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Groundwater, soil and compost, as possible sources of virulent and antibiotic-resistant Pseudomonas aeruginosa

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ABSTRACT

Pseudomonas aeruginosa is a major public health concern all around the world. In the frame of this work, a set of diverse environmental *P. aeruginosa* isolates with various antibiotic resistance profiles were examined in a *Galleria mellonella* virulence model. Motility, serotypes, virulence factors and biofilm-forming ability were also examined. Molecular types were determined by pulsed-field gel electrophoresis (PFGE). Based on our results, the majority of environmental isolates were virulent in the *G. mellonella* test and twitching showed a positive correlation with mortality. Resistance against several antibiotic agents such as Imipenem correlated with a lower virulence in the applied *G. mellonella* model. PFGE revealed that five examined environmental isolates were closely related to clinically detected pulsed-field types. Our study demonstrated that industrial wastewater effluents, composts, and hydrocarbon-contaminated sites should be considered as hot spots of high-risk clones of *P. aeruginosa.*

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Pseudomonas aeruginosa; virulence; biofilm; pulsed-field gel electrophoresis; antibiotic resistance

Introduction

Opportunistic *Pseudomonas aeruginosa* is a pathogen in diverse hosts including plants, animals and humans (Jander et al. 2000). In humans, it is responsible for severe nosocomial infections with the highest fatality rate of all Gram-negative bacteria (Aliaga et al. 2002). *P. aeruginosa* infections are multifactorial, the clinical outcome strongly depends on the interplay of cell-associated and extracellular virulence factors (Strateva and Mitov 2011; El Zowalaty et al. 2015). Intrinsic and acquired resistance to antibiotics is a co-factor in the mortality of *P. aeruginosa* infections (Cosgrove and Carmeli 2003). Swimming, swarming and twitching, the known motility forms of *P. aeruginosa* (Bradley 1980; Köhler et al. 2000), play a critical role in invasiveness (Drake and Montie 1988). Biofilms are well-documented as the source of infection (Kaiser et al. 2017), cause an increase in virulence (Schroeder et al. 2017) and are considered as hot spots for the accumulation and transfer of antibiotic resistance genes (ARGs) (Balcázar et al. 2015).

Due to its outstanding metabolic plasticity, *P. aeruginosa* can survive in diverse ecological niches including soil and aquatic habitats (Cabot et al. 2016), but it was usually observed in low abundance within these environments (Chatterjee et al. 2017). In contrast, *P. aeruginosa* was frequently isolated

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from hydrocarbon-contaminated sites (Kaszab et al. 2010) and can be dominant among hydrocarbon-degrading bacteria (Chaerun et al. 2004). The role of non-clinical environments as a reservoir or a transient recipient of *P. aeruginosa* is still under debate (Deredjian et al. 2014). Based on the absence of several virulence genes (Divya et al. 2018), or their non-haemolytic phenotype (Radhapriya et al. 2015), environmental *P. aeruginosa* isolates are sometimes considered as 'non-pathogenic' and are recommended for bioremediation (Ebadi et al. 2017), or plant protection purposes (Yasmin et al. 2017). Virulence models could provide tools for the reliable assessment of the actual virulence of these strains such as wax moth (*Galleria mellonella*), a non-mammalian test organism, introduced as an *in vivo* model for the study of *P. aeruginosa* (Velikova et al. 2016). Clinical *P. aeruginosa* is highly virulent to *G. mellonella* (Hill et al. 2014; Tsai et al. 2016) and the positive correlation between virulence of *P. aeruginosa* mutants in murine and *G. mellonella* models was verified (Jander et al. 2000). As the solo application of a non-mammalian model have its own limitations (Dubern et al. 2015), the simultaneous detection of virulence determinants, such as the presence of exotoxin and exoenzyme encoding genes or haemolytic activity, is proposed. Therefore, the overall aim of this research was to reach complex data on a set of environmental *P. aeruginosa* isolates with various antibiotic resistance profiles. To reach this goal, a virulence assay was performed in a *G. mellonella* model. Motility, serotype, virulence-related genes, haemolytic activity and biofilm-forming ability were also determined. To access genetic diversity, macrorestriction fragment patterns were created by PFGE.

Materials and methods

Bacteria isolation, identification, and screening for antibiotic resistance

Environmental samples were obtained from various environmental sources such as hydrocarbon (total petroleum hydrocarbons and/or benzene, toluene, ethylbenzene, xylene) contaminated groundwater and soil, compost, industrial wastewater effluents of Hungarian oil refineries and municipal sewage. *P. aeruginosa* isolates were cultivated by bacteriological standard methods and were identified with the PCR for the detection of species-specific variable regions V2 and V8 of 16S rDNA (Spilker et al. 2004). Antibiotic resistance profiles were determined with preliminary disc diffusion tests for 31 agents of 9 classes and quantitative Etest® (AB Biodisk, Solna, Sweden) for 9 chosen agents from the recommendation lists of the European Centre for Disease Prevention and Control (ECDC) and the Clinical Laboratory Standards Institute (CLSI) (EUCAST, 2018; CLSI 2018). Comparative isolates were *P. aeruginosa* ATCC-27853 (American Type Culture Collection, USA) and KPS3 (National Institute of Environmental Health, Hungary).

