MED. DOŚW. MIKROBIOL., 2018, 70: 57 - 65

Charakterystyka klinicznych izolatów *Pseudomonas aeruginosa* pod kątem tworzenia biofilmu, lekooporności oraz występowania genów dla toksyn wydzielanych w systemie sekrecji typu 3.

Characteristic of clinical isolates of *Pseudomonas aeruginosa* regarding biofilm formation, drug resistance and genes for toxins secreted by the type 3 secretion system

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Celem badania była analiza zdolności tworzenia biofilmu w kontekście lekooporności i wirulencji szczepów *P.aeruginosa* izolowanych z układu moczowego, krwi i zakażeń dolnych dróg oddechowych od pacjentów ambulatoryjnych oraz hospitalizowanych w szpitalach z południowej Polski. Tworzenie biofilmu przez poszczególne izolaty badano z wykorzystaniem metody z fioletem krystalicznym. Obecność genów kodujących toksyny wydzielane w systemie sekrecji typu 3 badano metodą łańcuchowej polimerazy (PCR). Lekooporność testowano zgodnie z wytycznymi EUCAST. Wśród 219 izolatów *P.aeruginosa* 46 zostało scharakteryzowanych jako szczepy wielolekooporne (MDR), a 26 jako szczepy o rozszerzonej oporności (XDR). Liczba szczepów produkujących biofilm odpowiedzialnych za zakażenia występujące u pacjentów hospitalizowanych (szczególnie na oddziałach intensywnej terapii) była dwukrotnie wyższa niż szczepów nietworzących biofilmu (odds ratio 0.5, 95%CI 0.31–0.97). Nie wykazano statystycznie znaczących różnic pomiędzy częstością występowania szczepów silniej tworzących biofilm, a poziomem ich lekooporności.

Słowa kluczowe: *Pseudomonas aeruginosa*, system wydzielania typu 3, lekooporność, biofilm, Polska

ABSTRACT

Introduction: The aim of this study was to analyze biofilm production in the context of antimicrobial resistance and virulence in *P. aeruginosa* (PA) isolates from hospitalized and non-hospitalized patients with urinary tract infections, bloodstream infections, or pneumonia in southern Poland.

Methods: We screened biofilm formation in the isolates using crystal violet staining. Strains were tested for antimicrobial susceptibility by disc diffusion method according to EUCAST guidelines. The presence of genes encoding the toxins secreted by the type III secretion system (T3SS) were detected by PCR method.

Results: Altogether, 216 isolates were investigated, 109 of which came from hospitalized patients. The number of biofilm-producing strains responsible for infections in the hospitalized patients was twice as high as the non-biofilm-producers (odds ratio 0.5, 95% CI 0.31–0.97). In the samples from intensive care units (ICUs), the biofilm-producing strains caused more infections than those lacking biofilm production (ICU vs. others OR 0.4 95% CI 0.19–0.78). Forty-six isolates contained multidrug resistant PA and 26 contained extensively-drug resistant PA. No statistically significant differences were observed in the frequency of strains resistant to particular antimicrobials or bearing T3SS proteins between the male and female patients, as well as between the various biofilm producer categories.

Conclusions: Most of the PA strains were able to form biofilms on abiotic surfaces, there was no difference in the frequency of biofilm producers among the strains that exhibited different levels of antimicrobial resistance.

Keywords: *Pseudomonas aeruginosa*, type 3 secretion system, drug resistance, biofilm, Poland

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Pseudomonas aeruginosa (PA) is one of the most important Gram-negative bacteria and one of the major pathogens responsible for a wide variety of severe nosocomial and community-acquired infections. According to the European Centre for Disease Prevention and Control (2), PA was one of the microorganisms most frequently isolated from hospital-acquired infections (HAIs) in 2014, especially from intensive care unit (ICU)-acquired infections, with bloodstream infections (BSIs) and urinary tract infections (UTIs) accounting for 8.2% and 14.1%, respectively. In adult patients in Poland,the prevalence of PA pneumonia (PNU) was 8.5%, while the prevalence of BSIs and UTIs was 0.7%, and 5.0%, respectively (4, 18). In children, the prevalence of PA in urinary tract infections was low at 2.1%, while in children <5 years of age it was 3.0% (17).

