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PREVALENCE OF VIRULENCE GENES AMONG BULGARIAN NOSOCOMIAL AND CYSTIC FIBROSIS ISOLATES OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

The aim of this study was to evaluate the prevalence of some virulence genes among 202 *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) patients (n=42) and non-CF in-patients (n=160) and to analyze the values according to the patient groups, infection localization and antimicrobial resistance. The following frequencies in all studied strains were established: *algD* (encoding GDP-mannose 6-dehydrogenase AlgD) – 91.1%, *pilB* (type IV fimbrial biogenesis protein PilB) – 23.8%, *nan1* (neuraminidase) – 21.3%, *lasB* (elastase LasB) – 100%, *plcH* (haemolytic phospholipase C precursor) – 91.6%, *exoS* (exoenzyme S) – 62.4%, and *exoU* (exoenzyme U) – 30.2%. The prevalence of *nan1* was significantly higher ($P<0.01$) in CF isolates (38.1%) than that in non-CF isolates (16.9%). The *nan1*–positive CF strains were cultured from 16 patients with recurrent lung exacerbations. This study revealed a statistically significant difference ($P<0.01$) between the portion of multidrug-resistant (MDR) nosocomial *P. aeruginosa* strains containing a large number (≥5) of virulence genes (38.1%) and the respective part of non-MDR isolates (17.6%). Moreover, *pilB*, *exoU* and *nan1* manifested a higher spread ($P<0.001$) among MDR than in non-MDR strains (respectively, 39.1% vs. 13.2%; 40.2% vs. 17.7% and 26.1% vs. 4.4%). In conclusion, the dissemination of *nan1* in CF isolates was moderate and correlated with the lower proportion of patients with lung exacerbations. The molecular-genetic detection of this gene may be used as an indirect measure of CF pulmonary disease evolution. Simultaneous determination of virulence factors and antimicrobial resistance is the contemporary approach for examination of the microbiological aspects of infections caused by *P. aeruginosa*.

Key words: *Pseudomonas aeruginosa*, virulence genes, polymerase chain reaction, cystic fibrosis isolates, non-cystic fibrosis isolates.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that is a common cause of hospital-acquired infections, particularly infecting patients with predisposing factors, such as burn victim, immunocompromised hosts, or those with metabolic disorders. In cystic fibrosis (CF) patients, *P. aeruginosa* is believed to be a major contributory factor to chronic lung infections, which could form biofilm and adhere to human mucin in the lower respiratory tract (23). The organism can

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only be eradicated in the early stage of colonization, while reduction of bacterial density is desirable during chronic colonization or exacerbations (3). It is not surprising that *P. aeruginosa* infections are associated with significant morbidity and mortality due to the organism's capacity to adapt easily to changes in the environment, to rapidly develop resistance to antibiotics, and to produce a variety of virulence factors.

P. aeruginosa possesses an "arsenal" of cell-associated and extracellular virulence factors. Many extracellular virulence factors have been shown to be controlled by a complex regulatory circuit involving cell-to-cell signaling (quorum sensing) systems that allow the bacteria to produce these factors in a coordinated, cell-density–dependent manner (22).

The aim of this study was to examine the prevalence of virulence genes (encoding adhesins, neuraminidase, invasins and type III effector proteins) in clinical isolates of *P. aeruginosa* and to analyze the values in respect to the patient groups, infection localization and antimicrobial resistance of the strains.

MATERIALS AND METHODS

Bacterial isolates and patients

A total of 202 clinical isolates of *P. aeruginosa* (including 160 nosocomial and 42 CF) was used in the present study. The nosocomial isolates were cultured during 2001-2007 from hospitalized non-CF patients (n=160) of different types of ward in six large hospitals in Sofia, Bulgaria. They were obtained from urine (n=62), tracheal aspirates (n=22), bronchial lavage (n=12), sputum (n=9), pleural fluid (n=2), surgical wounds or abscesses (n=26), blood (n=8), nose (n=8), throat (n=8), and bile (n=3). The CF *P. aeruginosa* isolates were recovered from sputa of 42 patients (25 females and 17 males) aged 6-27 years during the period 2005-2008. Bacterial identification was performed using a BBL Enteric/Nonfermenter ID system (Becton Dickinson).