Serological typing

Serotypes of *P. aeruginosa* isolates were determined by agglutination tests using serotype-specific polyvalent antisera in accordance with the manufacturer's instructions (Bio-Rad, France).

Pulsed-field gel electrophoresis (PFGE)

For PFGE, a slightly modified method of the previously described protocols (Bannerman et al. 1995; Deplano et al. 2005) was used with *spe*I rare cutting enzyme for digestion and CHEF DRII System (Bio-Rad) for the separation of fragments in 1% agarose gel. Electrophoresis was in a 0.5x concentration Tris-borate-EDTA buffer (TBE) for 25 h at 5.8 V, 14°C with the initial switch time of 10 sec and the final switch time of 45 sec. Gels for PFGE were stained in 500 ml aqueous solution containing 0.5 μg/mL final concentration of ethidium-bromide, were washed with distilled water for 30 min and were photographed under UV transillumination. PFGE-generated DNA profiles were analyzed by the Fingerprinting II version 3.0 Software (Bio-Rad) using 1.0% position tolerance and 1.0% optimization

settings. The universal size standard was the *Salmonella* Braenderup (H9812) strain. The quantitative differences among the banding patterns were defined by the Dice coefficient. Unweighted pair group method with arithmetic mean (UPGMA) was used for cluster analysis of the PFGE patterns. Interpretation of chromosomal DNA restriction patterns based on the criteria of Tenover et al. (1995) using the following categories: indistinguishable (homology higher than 95%), closely related (homology between 85-95%), possibly related (homology higher than 85%, but the criteria of Tenover were not fulfilled) and different (homology lower than 85%). PFGE results of environmental isolates were compared to the surveillance database of the National Center for Epidemiology, Hungary on hospital outbreaks.

Haemolytic activity and virulence genes

Haemolytic activity was determined on Columbia agar with 5% sheep blood (Chemium Ltd., Hungary) with incubation parameters recommended by the manufacturer (37°C, 22 h). Microbial DNA was isolated by UltraClean Microbial DNA isolation kit (MoBio), following the manufacturer's instructions. Virulence genes (effector protein-encoding genes *exoS* and *exoU*, elastase producing *lasB*, alginate encoding *algD*, alkaline phosphatase producing *aprA* and haemolytic phospholipase encoding *plcH*) were amplified using PCR with reaction parameters and primers described by Ullah et al. (2017), Badamchi et al. (2017) and Ajayi et al. (2003). Results were visualized by agarose gel electrophoresis. Positive controls were *P. aeruginosa* ATCC 27853 for *exoS, lasB, algD, aprA* and *P. aeruginosa* KPS-3 (a nosocomial strain isolated in Hungary) for *exoU* and *plcH* genes.

Galleria mellonella infection model

G. mellonella test was performed as it was suggested by Koch et al. (2014). Each test was performed in duplicate. Positive control was *P. aeruginosa* PA14 (DSM 19882), a virulent human clinical isolate, negative controls were injected with phosphate-buffered saline (PBS). After 24 and 48 h of incubation at 37°C, the rate of mortality was determined (a caterpillar was regarded as dead when no motion was observable). Virulence of the examined isolates was categorized after 48 h according to the survival of *G. mellonella* larvae into four groups: avirulent (with a survival rate of 75-100%), semi-virulent (survival rate: 50-74%), moderately virulent (survival rate: 25-49%) and virulent (survival rate: 0-24%).

Motility assays

Swimming, swarming and twitching were performed as it was previously described by Schmidt et al. (2011). In brief, overnight bacterial cultures transferred to Luria-Bertani (LB) medium, were grown to exponential phase (OD_{600} 1.5–2.5). Swimming was examined with Basal Medium 2 (BM2) containing 0.3% agar in 12 Well Nunc plates, inoculated with 0.5 µL liquid bacterial cultures. For swarming, BM2 was used with 0.5% agar content and an increased casamino acid concentration (0.5%) for a better flagellar synthesis. 1.0 µL of bacterial cultures were pipetted onto the prepared medium for inoculation. Twitching assay was performed on LB medium with 1% agar. Swimming and swarming plates were incubated overnight at 30°C, while the zone of twitching motility was marked after 48 h incubation at 37°C. The area was measured with the ImageJ program. The mean value of *P. aeruginosa* PA14 was calculated and was set as a threshold value for hypermotility. Categories and thresholds of motility tests are summarized in Table 1.

Biofilm and adherence assays

To assess biofilm-forming ability, a modified microtiter plate test was used with parameters and conditions described by Stepanović et al. (2000) in TSB (Tryptic Soy Broth) medium. Test was

Table 1. Categorization of bacterial isolates according to their motility with threshold values.