In modern medicine, acute infection such as BSIs, UTIs and PNU are often associated with medical devices such as tubes or catheters. The bacteria on these abiotic surfaces can often readily adaptive to a variety of niches, and some, like PA are capable of biofilm formation.

PA produces a large number of virulence factors and many of these factors are regulated by quorum sensing (21). An important feature of PA virulence is its type III secretion system (T3SS) (12). T3SS operation has been likened to a molecular syringe that injects bacterial effector proteins into host cells. PA has five effector proteins, namely ExoS, ExoT, ExoU, ExoY and FliC, and these protect the bacterium against phagocytosis, and also affect tissue invasion and systemic spread as well as modulating the inflammatory response. T3SS gene

expression is controlled by intrinsic and extrinsic regulatory mechanisms (6, 14). Global extrinsic regulation of T3SS gene expression is made via the Vfr (Virulence factor regulator) cAMP-dependent regulator. The role of T3SS in PA is well-established within the context of the acute infection, sepsis, and high mortality caused by this bacterium (10, 20). The change in growth conditions that occur during bacterial transition from an acute to chronic infection state correlates with alternations in PA behavior including increased antibiotic tolerance and the biofilm mode of growth switching (15). Biofilm formation is subject to a regulatory system involving the RetS and LadS sensor kinases. Both kinases have opposite effects on GacA system activation, a process that regulates biofilm formation through the control of polysaccharides as the key components of the biofilm matrix. Inverse regulation by RetS and LadS implies that T3SS gene expression and biofilm formation are considered as opposite events. However, it is now thought that such a model is an over-simplification and expression of T3SS proteins is possible during biofilm growth (6, 15). It has also been shown that during infections where biofilm is formed, antimicrobial resistance in the bacterium is an important feature, and such infections are persistent and difficult to eradicate (7).

The aim of this laboratory-based study was to analyze biofilm production in the context of antimicrobial resistance and the profile of virulence genesof *P. aeruginosa* strains isolated from different sites of infection in hospitalized and non-hospitalized patients in southern Poland.

MATERIALS AND METHODS

Ethics. This work was approved by the Bioethics Committee of Jagiellonian University Medical College (No. KBET/312/B/2012 and KBET/362/B/2012). All data were anonymized prior to conducting the analysis.

Samples. Non-repetitive samples from UTIs, BSIs, and patients with PNU were collected from hospitalized patients (12 hospitals), long-term care facility residents, and non-hospitalized patients (outpatients, diagnosed with infections, but treated in ambulatory care, not in hospital) in southern Poland. Samples were collected in collaboration with two microbiological laboratories: KORLAB NZOZ, which is located in Ruda Slaska, Poland, and the microbiological laboratory at St. Barbara's Regional Hospital in Sosnowiec, Poland, between 1 January 2013 and 31 December 2013. Relevant patient information such as age, gender, and whether a hospital, ICU, or home care was the place of treatment was collected. Patients with PA infections (inclusion criteria) were defined according to the diagnosis of the physicians.

DNA extraction and polymerase chain reactions (PCRs). DNA templates were extracted from liquid cultures of tryptic soy broth (BioCORP, Poland) grown at 37°C for 18 h using a Genomic Mini kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instructions. Isolates were screened for the presence of the major effector proteins genes of the T3SS (*exo*S, *exo*T, *exo*U, *exo*Y). PCR analysis was performed using primers based on the same sequences and reaction conditions described in a previously published method (1). PCR Master Mix (2X) (A&A Biotechnology, Poland) was used for the PCRs. PCR products, separated on 1.5% agarose gels for 60 min at 90V, were stained with ethidium bromide (Sigma-Aldrich, Munich, Germany) and detected using ultraviolet trans-illumination.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing of the strains was performed as described previously (18), with Mueller-Hinton agar plates, and following the current guidelines of the European Committee on Antimicrobial Susceptibility Testing for the disk diffusion method (clinical breakpoint table v.3.1; http://www.eucast.org/clinical_breakpoints/, accessed 5.06.2013). All disks were obtained from Oxoid (Basingstoke, UK). Multidrug-resistant (MDR) strains were defined as strains not susceptible to one antimicrobial in at least three different antimicrobial classes, and extensively drug resistant (XDR) strains were defined as strains susceptible to no more than two antimicrobial classes (13).