CF exacerbation diagnostic criteria

An acute exacerbation was defined, as per the 1994 Cystic Fibrosis Foundation (CFF) Microbiology Consensus Conference (4), as the presence of at least 3 of 11 new clinical findings, including increased cough, increased sputum production, fever, weight loss, school or work absenteeism, increased work of breathing, decreased exercise tolerance, or a deterioration in the chest exam, chest radiograph, forced expiratory volume in 1 s (FEV_1), or hemoglobin saturation.

Definition of multidrug-resistant (MDR) isolates

Multidrug-resistant (MDR) *P. aeruginosa* isolates were defined as resistant to at least three of the following antibiotics: ceftazidime, imipenem, gentamicin and ciprofloxacin (14), using the conventional serial agar dilution method. The minimal inhibitory concentrations were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) 2007 guide-line (5).

Polymerase chain reaction (PCR) amplification of virulence genes

The prevalence of virulence genes, encoding GDP mannose 6-dehydrogenase (alginate) (*algD*), type IV fimbrial biogenesis protein PilB (*pilB*), neuraminidase (*nan1*), elastase LasB (*lasB*), haemolytic phospholipase C precursor (*plcH*), exoenzyme S (*exoS*) and exoenzyme U (*exoU*), was determined by PCR. The virulence genes were amplified with specific primers (Alpha DNA) listed in Table 1. DNA from the isolates was extracted by boiling. PCR was carried out with 2 µl template DNA, 0.25 µM of each primer, 0.2 mM deoxyribonucleoside triphosphates, 1x reaction buffer, 2 mM $MgCl_2$ and 1.5 U Prime *Taq* DNA polymerase (GeNet Bio) in a total volume of 25 µl. The DNA was amplified using the following protocol: initial denaturation (94 ºC for 5 min), followed by 25–30 cycles of denaturation (94 ºC for 35–45 s), annealing (53–62 ºC, from 45 s to 1 min) and extension (72 ºC, from 45 s to 1 min 35 s), with a single final extension of 7 min at 72 ºC. PCR products were separated in 1% agarose gel for 50–110 min at 130 V, stained with ethidium bromide (0.5µg/ml) and detected by UV transillumination (wavelength 312 nm). Amplified genes were identified on the basis of fragment size shown in Table 1.

Table 1. Primers used for amplification of virulence genes.

Primers	Target gene	Sequence (5' – 3')	Product size (bp)	Position on *P. aeruginosa* PAO1 chromosome (locus)	Annealing temperature (ºC)	Source
algD-F algD-R	*algD*	ATG CGA ATC AGC ATC TTT GGT CTA CCA GCA GAT GCC CTC GGC	1310	PA3540	62	(15)
pilB-F pilB-R	*pilB*	ATG AAC GAC AGC ATC CAA CT GGG TGT TGA CGC GAA AGT CGA T	826	PA4526	60	(20)
nan1-F nan1-R	*nan1*	ATG AAT ACT TAT TTT GAT AT CTA AAT CCA TGC TCT GAC CC	1317	PA2794	53	(20)
lasB-F lasB-R	*lasB*	GGA ATG AAC GAG GCG TTC TC GGT CCA GTA GTA GCG GTT GG	300	PA3724	60	(15)
plcH-F plcH-R	*plcH*	GAA GCC ATG GGC TAC TTC AA AGA GTG ACG AGG AGC GGT AG	307	PA0844	60	(15)
exoS-F exoS-R	*exoS*	CTT GAA GGG ACT CGA CAA GG TTC AGG TCC GCG TAG TGA AT	504	PA3841	60	(15)
exoU-F exoU-R	*exoU*	GGG AAT ACT TTC CGG GAA GTT CGA TCT CGC TGC TAA TGT GTT	428	PS14 within *P. aeruginosa* PA14 pathogenicity island PAPI-2	60	(20)

PCR, polymerase chain reaction; F, forward; R, reverse.
algD, GDP-mannose 6-dehydrogenase AlgD (alginate)-encoding gene; *pilB*, type IV fimbrial biogenesis protein PilB-encoding gene; *nan1*, neuraminidase-encoding gene; *plcH*, haemolytic phospholipase C precursor-encoding gene; *lasB*, elastase LasB-encoding gene; *exoS*, exoenzyme S-encoding gene; *exoU*, exoenzyme U-encoding gene.

