifactorial and depends mainly on two types of virulence determinants:

• virulence factors involved in acute infections (usually secreted) and membrane bound factors;
• virulence factors involved in chronic infections (i.e. siderophores).

Virulence traits may vary between strains of PA; these traits vary with respect to the site of infection [5, 7, 22, 39].

PA has several known virulence factors. Elastase, encoded by the *las*B gene, is a metalloprotease that degrades elastin and collagen. Elastase inactivates human immunoglobulin G and several complement components [23]. Alkaline protease (AprA) is a zinc metalloprotease. AprA expression correlates with PA infections of the eye, gastrointestinal tract and wounds [23]. It can also degrade many biologically important proteins (i.e. casein, gelatin, and complement factors) [14, 19]. Type 4 fimbrial assembly protein, PilB, is essential for the synthesis of type IV pili. PilA is a type IV pilus protein. The type III secretion system is another important PA virulence determinant [10]. This system uses complex secretion and translocation machinery to inject a set of factors (effector proteins) directly into the cytoplasm of eukaryotic cells. Four of these effector proteins have been identified: ExoS, ExoT, ExoU, and ExoY. All have toxic effects on cells. ExoS has cytotoxic activity, induces apoptosis, and modulates bacterial phagocytosis and invasion into host cells [17, 37]. ExoT is involved in inhibiting the rate of wound healing and inhibits bacterial internalization functions of epithelial cells and macrophages [37]. ExoU is an acute cytolytic factor that mediates rapid cell lysis [36]. ExoY is an adenylate cyclase that causes rounding of certain cell types [36]. PA isolates that possess the *exo*U effector protein gene are referred to as having a cytotoxic phenotype and those with the *exo*S effector protein gene have an invasive phenotype [40]. It has been proved by Pena et al. that *exo*U+ genotype is independently associated with increased risk of early mortality of PA bloodstream infections, so detecting the presence of these virulence genes may lead to easier identification of potentially lethal infections [15, 24, 28].

Approximately 95% of PA strains produce pyocyanin, which is a blue-pigmented phenazine derivative that has a role in virulence [21]. Pyocyanin has a role in the pulmonary tissue damage that occurs during PA infection. Pyocyanin is produced from chorismic acid via the phenazine pathway, which consists of nine proteins encoded by a gene cluster [12]. Phenazine-1-carboxylic acid is the initial phenazine formed and is converted to pyocyanin in two steps that are catalysed by the enzymes PhzM and PhzS. PhzM is only active in the presence of PhzS [12].

Our previously published results indicated that some virulence factors may be associated with the age of the treated patients [26]. Additional samples are needed to study this relationship, because the characteristics of virulence traits among the PA strains isolated from different populations and sites of infection have not been comprehensively studied.

The aim of this laboratory-based study was to analyse the resistance and virulence of PA strains isolated from different types of infections in hospitalised and non-hospitalised

patients in southern Poland. We focused on the prevalence of particular virulence genes and their associations with the patient group, infection site, and the degree of antimicrobial resistance of individual PA strains.

MATERIALS AND METHODS

Ethics

This work was approved by the Bioethics Committee of Jagiellonian University Medical College (Chairperson Prof. Piotr Thor; no. KBET/312/B/2012 and KBET/362/B/2012). All data was anonymized before analysis.

Population description

Non-repetitive samples that represented different types of infections (urinary tract infections (UTIs), bloodstream infections (BSIs), and pneumonia (PNU)) were collected from hospitalised patients (12 hospitals), from residents of long-term care facilities, and from non-hospitalised patients in the south of Poland (Malopolska and Silesia). Patients were classified as: (1) long-term care facility (LTCF) residents, (2) patients hospitalized in intensive care units (ICUs), (3) patients hospitalized in non-ICU wards, or, (4) patients receiving home care (HC) (i.e. outpatients) whose infections were diagnosed by a physician.