	Swimming categories	Area (cm^2)*	Swarming categories	Area (cm^2)*	Twitching categories	Area (cm^2)*
Category 1	non-motile	0	non-motile	0	non-motile	0
Category 2	motile	0-1.10	motile	0-8.17	motile	0-1.82
Category 3	hypermotile	1.1- **	hypermotile	8.18- **	hypermotile	1.83- **

*Area was measured with the ImageJ image processing program
**The mean values of positive control PA14 were set as a threshold for hypermotile category

performed in triplicate, isolates were classified after 24, 48, 72 and 96 h as no biofilm producers (0), weak biofilm producers (+), moderate biofilm producers (++), or strong biofilm producers (+++). The cut-off OD (ODc) was determined as it was recommended by Stepanović et al. (2000).

Biofilm production was visualized by confocal laser scanning microscopy (CLSM) in Twincore Centre (Hanover, Germany) to acquire images of 48 h biofilms grown in static 96-well plate assays. The method of visualization was described previously (Van Duuren et al. 2017).

Results of environmental *P. aeruginosa* were challenged by a pairwise statistical analysis to clarify the possible relations among different features. Pearson's correlation (r) was calculated with a confidence interval of 95%.

Results

Antibiotic susceptibility

Based on their antibiotic resistance profiles, 44 non-duplicate environmental *P. aeruginosa* isolates were chosen for further investigations representing sensitive (n = 29) and resistant (n = 15) phenotypes. Resistant isolates were detectable in compost and hydrocarbon-contaminated groundwater or soil. Resistance was widespread against Cefotaxime (18.2%), Ceftriaxone (25.0%) and Imipenem (25.0%), while Cefepime, Ciprofloxacin and Gentamicin remained effective against nearly all examined isolates. Four environmental strains (P14, P43, P46 and P69) met the MDR (multidrug resistance) criteria defined by Magiorakos et al. (2012). Strains isolated from oil refinery sewage showed a sensitive phenotype. The origin and the antibiotic resistance profiles of environmental isolates are shown in Table 2.

Serotypes and PFGE patterns

Among environmental isolates, nine serotypes were determined with the dominance of O6 (29.5%), O1 (15.9%) and O3 (15.9%). The remaining 17 isolates were typed as O1, O4, O7, O9, O9, or were non-typable (ONT). Based on their PFGE banding patterns, 18 (40.9%) environmental isolates were considered as sporadic strains with a loose relationship to other environmental or nosocomial profiles (homology was lower than 85%). Three environmental isolates (P16, P80 and P174) showed 84-88% homology in PFGE-profiles with clinical isolates (originating from hospitals located in Budapest), and five of the examined 44 environmental isolates, (P59, P119, P124, P125 and P172) showed the same PFGE-types as a previously described nosocomial strain (with 85-95% homology). The common origin of these five environmental strains with isolates of hospital outbreaks is presumed. At the same time, none of the examined environmental isolates had 95% or higher homology with the evaluated nosocomial fingerprints (Figure 1.).

Environmental isolates showing 85-95% homology with clinical counterparts (marked with grey in Figure 1.) were sensitive to the examined antibiotics (except Imipenem and Cefotaxime-resistant P16), had clinically relevant serotypes O6 and O10 (with the exception of non-typable strain P124) and had various virulence gene profiles. The shared features of these clinically related environmental isolates were as follows: motility or hypermotility in all examined forms of motion, virulence (80-100% mortality) in the *G. mellonella* model, haemolytic activity and biofilm-forming capability. Three of these environmental isolates originated from industrial sewage effluents.

Table 2. The origin and antibiotic resistance profiles of environmental *P. aeruginosa* isolates.