Statistical analysis

The distribution of virulence genes with respect to isolate origin was compared using the chi-square test. A *P* value below 0.05 was considered to be statistically significant.

RESULTS

Prevalence of virulence genes among all studied *P. aeruginosa* isolates

The frequencies of occurrence of virulence genes in all studied strains (n=202) were as follows: *algD* – 91.1%, *pilB* – 23.8%, *nan1* – 21.3%, *lasB* – 100%, *plcH* – 91.6%, *exoS* – 62.4%, and *exoU* – 30.2%.

Prevalence of virulence genes among CF and non-CF *P. aeruginosa* isolates

The comparative frequencies of virulence genes in nosocomial non-CF isolates (n=160) and CF *P. aeruginosa* isolates (n=42) were as follows: *algD* – 92.5% vs. 85.7%, *pilB* – 26.9% vs. 9.5%, *nan1* – 16.9% vs. 38.1%, *lasB* – 100% for both type of isolates, *plcH* – 96.9% / 71.4%, *exoS* – 65.0% / 52.4%, and *exoU* – 30.6% / 28.6%.

(i) A statistically significant difference was not found between the dissemination of *algD*, *lasB*, *exoS* and *exoU* among the two groups of patients monitored.

(ii) The prevalence of *nan1* was significantly higher in CF isolates (38.1%) than that in non-CF isolates of *P. aeruginosa* (16.9%) – $P<0.01$. The *nan1*–positive CF isolates (n=16) were obtained from 16 patients with recurrent pulmonary exacerbations. Exacerbations were manifested by: weight loss of at least 1 kg; acute changes in respiratory signs and symptoms, such as increased cough frequency, sputum production and dyspnea, new auscultation findings (crackles, wheezing, rales or ronchi); inflammation changes according to the complete blood count (elevated peripheral leukocyte

count (more than 9.5 x 10^9 white blood cells/L) and erythrocyte sedimentation rate over 17 mm/h) and increased serum C-reactive protein levels (higher than 3.9 mg/L); new changes on X-ray, such as pneumonia and emphysema (found in 81% of the patients); and elevated levels of polynucler cells and metaplasia on sputum cytology (in 75% of them). In addition, the drop of forced vital capacity (FVC) and FEV_1 was drastic and hardly came back at baseline after the treatment. Unfortunately, in 2008 two children died after severe lung exacerbations.

(iii) The detection of *pilB* and *plcH* among CF isolates was significantly lower than that in non-CF nosocomial strains ($P<0.01$ and $P<0.001$, respectively).

Prevalence of virulence genes with respect to the infection localization in non-CF patients

The distribution of virulence genes encoding adhesins (alginate, type IV pili), neuraminidase, invasins (elastase LasB, haemolytic phospholipase C) and type III effector proteins (ExoS, ExoU) varied in respect to the infection localization in non-CF patients (Table 2).

(i) The highest spread of *pilB* was established among *P. aeruginosa* isolates from blood (75.0%) and urines (35.5%), which was significantly higher than that in isolates from in-patients with upper respiratory tract infections (URTIs) (12.5%) and lower respiratory tract infections (LRTIs) (11.1%) – $P<0.001$ and $P<0.05$, respectively.

(ii) The *nan1* gene revealed a widespread dissemination in *P. aeruginosa* isolates from blood (62.5%), which was significantly higher than that among the isolates from urines (19.4%) – $P<0.02$; wounds (15.4%) – $P<0.02$; URTIs (12.5%) – $P<0.01$; and LRTIs (6.7%) – $P<0.01$.

(iii) The presence of *exoS* was the highest among *P. aeruginosa* isolates from blood (87.5%) and significantly different only with those obtained from in-patients with LRTIs (55.6%) – $P<0.05$.

(iv) The *exoU* frequencies were the most expressive in the wound (46.2%) and LRTIs *P. aeruginosa* isolates (40.0%). They were significantly higher than those of the isolates from urines (17.7%) – $P<0.01$ and $P<0.02$, respectively; and blood (12.5%) – $P<0.05$.