Samples were collected in collaboration with the KORLAB NZOZ, Ruda Slaska, Poland and the microbiological laboratory of St. Barbara's Regional Hospital in Sosnowiec, Poland, between 1 January 2013 and 31 December 2013. Relevant patient information (e.g. age, sex, type of infection (polymicrobial or monomicrobial for UTI and PNU), and place of treatment of the infection) was also collected.

Bacterial isolates

Specimens were collected from patients when symptoms of infection first occurred. Microbiological examinations were performed on urine, blood, sputum, tracheobronchial aspirates, and bronchoalveolar lavage fluid when these types of samples were available. Standard methods and the semi-automatic Phoenix Automated Microbiology System (Becton-Dickinson BD, Franklin Lakes, NJ, USA) were used to identify microorganisms.

DNA extraction

DNA templates were extracted from liquid culture in tryptic soy broth (BioCorp) (37°C, 18 h) using a Genomic Mini kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instructions.

Screening for the virulence genes

Isolates were screened for the presence of major virulence factors that are often found in PA (i.e. *las*B, *exo*S, *exo*T, *exo*U, *exo*Y, *pil*A, *apr*A, *pil*B, *phz*M, *phz*S). PCR analysis was performed using previously published primers and conditions [2, 9]. The 2xPCR Master Mix (A&A Biotechnology, Poland) was used for PCR reactions. PCR products were separated on 1.5% agarose gel for 60 min at 90V, stained with ethidium bromide (Sigma-Aldrich, Munich, Germany) and detected using ultraviolet transillumination.

Susceptibility testing

Antimicrobial susceptibility testing was performed for all strains using Mueller-Hinton agar plates and following the current guidelines of the European Committee on Antimicrobial Susceptibility testing for the disc diffusion method (clinical breakpoint table v.5.0; http://www.eucast.org/clinical_breakpoints/, accessed 5.06.2015). Gentamycin, tobramycin, amikacin, netilmicin, imipenem, meropenem, doripenem, ceftazidime, cefepime, ciprofloxacin, levofloxacin, ticarcillin/clavulanic acid, and piperacillin/tazobactam discs were used for the testing. All discs were obtained from Oxoid Limited (Hampshire, UK). Resistant and intermediate strains were grouped together as drug-resistant. Metallo-beta-lactamases (MBLs) were detected using the imipenem-EDTA double-disc synergy method. Enlargement zone around IMP disc in the direction of EDTA disc indicated the presence of MBLs [18]. Multi-drug resistant (MDR) strains were defined as those strains that were not susceptible to at least one antimicrobial in at least three different antimicrobial classes. Extensively-drug resistant (XDR) strains were defined as those strains that were susceptible to no more than two antimicrobial classes [20].

STATISTICAL ANALYSES

Analyses were performed using Statsoft Statistical software (version 10, StatSoft Inc., Tulsa, OK, USA). The distributions of continuous variables were tested for normality using the Shapiro-Wilk test. Because the values for the tested variables did not follow normal distribution, the data was summarised as median, and as the 25^{th} (Q1) and the 75^{th} (Q3) percentile values, and were tested using the nonparametric Mann-Whitney U test. For dichotomous variables, a Chi-square test was used for expected frequencies >10, a Chi-square test with Yates' correction was used for expected frequencies between 5 and 10, and a Chi-square test with confirmation by Fisher's exact test was used for expected frequencies ≤5. Logistic regression analysis was used to evaluate the effects of factors on the presence or absence of MDR/XDR infection. The model was created using a backward stepwise regression method with v-fold cross-evaluation. We began with a set of 17 variables, including sex, age, site of infection (BSI, UTI, or PNU), sample source (HC – home care patients, ICU, hospitalised on non-ICU, or LTCF), and all of the studied virulence genes (present vs. absent). Age was included as a categorical variable because the relationship between the logarithm of the chance and continuous predictor was non-linear (p(LR) =

0.02). The model contained only two statistically significant variables and the distribution of probabilities predicted by the model did not differ significantly from the results observed from the sample (Hosmer Lemeshow test = 0.5189, p-value = 0.4712). A p-value < 0.05 was considered to indicate a statistically significant result.