Designation of *P. aeruginosa* isolates	Antibiotic resistance profile										Sampling site	Type of sample	Year of sampling
	CPS	CT	TZ	TX	PM	PC	IP	CI	OF	GM			
P2*	S	S	S	S	S	S	S	S	S	S	Diósd	HCS	2002
P3	S	I	S	S	S	S	S	S	S	S	Őrbottyán	C	2008-2009
P9*	S	S	S	S	S	S	**R**	S	S	S	Diósd	HCG	2003
P10*	S	8	S	S	S	S	S	S	S	S	Diósd	HCG	2003
P14*	S	**R**	**R**	**R**	S	**R**	I	S	S	**R**	Tököl	HCS	2003
P16*	S	**R**	S	I	S	S	**R**	S	S	S	Tököl	HCS	2003
P18*	S	S	S	S	S	S	S	S	S	S	Tököl	HCS	2003
P28*	S	I	S	**R**	S	S	**R**	S	S	S	Püspökladány	HCS	2003
P30*	S	I	S	**R**	S	S	**R**	S	S	S	Szabadszállás	HCS	2004
P32*	S	**R**	S	**R**	S	S	**R**	S	S	S	Komádi	HCG	2004
P39*	S	I	S	**R**	S	S	**R**	S	I	S	Algyő	HCS	2004
P42*	S	I	S	S	S	S	S	S	S	S	Mezőtúr	HCS	2005
P43*	S	**R**	**R**	**R**	I	**R**	**R**	S	S	**R**	Ópusztaszer	HCG	2005
P46*	S	**R**	S	**R**	S	**R**	**R**	S	S	S	Budapest	HCG	2005
P53*	S	**R**	S	**R**	S	S	**R**	S	S	S	Zalaegerszeg	HCG	2006
P59	S	S	S	I	S	S	S	S	S	S	Gödöllő	C	2008-2009
P60	S	I	S	S	S	S	S	S	S	S	Gödöllő	C	2008-2009
P62*	S	I	S	I	S	S	S	S	S	S	Szarvas	HCG	2006
P66*	S	S	S	I	S	S	S	S	S	S	Zalaegerszeg	HCG	2006
P69*	I	**R**	I	**R**	S	**R**	**R**	S	**R**	S	Nagyszénás	HCS	2007
P71*	S	I	S	I	S	**R**	S	S	S	S	Debrecen	HCG	2007
P77*	S	I	S	I	S	S	S	S	S	S	Zalaegerszeg	HCG	2007
P78*	S	I	S	I	S	S	S	S	S	S	Szarvas	HCG	2007
P79*	S	S	S	S	S	S	S	S	S	S	Szarvas	HCG	2007
P80	S	S	S	S	S	S	S	S	S	S	Százhalombatta	ORS	2007
P104	S	I	S	**R**	S	S	S	I	**R**	S	Kecskemét	C	2008-2009
P106	S	I	S	S	S	S	S	S	S	S	Kecskemét	C	2008-2009
P108	S	I	S	S	S	S	S	S	S	S	Kecskemét	C	2008-2009
P113	S	I	S	S	S	S	S	S	S	S	Balatonfűzfő	C	2008-2009
P114	S	**R**	**R**	**R**	I	**R**	S	S	S	S	Kecskemét	C	2008-2009
P119	S	I	S	I	S	S	S	S	S	S	Tiszaújváros	ORS	2009
P124	S	I	S	I	S	S	S	S	S	S	Zalaegerszeg	ORS	2009
P125	S	I	S	I	S	S	S	S	S	S	Dunaújváros	ORS	2009
P135	S	I	S	I	S	S	**R**	S	I	S	Pétfürdő	HCS	2009
P144	S	I	S	S	S	S	S	S	S	S	Pétfürdő	HCS	2010
P152	S	I	S	S	S	S	S	S	S	S	Kecskemét	C	2010
P160	S	I	S	I	S	S	S	S	S	S	Balassagyarmat	HCG	2010
P164	S	I	S	I	S	S	I	S	S	S	Varsád	MS	2010
P169	S	I	S	S	S	S	S	S	S	S	Orosháza	HCG	2011
P170	S	I	S	S	S	S	I	S	S	S	Polgár	HCG	2011
P172	S	S	S	S	S	S	S	S	S	S	Békéscsaba	HCG	2011
P174	S	I	S	I	S	S	S	S	S	S	Békéscsaba	HCG	2011
P177	S	I	S	S	S	S	S	S	S	S	Miskolc	HCG	2011
P183	S	I	S	S	S	S	S	S	S	S	Siklós	HCG	2011
KPS-3	S	I	S	S	S	S	S	S	S	S	Budapest	N	2001-2007
ATCC 27853	S	I	S	S	S	S	S	S	S	S	Boston, USA	N	1971

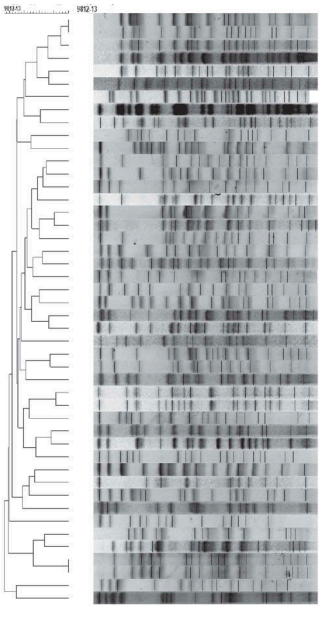
*antibiotic resistance, serotype, biofilm and *exoU/exoS* data of these isolates were partially published previously (Kaszab et al. 2010; Radó et al. 2017). R (bold font), resistant; I, intermediate; S, sensitive to antibiotics.

CPS, Cefoperazone-sulbactam; CT, Cefotaxime; TZ, Ceftazidime; TX, Ceftriaxone; PM, Cefepime; PC, Piperacillin; IP, Imipenem; CI, Ciprofloxacin; OF, Ofloxacin; GM, Gentamicin.

C, compost; HCG, hydrocarbon-contaminated groundwater; HCS, hydrocarbon-contaminated soil; MS, municipal sewage; N, nosocomial; ORS, oil refinery sewage.