Prevalence of virulence genes among MDR and non-MDR *P. aeruginosa* isolates from non-CF patients

Of the 160 nosocomial non-CF *P. aeruginosa* isolates, 92 were MDR. Three of the genes (*pilB*, *nan1* and *exoU*) manifested a significantly higher spread ($P<0.001$) among MDR than in non-MDR strains of *P. aeruginosa* (respectively, 39.1% vs. 13.2%; 26.1% vs. 4.4% and 40.2% vs. 17.7%) – Figure 1.

The proportion of MDR nosocomial *P. aeruginosa* isolates containing a large number of virulence genes (≥5) (38.1%) was higher than the proportion of non-MDR isolates with a large number of virulence genes (17.6%) – $P<0.01$ (Table 3).

Table 2. Prevalence (as percentage) of virulence genes in non-cystic fibrosis *P. aeruginosa* isolates (n=160).

Gene	Non-cystic fibrosis isolates					
	Urine (n=62)	LRTIs (n=45)	URTIs (n=16)	Wounds (n=26)	Blood (n=8)	Total[a] (n=160)
algD	93.5	95.6	93.8	84.6	100.0	92.5
pilB	35.5	11.1	12.5	26.9	75.0	26.9
nan1	19.4	6.7	12.5	15.4	62.5	16.9
lasB	100.0	100.0	100.0	100.0	100.0	100.0
plcH	96.8	97.8	100.0	96.2	100.0	96.9
exoS	66.1	55.6	75.0	65.4	87.5	65.0
exoU	17.7	40.0	37.5	46.2	12.5	30.6

[a] Frequency of the virulence gene in all non-cystic fibrosis isolates of *P. aeruginosa*, including three bile isolates.
LRTIs, lower respiratory tract infections; URTIs, upper respiratory tract infections; *algD*, GDP-mannose 6-dehydrogenase AlgD (alginate)-encoding gene; *pilB*, type IV fimbrial biogenesis protein PilB-encoding gene; *nan1*, neuraminidase-encoding gene; *plcH*, haemolytic phospholipase C precursor-encoding gene; *lasB*, elastase LasB-encoding gene; *exoS*, exoenzyme S-encoding gene; *exoU*, exoenzyme U-encoding gene.

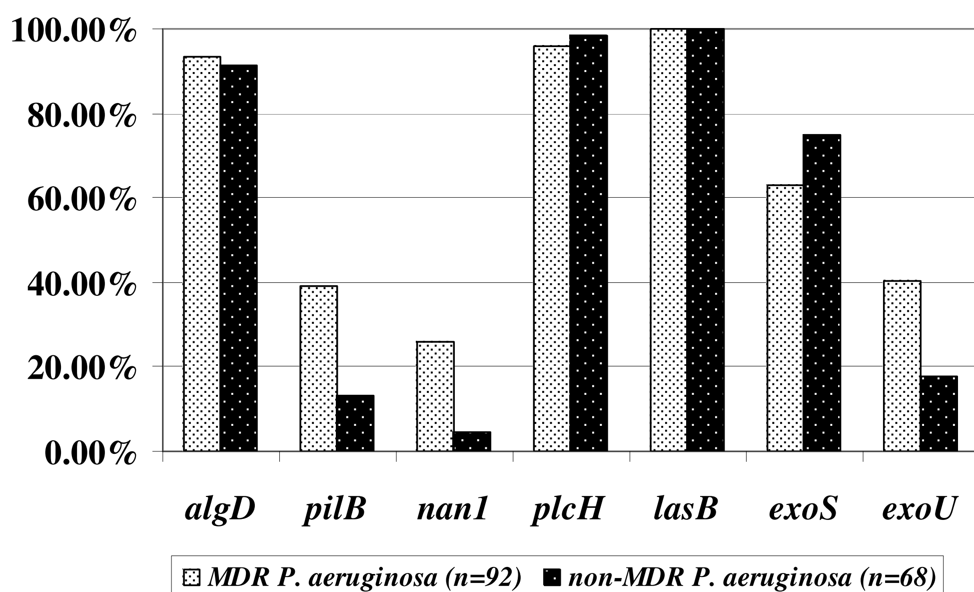

Figure 1. Prevalence (as percentages) of virulence genes among MDR and non-MDR *P. aeruginosa* isolates from non-cystic fibrosis patients. MDR, multidrug-resistant; *algD*, GDP-mannose 6-dehydrogenase AlgD (alginate)-encoding gene; *pilB*, type IV fimbrial biogenesis protein PilB-encoding gene; *nan1*, neuraminidase-encoding gene; *plcH*, haemolytic phospholipase C precursor-encoding gene; *lasB*, elastase LasB-encoding gene; *exoS*, exoenzyme S-encoding gene; *exoU*, exoenzyme U-encoding gene.