RESULTS

Population description

The study population consisted of 232 patients. From these patients, 232 non-repetitive strains of PA were isolated (Table 1). We included in the study one strain for each patient, in case where a second or a third strain was isolated from the same patient, these repetitive strains were identical to the first isolate and rejected from the study.

The analysis of the population data revealed that the median (Q1; Q3) age was 64 years (47; 72) (Table 1). Thirty-one percent of the population was female. Inpatients accounted for 67% (n=155) of the population; 19% of these were hospitalised in ICUs (n=45), and 4% were the residents of LTCFs (n=10).

Most of the samples from both sexes originated from patients hospitalized in regular hospital wards, and relatively more women than men were hospitalised in those settings (52.0% women vs. 39.8% men). More women than men were residents of LTCFs (5.6% women vs. 3.7% men). In contrast, a greater proportion of male patients were hospitalised in ICUs (24.2% men vs. 8.5% women). Isolates originating from blood infections represented the minority of the studied isolates, but they were more common in men than in women (5.6% men vs. 2.8% women). The results were similar for PNU infections (34.2% men vs. 19.7% women). The opposite trend was observed for UTIs, which were present in 77.5% of the female patients, and in only 60.3% of the male patients. The frequencies of samples of different origin and from different infection sites were distributed unevenly within each sex group in a statistically significant manner (p = 0.024 and 0.038, respectively).

Bacterial isolates

Bacterial isolates accounted for: 152 urine isolates (65.6%), 69 pneumonia isolates (29.7%), and 11 isolates from bloodstream infections (4.7%) (Table 1).

Susceptibility testing

MBLs were detected in 24 (10.3%) isolates: in 10 isolates from UTI (4.3%), 12 from PNU (5.2%) and 2 from BSI (0.8%).

Isolates were classified as MDR, XDR, or as not resistant. Fifty-one (22%) of the strains were classified as MDR, 23 (10%) as XDR, and 158 (68%) as not resistant. MDR and XDR strains occurred more frequently in males compared with females (36% vs. 22.5%, respectively, p=0.04; odds ratio (OR) 95% confidence interval (95%CI) = 1.94; (1.02; 3.68)).

MDR and XDR strains were more frequently isolated from PNU (44.9% of the strains) samples compared with samples from other types of infections (27.0% of urine strains, 18.2% of blood strains; p=0.019; OR (95%CI) = 2.28 (1.26; 4.10)).

Table 1. Population data. Differences between strains isolated from different sites of infection

Infection site/ Characteristics	BSI n=11 [number/%]		UTI n=152 [number/%]		PNU n=69 [number/%]		P-value
Age (years), median; Q1-Q3	50; 39-68		64; 46-76		64; 54-71		
Age categories							
0-17	1	9.1	27	17.8	0	0	<0.001
18-60	5	45.5	41	27.0	24	34.8	
61-80	4	36.4	64	42.1	40	58.0	
>80	1	9.1	20	13.2	5	7.2	
Origin							
HC	0	0	69	45.4	7	10.1	<0.001
LTCF	0	0	5	3.3	5	7.2	
Hospital, excluding ICU	2	18.2	69	45.4	30	43.5	
ICU	9	81.8	9	5.9	27	39.1	
Sex							
Male	9	81.8	97	63.8	55	79.7	0.038
Female	2	18.2	55	36.2	14	20.3	

BSI – bloodstream infection, UTI – urinary tract infection, PNU - pneumonia, Q1-Q3 – quartile, HC – home care, LTCF – long term care facility, ICU – intensive care unit

MDR and XDR strains occurred less frequently among younger patients (<17 years of age; 7.1%; p = 0.003) compared with older patients (OR (95%CI) = 0.14 (0.03; 0.61)). These strains were also isolated less frequently from patients in home-care (25.0%), compared with hospitalised patients (both ICU and non-ICU; 33.5%) or residents of LTCFs (60.0%).