Virulence genes

The frequency of the examined virulence-related genes among environmental *P. aeruginosa* was as follows: *lasB* (100.0%), *algD* (95.3%), *aprA* (95.3%), *plcH* (88.3%), *exoS* (72.7%) and *exoU* (22.7%). All environmental isolates harboured at least two of the examined six virulence-related genes and 34 (79.0%) isolates showed a profile with five simultaneously detectable virulence genes. Regarding *exoU*/*exoS*, the most variable traits among virulence genes (Ahmed



PFGE type	Designation of strains	Type	Sero-type	Virulence in *G. mellonella* model		Motility			Virulence markers**							Biofilm-forming (48h)****
				48h mortality (%)	Category	Swimming g (cm²)	Swarming g (cm²)	Twitching g (cm²)	*exoS*	*exoU*	*lasB*	*algD*	*aprA*	*plcH*	Hemolytic activity (24h)***	
Psa213	P66	HCG	O11	95	virulent	HM	M	HM	+	-	+	+	+	+	+++	+++
	P78	HCG	O7	90	virulent	HM	M	M	+	-	+	+	+	+	+++	+
	P77	HCG	O8	85	virulent	M	M	M	+	-	+	+	+	+	+	++
	P106	C	O8	100	virulent	HM	NM	M	+	-	+	+	+	+	+	+++
Psa220	**P28**	HCS	O1	5	avirulent	M	M	M	+	-	+	+	+	+	+	+++
Psa221	P2	HCS	ONT	65	middle virulent	NM	NM	M	+	-	+	+	+	-	-	+++
Psa217	P164	MS	O11	100	virulent	M	NM	M	+	-	+	+	+	-	+++	-
Psa256	**P9**	HCG	ONT	25	avirulent	NM	NM	M	-	+	+	+	+	+	-	++
Psa257	P10	HCG	O3	40	middle avirulent	M	NM	M	+	-	+	+	+	+	-	+
Psa214	**P32**	HCG	O7	55	middle virulent	M	NM	M	+	-	+	+	+	+	++	+++
Psa215	P170	HCG	O1	100	virulent	HM	NM	M	+	-	+	+	+	-	-	++
Psa025	P172	HCG	O6	100	virulent	HM	M	M	+	-	+	+	+	-	+	+
Psa211	P177	HCG	O6	70	middle virulent	HM	M	HM	+	-	+	+	+	+	++	+
Psa163	P80	ORS	O6	100	virulent	NM	M	M	+	-	+	+	+	+	+++	++
Psa226	P3	C	ONT	80	virulent	HM	M	HM	+	-	nd	nd	nd	nd	+	+
Psa238	P79	HCG	O6	80	virulent	HM	M	M	+	-	+	+	+	+	++	+++
	P46	HCG	O6	70	middle virulent	M	M	M	+	-	+	+	+	+	+	+++
Psa046	P59	C	O6	80	virulent	HM	M	M	+	-	+	+	+	+	+++	+++
Psa201	ATCC 27853*	N	O6	85	virulent	ND	ND	ND	+	-	+	+	+	+	+	++
Psa231	**P71**	HCG	O1	95	virulent	M	M	HM	-	+	+	+	+	+	++	+
Psa228	**P104**	C	O4	100	virulent	HM	M	M	+	-	+	+	+	+	+++	++
Psa208	**P16**	HCS	O9	50	middle avirulent	HM	NM	M	+	-	+	+	+	-	+++	+++
Psa065	P124	ORS	ONT	100	virulent	HM	HM	M	+	-	+	+	+	+	+++	+
Psa240	P60	C	O6	95	virulent	M	M	HM	+	-	+	+	+	+	+++	+++
	P42	HCS	O6	90	virulent	HM	M	HM	+	-	+	+	+	+	+	++
Psa232	**P114**	C	O1	95	virulent	HM	HM	HM	+	-	+	+	+	+	+++	+
Psa016	P113	C	O10	80	virulent	HM	M	M	+	-	+	+	+	+	+	+
	P169	HCG	O10	95	virulent	HM	M	M	+	-	+	+	+	+	+	++
Psa252	**P69**	HCS	O6	50	middle avirulent	M	M	M	+	-	+	+	+	+	++	-
Psa243	**P14**	HCS	O3	5	avirulent	HM	NM	M	+	-	+	+	+	+	+/-	+++
	P18	HCS	O3	10	avirulent	M	M	M	+	-	+	+	+	-	+/-	+
Psa203	P144	HCS	O1	100	virulent	HM	M	HM	-	+	+	-	-	-	-	-
Psa229	**P62**	HCG	O3	100	virulent	M	M	HM	+	-	+	+	+	+	-	+
	P30	HCS	O3	75	middle virulent	M	M	M	+	-	+	+	+	+	-	++
Psa205	P152	C	O1	100	virulent	HM	M	M	+	-	+	+	+	+	-	+
Psa212	P183	HCG	O6	95	virulent	M	M	HM	+	-	+	+	+	+	+++	+++
Psa253	**P39**	HCS	O6	90	virulent	M	M	M	+	-	+	+	+	+	+++	++
Psa209	P174	HCG	O1	100	virulent	HM	M	HM	+	-	+	+	+	+	+++	+
Psa255	**P53**	HCG	O6	90	virulent	M	M	HM	+	-	+	+	+	+	+	-
Psa204	P160	HCG	O6	100	virulent	HM	M	M	+	-	+	+	+	+	+	+
Psa216	KPS-3*	N	O10	65	middle virulent	ND	ND	ND	-	+	+	+	+	+	+	+++
Psa223	**P43**	HCG	ONT	10	avirulent	M	M	M	-	+	+	+	+	+	+++	+
Psa024	P119	ORS	O10	100	virulent	HM	M	M	-	+	+	+	+	+	+	+++
	P125	ORS	O10	100	virulent	HM	M	M	-	+	+	-	-	-	+	++
Psa207	**P135**	HCS	O3	30	middle avirulent	HM	M	HM	-	-	+	+	+	+	+++	+
Psa242	P108	C	O3	40	middle avirulent	HM	M	NM	-	-	+	+	+	+	++	+++