Table 3. Presence of virulence genes among MDR and non-MDR nosocomial *P. aeruginosa* isolates.

Number of virulence genes	Number (%) of MDR isolates (n=92)	Number (%) of non-MDR isolates (n=68)
1	1 (1.1)	1 (1.5)
2	1 (1.1)	0 (0.0)
3	13 (14.1)	7 (10.3)
4	42 (45.7)	48 (70.6)
5	16 (17.4)	10 (14.7)
6	17 (18.5)	2 (2.9)
7	2 (2.2)	0 (0.0)

MDR, multidrug-resistant isolate.

DISCUSSION

The established prevalence of some of the genes (*algD*, *lasB* and *plcH*) among all studied *P. aeruginosa* isolates (n=202) was similar to their widespread dissemination (100%) in French *P. aeruginosa* isolates (n=162) obtained from sputa of CF patients, clinical samples from patients without inherited diseases and plants (15). In our study the frequencies of virulence genes encoding type III secretion proteins – ExoS (62.4%) and ExoU (30.3%), were similar to those ascertained by Feltman *et al.* (8) – respectively, 72% and 28% among CF and non-CF *P. aeruginosa* isolated in USA during 1999-2000. The part of our *P. aeruginosa* strains containing either the *exoS* (51%) or the *exoU* gene (18.8%) was higher than that of isolates having both genes (*exoS*+*exoU*) – 11.4%. Previously, Feltman *et al.* (8) reported that of 115 *P. aeruginosa* clinical

and environmental isolates, 82 contained the *exoS* but not the *exoU* gene, 31 contained the *exoU* but not the *exoS* gene and a single contained both genes. However, recent studies established that the simultaneous *exoS*+*exoU* detection was a frequent finding in clinical and environmental *P. aeruginosa* isolates. Finnan *et al.* (9) found 75% dissemination of both genes.

It was not surprising that the detection of *nan1* among the investigated CF *P. aeruginosa* was significantly higher than that in non-CF isolates. Three decades ago, neuraminidase production by a CF *P. aeruginosa* isolate was described and suggested to contribute to lung infection (16). The spread of *nan1* in CF *P. aeruginosa* isolates increases when the clinical state of patients worsens, suggesting the possible role of neuraminidase in CF pulmonary disease evolution (15, 21). The prevalence of *nan1* among our CF isolates was significantly lower ($P<0.02$) than that in French CF *P. aeruginosa* isolates examined by Lanotte *et al.* (15) – 38.1% / 61.7 %, which was related to their predominant stable clinical status. On auscultation they had a clear vesicular murmur (with bronchial character in the places where bronchiectasis was formed) without any rales or ronchi. All data from complete blood count were within normal limits or on the upper limit. There were no additional new changes on X-ray findings. The spirometry showed steady levels of forced vital capacity and FEV_1, slightly decreasing with the age. The sputum cytology manifested steady levels of polynuclear cells and the rate of metaplasia was not increasing significantly with check-ups.

In the present study, *exoS* gene was found more rarely in CF isolates than in the other clinical isolates (52.4% vs. 65.0%), which contrasted with the reported higher secretion of ExoS by *P. aeruginosa* strains from sputa of CF individuals (6, 8, 15). According to the data published by Lanotte *et al.* (15), the prevalence of *exoS* among CF *P. aeruginosa* isolates was significantly higher than that in non-CF isolates (93.8% vs. 75.3%; P=0.002). Feltman *et al.* (8) reported 85% distribution of *exoS* among isolates from sputa of CF patients and a variable frequency of the gene in the clinical strains from samples of patients without inherited diseases (from 60% to 75%).

The frequency of *pilB* among the studied CF *P. aeruginosa* was low (9.5%) which suggested the role of non-pilus adhesins (such as alginate) in this group of isolates. Recently, it was ascertained an absolute absence of *pilB* among CF *P. aeruginosa* strains from Ireland (9).