Virulence factor screening

The most prevalent virulence genes were *phz*M, and *exo*T, which were detected in 221 (95.2%) and 219 (94.4%) of the isolates, respectively. The prevalence values for the remaining four genes for effector proteins were 82.8% (n=193) for *exo*S, 89% (n=206) for *exo*Y, and 24% (n=56) for *exo*U. Approximately 11% of the strains contained both *exo*S and *exo*U genes. *exo*U and *exo*S simultaneously were present in 16 UTI strains (10.5%), 5 strains coming from BSI (45.5%) and 4 strains from pneumonia (6%). The simultaneous detection of all four effector proteins was confirmed in 10.4% of the strains.

*apr*A was present in 169 (73%) of the isolates and was more prevalent in isolates from patients with BSI (p=0.0007). *pil*B, which confers twitching motility, was confirmed in 7 (3%) of the isolates. *pil*A was found in 59 (25%) of the isolates. *pil*B was more prevalent in isolates from older patients (age >80 years, p=0.013). *phz*S was present in 86% (n=199) of the isolates. The two phenazine genes (*phz*M and *phz*S) were also detected more frequently in the isolates from BSI (p=0.001 and p=0.0004, respectively). *las*B occurred more frequently in the isolates originating from PNU (p=0.048) (Table 2). The gene for this factor, which is involved in pathogenesis via its degradation of human immunologically-competent molecules, was present in 79% (n=183) of the isolates.

The presence of *pil*B gene was associated with patient age (Table 3). Three of the genes were more prevalent in isolates from patients hospitalised in ICUs (i.e. *las*B (p=0.017), *apr*A (p=0.022), and *phz*S (p=0.039)). The frequency of the *exo*U and *exo*S genes was higher among inpatients and outpatients (p=0.018) (Figure 1).

The presence of a specific virulence gene was not correlated with MDR or XDR phenotype. The odds of having an MDR or XDR infection were also two times greater if the isolate was from a PNU compared with a UTI (OR (95%CI) = 2.105 (1.12; 3.94)). This association was not present in blood infections (OR (95%CI)= 0.55 (0.19; 1.59)).

No correlation was found between the presence of any of the type III secretion proteins and the presence of MBL phenotype nor was there any resistance found to imipenem or ciprofloxacin.

DISCUSSION

Non-fermentative, Gram-negative bacilli inhabit many reservoirs in hospitals and have developed resistance to many commonly used antibiotics. These characteristics have led to these bacteria becoming prevalent in hospital-acquired infections (HAI) during the past 50 years. The virulence and frequency (10–22% of all HAI, independent of patient age) of PA have been important factors associated with the increased use of antipseudomonal beta-lactam antibiotics and quinolones as presumptive therapies in cases in which PA is the suspected aetiology [11, 33].

Table 2. Distributions of virulence genes of *P. aeruginosa* strains with respect to site of infection

	Site of the infection							
	BSI (n = 11)		UTI (n = 152)		PNU (n = 69)		P value	Total (n = 232)
	n	%	n	%	n	%		n (%)
***pil*A**	2	18%	35	23%	22	32%	0.35	59 (25.4)
***pil*B**	0	0%	7	5%	0	0%	0.16	7 (3.0)
***las*B**	8	73%	114	75%	61	88%	<0.05	183 (78.9)
***exo*S**	11	100%	137	90%	57	83%	0.15	205(88.4)
***exo*T**	11	100%	146	96%	62	90%	0.19	219 (94.4)
***exo*U**	5	45%	38	25%	19	28%	0.32	62 (26.7)
***exo*Y**	11	100%	135	89%	60	87%	0.58	206 (88.4)
***apr*A**	10	91%	99	65%	60	87%	<0.01	169 (72.8)
***phz*M**	11	100%	150	99%	60	87%	<0.01	221 (95.2)
***phz*S**	11	100%	121	80%	67	97%	<0.01	199 (85.8)

BSI – strains from bloodstream infections; UTI - strains from urinary tract infections; PNU – strains from pneumonia; *pil*A - type IV pilus protein; *pil*B - type IV pili; *las*B – elastase; *exo*S, *exo*T, *exo*U, *exo*Y - the type III secretion system; *apr*A - alkaline protease; *phz*M, *phz*S – enzymes that catalyse the conversion of phenazine-1-carboxylic acid into pyocyanin.