To detect metallo-β-lactamases (MBL, strains resistant/intermediate to imipenem/meropenem and resistant to ticarcillin/clavulanate), the disc method where ceftazidime (30 μg) and imipenem (10 μg) were placed 20 mm apart from the disc area with EDTA (10 μl 0.5M EDTA, pH 7.3–7.5) was used. EDTA is an inhibitor of MBLs, and the test is considered to be positive if the inhibition zone around the disc with ceftazidime and/or imipenem is enhanced (11).

Biofilm screening. Biofilm formation in the isolates was screened using the method of O'Toole with crystal violet staining (16). The strains were grown in M63 minimal medium containing 13.6 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 0.5 mg/L $FeSO_4 \cdot 7H_2O$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, supplemented with 0.2% glucose and 0.5% casein (Oxoid, Basingstoke, UK), and the optical density (OD) of each strain was measured at 630 nm. Biofilm assays were performed in triplicate. Uninoculated M63 medium (Amresco, USA) with casein (Oxoid) was used as a negative control. The OD cut-off (ODc) was defined as the mean OD value of the negative control. Based on the OD value, the strains were classified as follows: not biofilm producers (OD<ODc), weak biofilm producers (ODC<OD< 2xODc), moderate biofilm producers (2xODc<OD<4xODc) or strong biofilm producers (4xODc<OD), according to the description of Borges et al. (3).

Statistics. Statistical analyses were performed using Statsoft Statistical software (version 10, StatSoft Inc., Tulsa, OK, USA). The distributions of the continuous variables were tested for normality using the Shapiro-Wilk test. Because the values of the variables tested did not follow a normal distribution, the data, which we summarised as median, 25th (Q1), and 75th (Q3) percentile values, were tested using the nonparametric Mann-Whitney U test. For dichotomous variables, a Chi-square test was used for expected frequencies of ≤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 of ≤ 5. A *P*-value of < 0.05 was considered to indicate a statistically significant result.

RESULTS

Altogether, 216 strains were studied and 109 of them were isolated from hospitalized patients. Sixty-nine percent of the strains came from males and the median patient age was 64 (Quartile 1: 47; Quartile 3: 72), although 26 specimens came from under 17 year-olds. 148 of the isolates came from UTIs, 7 from BSIs and 61 from people with PNU. ICUs were responsible for 39 diagnosed infection cases (18.1% of all the PA infections that were studied), including 5 cases of BSI (71.4% of all BSIs), 25 cases of PNU (41.0%) and 9 UTI cases (6.1%).

Twenty six isolates were considered to be extensively drug-resistant, while 46 were multidrug resistant. All the strains isolated from BSIs (n=7) were MDR (n=1) or XDR

(n=6) strains. Strains isolated from UTI cases were 50% less drug-resistant (MDR and XDR vs. others) than the strains isolated from other cases (OR 0.4; 95%CI 0.23-0.76). Drug-resistant strains (XDR and MDR) were not common among the ICU and non-ICU hospitalized patients, or among patients with PNU. In the non-hospitalized patients, the number of drug-resistant strains (MDR and XDR) was almost two times lower than in the hospitalized patients (ICU and non-ICU together) (OR 0.5; 95% CI 0.28-1.01). Twenty-one strains were able to produce MBLs.

Among the isolates, 67% were classified as moderate or strong biofilm producers. The number of biofilm-producing strains responsible for infections in the hospitalized patients was two times higher than the non-biofilm-producers (OR 0.5, 95% CI 0.31-0.97). In the samples from ICUs, the biofilm-producing strains caused half of the infections of the biofilm-producing strains from non-ICU patients and from non-hospitalized patients together (ICU vs. others OR 0.4 95% CI0.19-0.78) (Table 1).