Figure 1. The characteristics of environmental *P. aeruginosa* isolates in order of their localization on PFGE dendrogram. grey coloured, same pulsed-field types as clinical counterparts; bold font, multidrug resistance (MDR); nd, no data; *clinical reference strains; ** + positive PCR; – negative PCR; ***- no haemolysis; + moderate haemolysis, ++ normal haemolysis, +++ intensive haemolysis; **** Biofilm-forming in modified microtiter assay: – no biofilm producer; + weak biofilm producer; ++ moderate biofilm producer; +++ strong biofilm producer.

et al. 2019), the commonly determined environmental profile is possessing *exoS*. However, environmental isolates carrying *exoU* (isolated from hydrocarbon-contaminated soil, groundwater and wastewater) and isolates lacking both *exoU*/*exoS* (isolated from compost samples) were also detected. Regarding haemolytic features, 15 isolates (34.1%) showed intensive beta-haemolytic activity and only seven (15.9%) of the environmental isolates were non-haemolytic (Figure 1.).

Virulence in G. mellonella model

In the applied *G. mellonella* virulence model, the majority (65.9%) of the examined environmental isolates was virulent with a mortality rate of 75-100%. Five of the examined 44 environmental *P. aeruginosa* isolates were proved to be moderately virulent (50-75%), five isolates were semi-virulent (25-50%) and five strains were avirulent (0-25% mortality). The survival slope variations between virulent and not virulent (moderately, semi, or avirulent) phenotypes suggesting a significant difference in virulence after 24 h injection (Figure 2.).

Regarding motility assay, all examined environmental isolates showed at least one type of motility and one strain (*P. aeruginosa* P114 originated from compost) was proved to be hypermotile in all examined motility forms (Figure 1.). Twenty-five (56.8%) environmental isolates were hypermotile in swimming and 13 (29.5%) strains showed hypermotility in twitching. Swarming was moderate in frequency since only two (4.5%) isolates were proved to be hypermotile.

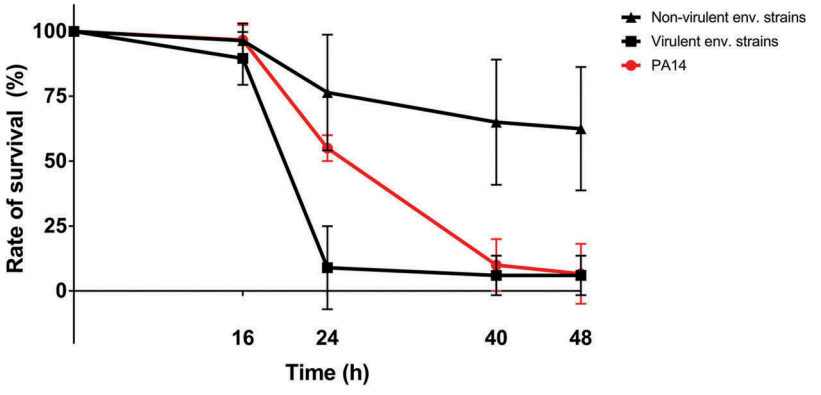


Figure 2. *Galleria* survival after inoculation with bacterial samples. Non-virulent environmental (48 h mortality between 0-75%); virulent environmental isolates (48 h mortality between 75-100%).

Biofilm-forming ability

Regarding biofilm-forming ability, different incubation times (24, 48, 72 and 96 h) lead to huge differences in the detected level of adherence. Therefore, the recommended 48 h incubation (Van Duuren et al. 2017) was used for further evaluation in the applied microtiter-plate test. After 48 h, only three (6.8%) of the examined environmental isolates were non-adherent, 61.3% was weak or moderate biofilm producer and 14 (31.8%) was proved to be a strong biofilm producer. CLSM revealed, that 14 (34.1%) of the biofilm-producing *P. aeruginosa* isolates (of which four were strongly adherent) did not form structured biofilms: the detected OD values were a result of the massive accumulation of planktonic cells in pellicles (Figure 3.). Twenty-seven (61.3%) of the examined 44 environmental isolates showed biofilm-forming ability with both applied techniques of which 10 (22.7%) was a strong biofilm producer.