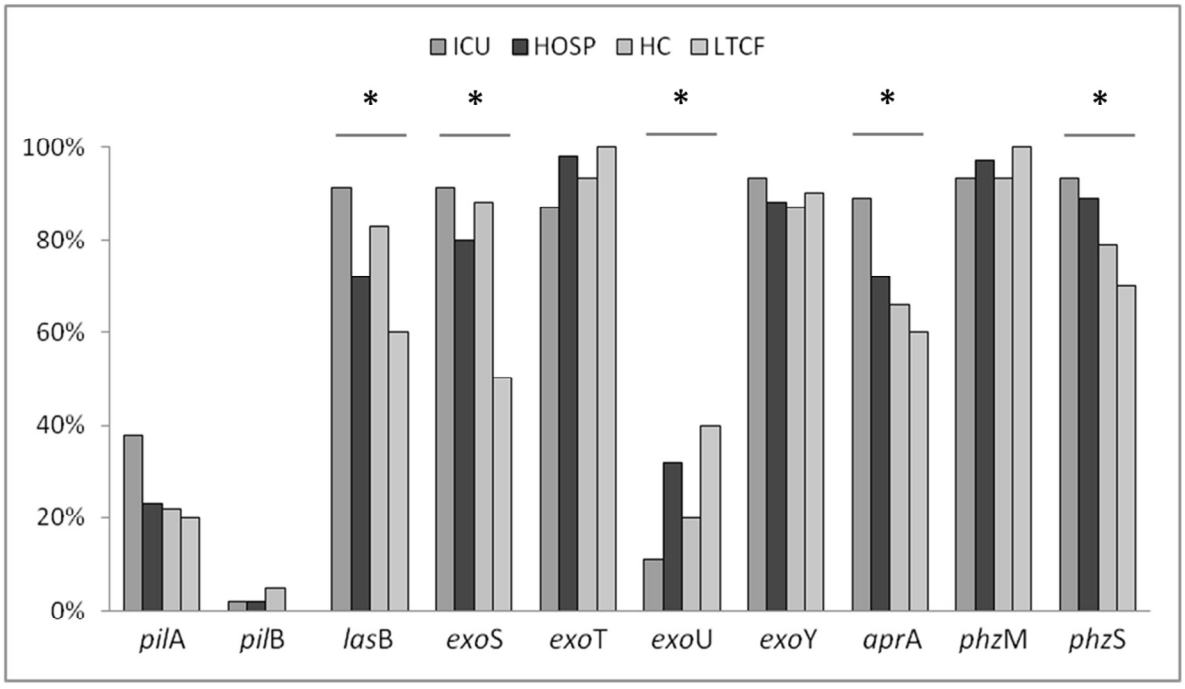

Fig. 1. Distribution of virulence genes, by type of patient care. (ICU: intensive care unit; HOSP: patient hospitalised, non-ICU; HC: home care; LTCF: long term care facility) * indicates a statistically significant result

Table 3. Distribution of virulence genes of *P. aeruginosa* strains, with respect to age group

	Age groups [years]								
	0-17 (n = 28)		18-60 (n = 70)		61-80 (n = 108)		>80 (n = 26)		P value
	n	%	n	%	n	%	N	%	
***pil*A**	6	21%	25	36%	20	19%	8	31%	0.11
***pil*B**	0	0%	1	1%	2	2%	4	15%	0.01
***las*B**	23	82%	60	86%	81	75%	19	73%	0.45
***exo*S**	23	82%	55	79%	91	84%	24	92%	0.46
***exo*T**	25	89%	66	94%	102	94%	26	100%	0.41
***exo*U**	5	18%	16	23%	30	28%	5	19%	0.67
***exo*Y**	27	96%	65	93%	92	85%	22	85%	0.21
***apr*A**	18	64%	54	77%	78	72%	19	73%	0.76
***phz*M**	28	100%	67	96%	100	93%	26	100%	0.31
***phz*S**	23	82%	59	84%	94	87%	23	88%	0.86