Table I. Characteristics of the *Pseudomonas aeruginosa* isolates. Strains were classified as follows: no biofilm production (OD<ODc), weak production (ODc<OD< 2xODc), moderate production (2xODc<OD<4xODc) or strong biofilm production (4xODc<OD), according to Borges et al (8).

	Biofilm formation level				
	Moderate or strong*, N=145	Weaker or absent*, N=71	OR	95%CI	p-value
Patient age, median; 1Q, 3Q (year)	63; 51-72	64; 43-75	n/a		0,7
Patient gender, male (n; %)	93 (64.1%)	55 (77.5%)	0.5	0.27-0.99	0.048
Hospitalized patients(n; %)	66 (45.5%)	43 (60.6%)	0.5	0.31-0.97	0.017
ICU patients(n; %)	19 (13.1%)	20 (28.2%)	0.4	0.19-0.78	0.017
Type of infection (n; %)					
Pneumonia	42(29.0%)	19(26.8%)	1.1	0.59-2.11	0.8
Bloodstream infections	4(2.8%)	3 (4.2%)	0.6	0.14 -2.95	
Urinary tract infections	99(68.3%)	49 (69%)	1.0	0.52-1.78	
MBL(n; %)	11(7.6%)	10 (14.1%)	0.5	0.21-1.24	0.2
XDR or MDR (n; %)	44 (30.3%)	28 (39.4%)	0.7	0.37-1.21	0.2
Colistin resistance, median; 1Q, 3Q (MIC)	0.5; 0.4-1	0.5; 0.4-1	n/a		0.8
Virulence factor (n; %)					
*exo*S	123 (84.8%)	64 (90.1%)	0.6	0.25-1.51	0.4
*exo*T	138 (95.2%)	66(93.0%)	1.5	0.46-4.88	0.5
*exo*U	38(26.2%)	15(21.1%)	1.3	0.67-2.62	0.4
*exo*Y	128(88.3%)	65(91.5%)	0.7	0.26-1.85	0.6

1Q, 3Q 25th (Q1) and 75th (Q3) percentiles; CI, confidence interval; ICU, intensive care unit; MBL, metallo beta-lactamase resistance; MDR, multidrug resistance; MIC, minimal inhibitory concentration; OD, optical density;ODc, optical density cut-off; OR, odds ratio; XDR, extensively-drug resistant; (*exo*S, *exo*T, *exo*U, *exo*Y). *Moderate or strong biofilm producers and weaker and absent biofilm producers are shown together as separate categories.

Among genes encoding T3SS proteins, the most widespread was *exo*T, which was present in 204 strains, while *exo*Y (n=193) and *exo*S (n=187) were slightly less prevalent. *exo*U was found in 53 strains (including 26.2 %; n=16 from PNU infections). Furthermore, at least one of these virulence genes was found in 99% of the samples, with 188 isolates bearing three or more virulence factors. There were significantly more moderate to strong biofilm producers (77% vs 64%, p=0.048) in the strains from the female patients, with lower numbers of female patients in the ICU (7% vs 23%, p=0.018). Additionally, strains from the female patients were significantly more frequently XDR or MDR types (76% vs 62%, p=0.038); these differences may be related to the higher median age of the female group (female 64.5, range 47–76; vs. male 63, range 49–72). Also, we did not observe any statistically significant differences in the frequency of strains resistant to particular antimicrobials or bearing T3SS proteins between males and females, or between the biofilm producer categories (Table 1). No relationship was found between the ability to form biofilm and the presence of virulence genes, or between non-hospitalized vs. hospitalized patients.