Statistical analysis

The statistical analysis revealed that Minimal Inhibitory Concentrations (MICs) against six different agents (Gentamicin, Imipenem, Cefepime, Ceftazidime, Cefotaxime and Cefoperazone-sulbactam) showed inverse correlation with the rate of 48 h mortality in *G. mellonella* test: the higher MICs (increasing resistance) lead to a lower rate of *in vivo* mortality (Figure 4. part A). Despite the negative correlation, simultaneously virulent and antibiotic-resistant phenotypes were detectable among isolates originating from hydrocarbon-contaminated groundwater (P53, P62, P71, P77) soil (P16, P42) or compost (P114). The presence or absence of the examined virulence-related genes *lasB, algD, aprA, plcH, exoS* and *exoU* and the phenotypically determined haemolytic activity did not show significant correlation with *G. mellonella* mortality results. The only exception was the simultaneous lack of *exoS* and *exoU* genes showing a significantly lower rate of mortality in the applied virulence model. Twitching was a possible motility-related contributor in virulence on *G. mellonella*, since it was the only examined motility form showing a significant correlation with the rate of mortality (Figure 4. part B). The cumulative ability for motion also significantly enhanced the rate of virulence, which underlines that the critical role of twitching in *G. mellonella* infection can be at least partially replaced by swimming and/or swarming. Hypermotility tends to be a lacking ability of avirulent environmental isolates with one exception (P14) only.

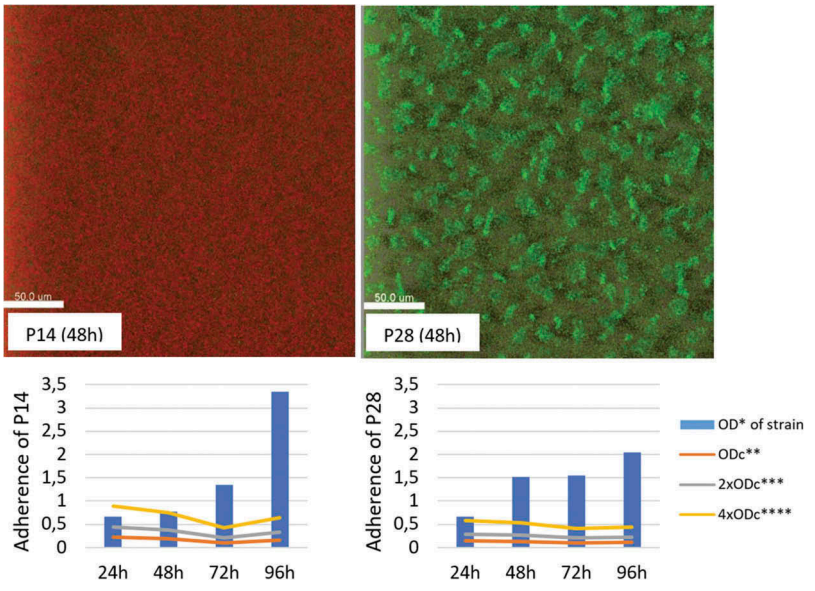


Figure 3. The biofilm-forming ability of environmental *P. aeruginosa* isolates P14 and P28 based on their adherence in microtiter-plate test and their image by confocal laser scanning microscopy (CLSM). *OD – optical density; **ODc – cut-off OD, threshold of weak-adherence; ***2xODc – threshold of moderate adherence; ****4xODc – the threshold of strong adherence.

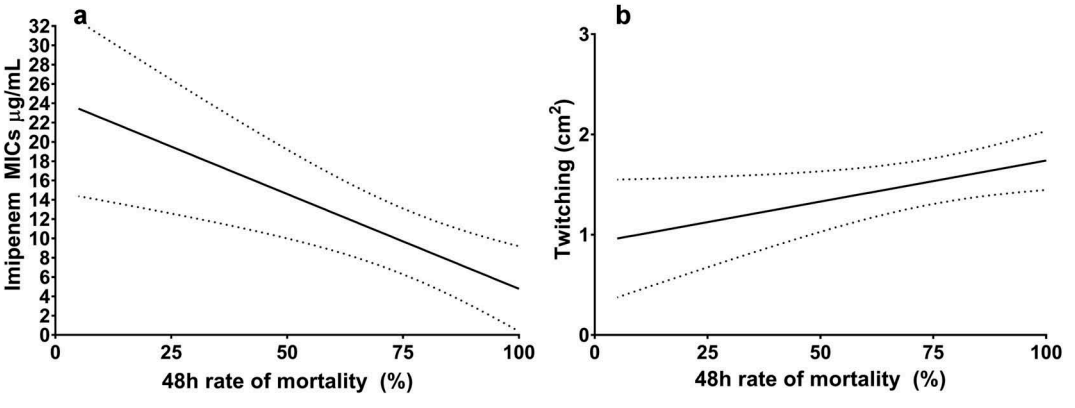


Figure 4. The correlation between Imipenem resistance and mortality (part A), and between twitching motility and mortality (Part B) determined in *G. mellonella* model (48 h).