*pil*A - type IV pilus protein; *pil*B - type IV pili; *las*B – elastase; *exo*S, *exo*T, *exo*U, *exo*Y - the type III secretion system; *apr*A - alkaline protease; *phz*M, *phz*S – enzymes that catalyse the conversion of phenazine-1-carboxylic acid into pyocyanin.

The proportion of PA as a cause of nosocomial BSI has increased by nearly 30% in recent years. According to the WHO National Health and Nutrition Survey, the proportion of PA infections was approximately 3% between 1992 and 1997 in the U.S., and was 3.8% during 2009-2010. PA is one of the most important Gram-negative bacteria. It is the second most common etiological agent isolated from BSIs. The proportions of PA appear to be stable for UTI and decreased for pneumonia (21% in 1997 and 17% in 2010) nosocomial infections [27, 32]. The total prevalence of PA in ICU-acquired pneumonia cases was 16.6% in 2014. According to the European Centre for Disease Prevention and Control (ECDC) (Annual Epidemiological Report 2014 - Antimicrobial resistance and healthcare-associated infections; http://ecdc.europa.eu/), PA was the microorganism most frequently isolated from

HAIs in 2014. It was also frequently isolated from ICU-acquired BSIs (8.2%) and UTIs (14.1%) that occurred in hospitals in European countries.

The results of our study indicated that there were significant correlations between some virulence genes and sources of infection. More studies are needed to determine the specific roles of these genes in the different clinical infections caused by PA. The differences in the distributions of some genes suggested that some strains adapted more effectively to the specific conditions that were present during the infection of a specific site. However, the results of the statistical analysis also indicated that variations in the distribution of virulence genes with respect to origin were not significant. These results were similar to the results obtained by other investigators [13, 19, 29].

The correlation between high drug-resistance and highly virulent phenotypes is controversial [6]. There is an interaction between virulence and resistance within bacteria, and the associated biological costs depend on factors including the bacterial species involved, virulence and resistance mechanisms, the ecological niche, and host characteristics [3]. Our results indicated that the percentages of all virulence genes for the MDR or XDR phenotypes and the non-resistant strains were similar. These results supported the hypothesis that virulence and resistance are unrelated; however, our observation does not apply to the whole spectrum of virulence, only to the examined 10 virulence genes.

The PA strains isolated from blood cultures were more virulent compared with the strains isolated from other sites of infection. In our study, the high incidence of virulence genes among PA isolates from bloodstream infections may be due to the relatively small number of these isolates. Generally, bacteria might have been exposed to stressful conditions within the host's vascular system [35]. Enhanced virulence might be a viable defence strategy in this environment of activated immune cells, other host-protective mechanisms, and limited major nutrients (i.e. iron).

The frequency of the gene that confers twitching motility (i.e. *pil*B) was very low in all of the infection sites. This result suggested that non-pilus adhesins had an important role in these PA infections. This gene (*pil*B) was only detected in strains from UTIs and only from a small number of strains. Our results were not similar to the results of Mitov et al. who found that the prevalence of *pil*B is much higher in isolates from urine, blood, and the respiratory tract [22]. In our study, the *pil*B gene was more prevalent among the strains isolated from older patients. Older patients have a higher risk of development of UTI; strains of PA that are associated with UTIs tend to form biofilms and *pil*B-positive strains produce biofilms [4].

Kiewitz and Tummler's results suggested that the *pil*A gene is located in the chromosome regions of extended interclonal variability and has no high codon adaptation indices [16]. Therefore, the expression of this gene may be relatively low, additionally this gene has proved to be relatively rare: approximately one-fourth of the isolates in our study possessed the *pilA* gene.