As it was described above, the distribution of virulence genes varied in respect to the site of infection among non-CF in-patients. The *nan1*-detection (from 6.7% (LRTIs) to 62.5% (blood)) was lower than that in similar French clinical strains of *P. aeruginosa* (40.7% – urines, 48% – LRTIs, 41.2% – wounds) (15).

In this study, the prevalence of *exoS* among LRTIs isolates (55.6%) was lower than that in LRTIs *P. aeruginosa* isolates from the studies mentioned above (Lanotte *et al.* – 80%, and Feltman *et al.* – 75%) (8, 15). ExoS is responsible for direct tissue destruction in lung infection and may be important for bacterial dissemination (22).

exoU was found predominantly in *P. aeruginosa* isolates from LRTIs and wounds. Its prevalence in our wound isolates (46.2%) was nearly identical with that found by Feltman *et al.* in USA (40.0%) (8). It is known that 90% of ExoU-producing *P. aeruginosa* strains are associated with severe infections (11). Of the type III secretion proteins, ExoU is the most cytotoxic. Secretion of ExoU is a marker for highly virulent *P. aeruginosa* isolates obtained from patients with hospital-acquired pneumonia (18).

Our work revealed a significant difference between the portion of MDR nosocomial *P. aeruginosa* strains containing a large number (≥5) of virulence genes and the respective part of non-MDR isolates. Numerous reports have documented that the rise in MDR nosocomial *P. aeruginosa* continues to threaten hospitalized patients despite various countermeasures including isolation techniques and antibiotic de-escalation therapy (2, 13). Whether MDR *P. aeruginosa* strains necessarily express a more virulent phenotype continues to remain a controversial issue (7). Recently, Zaborina *et al.* screened consecutively isolated MDR *P. aeruginosa* clinical strains for their ability to disrupt the integrity of human

cultured intestinal epithelial cells (Caco-2) and correlated these finding to related virulence phenotypes such as adhesiveness, motility, biofilm formation and cytotoxicity (24). These strains were characterized and found to harbor the *exoU* gene and to display high swimming motility and adhesiveness.

It is known that bacteria growing in biofilms are more resistant to antimicrobial agents than their planktonic counterparts are (12). Various hypotheses have been put forward to explain biofilm resistance (poor antibiotic penetration, nutrient limitation and slow growth, high cell density, adaptive stress responses etc.), but to date, there are no data that entirely explain this phenomenon (19). Due to the success of a genetic screen that Mah *et al.* carried out, some additional mechanisms are being proposed. The locus identified in their screen, ndvB, is required for the synthesis of periplasmic glucans. These glucose polymers may prevent antibiotics from reaching their sites of action by sequestering these antimicrobial agents in the periplasm (17).

We presented a significantly higher spread of some virulence genes (*exoU*, *pilB* and *nan1*) among MDR nosocomial *P. aeruginosa* isolates. In a recent accomplished study, Garey *et al.* determined the prevalence of *exoU* and *exoS* from bloodstream isolates of hospitalized patients with *P. aeruginosa* bacteremia and ascertained that the isolates containing the *exoU* gene were significantly more resistant ($P<0.05$) to different classes of antimicrobials: β-lactams (piperacillin/tazobactam, ceftazidime, cefepime, carbapenems), fluoroquinolones, and aminoglycosides (gentamicin) (10).

In conclusion, the present study established a higher frequency of spread of *nan1* among CF *P. aeruginosa* isolates compared with that in non-CF isolates. This prevalence was moderate and correlated with the lower proportion of patients with lung exacerbations. The molecular-genetic detection of the *nan1* gene may be used as an indirect measure of CF pulmonary disease evolution. The distribution of virulence genes varied in respect to the site of infections among non-CF hospitalized patients. For the first time, it was found that the frequencies of *pilB*, *exoU* and *nan1* were significantly higher in MDR strains than those in non-MDR *P. aeruginosa* strains. Moreover, the part of MDR isolates containing a large number of virulence factors (≥5) was bigger than the respective partition of non-MDR *P. aeruginosa*. The exact reasons for the frequent presence of virulence-related genes in MDR isolates are still unknown therefore a further work is necessary. Simultaneous determination of virulence factors and antimicrobial resistance is a contemporary approach for examination of the microbiological aspects of infections caused by *P. aeruginosa*.

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