DISCUSSION

The subject of our study was PA, a bacterium consistently associated with nosocomial infections. The number of BSIs caused by PA is growing, and in 2009–2010 around 3.8% of BSIs were caused by this bacterium compared with only 3% between 1992 and 1997. The number of cases of PNU caused by PA was 17% in 2010, a figure lower than in 1997 (21%) (19). PA had a prevalence of 13% in device-associated infections (DAIs) in UTIs in Poland (9). In contrast, the prevalence of PA-associated BSIs in very low birth weight newborns was only 1.3%, but this condition was associated with an extremely high fatality rate of 80% (25). A major problem faced by modern medicine is the need for medical devices to be used for patient therapy, especially in ICUs, as these devices are responsible for large numbers of hospital-acquired infections, particularly in critically ill patients. In this population, a significant number of UTIs and BSIs are associated with DAIs (19). In Poland, the number of such infections can be larger than in other countries. Indeed, the incidence of catheter-associated UTIs in adult patients was 7.2/1000 catheter-days in 2014 (9), a figure higher than that in the NHSN/CDC (2012) report (8). Furthermore, the device utilization ratio in ICUs in Poland is high; therefore the risk of DAIs is correspondingly high (9, 24, 25). However, our laboratory-based study has no data on the use of medical devices; unfortunately, device-related infections result from the multifaceted interaction of PA, the device, and patient, but bacterial factors probably play the most important role in the pathogenesis of DAIs. Also, it has been reported that different bacteria use different mechanisms to colonize medical devices (23).

How severe an infection with PA becomes is multifactorial, but depends mainly on the presence of the following two virulence determinant types in this bacterium: 1) virulence factors involved in acute infections (usually secreted) and membrane bound factors and, 2) virulence factors involved in chronic infections (i.e., siderophores, alginate biosynthesis) (6). Biofilm production is also a very important virulence trait. A bacterium's ability to form a biofilm is linked to multiple factors including its ability to move and its possession of specific proteins. Whether biofilm-producing strains have the ability to produce effector proteins, such as Exo is an interesting question.

For our strains, we saw no difference in the presence of genes encoding effector proteins between the biofilm-producers and the non-producers. The prevalence of effector proteins was high for *exo*T, and *exo*Y, which were present in almost all of the strains. *Exo*S was somewhat less prevalent (found in 82.8% of the strains). Only the *Exo*U gene, which encodes the most cytotoxic of the type III secretion proteins, was less prevalent, being detected in about one-quarter of the isolates from patients with PNU, but this level is still worrisome. According to other studies, ExoU secretion is a marker for highly virulent strains in patients with hospital-acquired PNU (5, 22). Strains with the ability to produce biofilms and possessing the *exo*U gene are considered to be highly virulent and may cause therapeutic problems. However, it should be emphasized that only the absence of a gene provides clear evidence that the gene is not functional, as the presence of a gene does not necessarily mean that the gene is expressed. In our research, the genetic potential of PA strains affecting their virulence was determined. Certainly, the lack of experiments on the expression of genes was a limitation in this article. For that reason,further studies are necessary to show that the four *exo* genes are functional. Similarly, the study of the ability to biofilm formation created in vitro allowed us to assess the possibility of a strain and not its traits actually presented during the infection

As was expected in this study, most of the PAstrains tested were able to form biofilms on abiotic surfaces (96-well plates), especially the strains isolated from ICU infection cases where the number of biofilm producers was 4-times higher than that of the non-hospitalized patients; however, surprisingly, there was no difference in the frequency of biofilm producers among strains that exhibited different levels of antimicrobial resistance. Furthermore, while most of the strains possessed at least one T3SS gene, no association was observed between the strains carrying T3SS genes and biofilm production. It was also not expected that the strains isolated from ICUs were characterized by a reduced ability for biofilm formation, compared with the strains obtained from the other sources.

Our previous data indicate serious potential therapeutic problems related to high antibiotic resistance in non-fermentative bacilli in Polish hospitals. The growing prevalence of MDR and XDR bacteria is challenging for clinicians as the treatment options are limited for patients with such infections (4). Antibiotic resistance of biofilm is related to decreased growth rate and decreased penetration of drug through the biofilm matrix, rather than having a particular antibiotic resistance genes of biofilms producers. Therefore, it will be interesting to consider the relationship between drug resistance and the ability to form biofilms in a comprehensive and thorough future study.

Conflict of interest

The authors declare no competing financial interests.

Funding

This work was supported by two grants from the National Science Centre (No.UMO-2012/05/B/NZ7/02880 and No 2012/05/N/NZ7/00786). The sponsor provided the funding for the project only.

The authors contributed equally to this work.

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Otrzymano: 18 XII 2017 r.
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