Elastase, which is encoded by *las*B, is a secreted proteolytic enzyme with a wide range of substrates. The frequency of the *las*B gene was quite high (almost 80% of the isolates), and this gene was more frequent among the PNU strains. Other investigators have found that even higher percentages of PA strains are positive for this gene. For example, Mitov et al. found that up to 100.0% of PA strains are positive for the *las*B gene, independent of the site of isolation [22]. Our results indicated that patients undergoing hospitalization in ICUs had a higher risk of infection with *las*B gene strains, compared with outpatients or patients from regular hospital wards.

The prevalence of effector proteins was quite high for *exo*T, and *exo*Y, which were present in almost all of the strains that were isolated from the study population. ExoS was a bit less prevalent (found in 82.8% of the strains). Only the gene for ExoU, which is the most cytotoxic of the type III secretion proteins, was less prevalent - detected in about one-fourth of the isolates, but it may be worrisome. This frequency is higher than reported by other researchers [8, 24].

ExoU gene is associated with ST235 strains [38]. Studies performed in the United States revealed the presence of associations between type III secretory proteins and morbidity and mortality in PA-infected patients [28]. Secretion of ExoU is a marker for highly virulent strains obtained from patients with hospital-acquired pneumonia [31]. Detection of these genes or their products during an infection may be clinically meaningful, and knowledge of this feature of infections characteristic for specific geographic areas appears to be particularly important for patient prognosis. We found that the *exo*U gene was more frequent in the strains isolated from BSIs. It has been proven in 2015 by Pena et al. that *exo*U+ genotype is associated with increased risk of mortality of bloodstream infections caused by PA [24]. What is more, it has been proven that highly virulent strains containing the *exo*U gene of the type III secretion system are more likely to be FQ-resistant than strains containing the *exo*S gene [1].

All tested strains contained at least one gene for the effector protein and approximately 11% of the strains contained both the *exo*S and *exo*U genes. Published results for the frequency of this genetic pattern are inconsistent. Feltman found that simultaneous detection of *exo*S and *exo*U was rare [7]. Others have found that it is frequent [9, 22]. The ubiquity of these genes is consistent with their important roles in PA virulence. An increase in the prevalence of strains with these characteristics might present a potential clinical threat to the health of hospital populations. Our previous data suggest, that the presence of *exo*U gene is associated with ST235 strains [25]. But in the case of 232 strains, we were not able to perform MLST on such scale. We also found

no correlation between the presence of *exo*U and XDR phenotype, nor did we find any resistance to ciprofloxacin and imipenem, as other authors suggested [24, 34].

Alkaline protease is an important virulence factor, because it promotes the development of bacteria within the infected host and interferes with immune system [30]. The gene encoding this protease was prevalent in the strains isolated from patients with PNU or a BSI, but was less prevalent in the strains isolated from patients with a UTI. This result suggested that alkaline protease may not be significantly involved in the pathogenesis of UTI. The urinary tract may not provide the substrate required for expression of the *apr*A gene [30].

Most of the clinical isolates carried genes involved in phenazine production. The two phenazine genes (*phz*M, *phz*S) were detected more frequently among isolates from BSI samples, compared with isolates from UTI or PNU samples. None of the virulence factors genes were associated with patient age.

Rapid tests that detect virulence genes may help to resolve the increasing problem of the association between virulence and resistance. These tests may be very helpful for clinicians because they could be used to confirm that the virulence markers are present (i.e. in blood) before the bacteria can be isolated.

This study revealed that there was a high prevalence of different virulence genes among clinical strains of PA in southern Poland. These genes may be associated with severe infections. Strains isolated from different types of infections exhibited variant virulence gene patterns. The strains isolated from patients with BSIs were especially likely to have characteristics associated with high virulence. Understanding the contribution of selected virulence genes to the outcome of an infection may be important for therapeutic management of patients infected with PA. Future studies should address these mechanisms of virulence and the relationships between virulence and resistance.

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