Discussion

According to the scientific literature, the detected rate of resistance against Piperacillin (13.6%) and especially Imipenem (25.0%) was higher than as it was recently reported by Liew et al. (2019) on freshwater *P. aeruginosa* isolates. In the present study, Imipenem-resistant isolates were exclusively originated from hydrocarbon-contaminated samples verifying the importance of contaminated areas as a possible source of carbapenem-resistant cell lines (Kaszab et al. 2010). Regarding dominant serotypes, the detection rate of O6 (29.5%) was lower, than as it was previously reported in marine habitats (95.8%) (Bouhaddioui et al. 2002), which emphasize that groundwater, soil and compost can be more diverse in *P. aeruginosa* serotypes than marine environments. Clinically relevant serotypes determined by Lu et al. (2014) were detected in 26 cases, meaning that 59.0% of the examined environmental isolates were in a clinically important serogroup (O1, O6, O10 or

O11). At the same time, O12, the predominant MDR serotype in clinical settings (Thrane et al. 2015) was not detectable. *G. mellonella* virulence test revealed, that environmental strains with clinically relevant serotypes have a higher rate of virulent isolates (84.6%) than strains with non-clinical (O3, O4, O7, O8, O9 and ONT) serotypes (33.3%).

Based on our PFGE results, PFGE profiles did not show a significant correlation with virulence, biofilm or motility properties of the examined isolates: virulent and antibiotic-resistant strains were localized randomly on dendrogram. Although some studies previously reported a correlation between PFGE types and antibiotic resistance (Salimi et al. 2010), our findings agree with the reports of no correlation between these features (Tokajian et al. 2012; Karami et al. 2018). This result emphasizes the lack of discriminatory power of phenotypic methods and support the necessity of genomic studies in this field.

Among the examined virulence genes, the determined frequency of invasive *exoS* (72.7%) and cytotoxic *exoU* (22.7%) are within the recently observed ranges in the environmental (Rutherford et al. 2018; Ahmed et al. 2019) and clinical (Fazeli and Momtaz 2014) settings. Isolates harbouring both *exoS* and *exoU* virulence genes were not detectable, since these virulence genes are nearly always mutually exclusive (Shaver and Hauser 2004). The role of virulence genes and their effector proteins in *G. mellonella* killing was previously examined and it was concluded that only the secretion of ExoT and ExoU plays a significant role (Miyata et al. 2003). Here we've verified that *exoS* is also an important contributor in wax moth killing and only the simultaneous absence of *exoS* and *exoU* leading to a decreased rate of mortality. Considering the low number of isolates simultaneously lacking *exoU* and *exoS*, further investigations and gene expression analyses are required to verify these results.

Regarding extracellular protease encoding *lasB* (elastase LasB) and *aprA* (alkaline protease AprA), *lasB* was detected in a higher rate (100.0%) than in clinical settings (Roshani-Asl et al. 2018). Due to this exclusiveness, it was not possible to evaluate the previously reported relationship between the presence of *lasB* gene and biofilm-forming ability or Imipenem, Piperacillin/Tazobactam resistance (Roshani-Asl et al. 2018). Regarding *aprA*, the previously reported clinical frequencies were 27.2% among patients with otitis media (Hassuna 2016), 28.6% among burned patients (Fazeli and Momtaz 2014) and 100% in intensive care units (ICUs) (Bradbury et al. 2010). The rate of *aprA* detection (95.3%) among our environmental isolates is similar to its frequency among clinical strains isolated from ICUs. *AlgD* (97.7%) and *plcH* (86.6%) were both detected in the same values as in clinical environments (92.3–98.0% and 84.6%, respectively) (Taee et al. 2014; Georgescu et al. 2016). Interestingly, there was no significant correlation between the presence of *plcH* gene, encoding a haemolytic phospholipase C precursor, and the phenotypically determined haemolytic activity of environmental strains. The answer to this insignificant correlation may be the role of other cytolytic factors in haemolysis such as the production of exotoxin A or elastase (Lee et al. 2006).

Our *G. mellonella* assay verified that virulence is a dominant phenotypic trait in the environment. Virulent isolates with a notable set of invasive and cytotoxic factors were isolated from all examined environmental media (groundwater, soil, compost, sewage effluents). This finding supports the opinion that all *P. aeruginosa* strains are potential pathogens (Pirnay et al. 2009; Silby et al. 2011) due to their invasive and toxigenic features (Mesaros et al. 2007; Kumar et al. 2013). Considering that, due to host-specific virulence factors (Choi et al. 2002), virulence in *G. mellonella* model can significantly differ from other test organisms, additional virulence tests should be performed in the future.

Based on our motility results, the previously drawn connections between twitching and biofilm formation (O'Toole and Kolter 1998) or antibiotic resistance (Shehata and Sayed 2011) were not verified since twitching motility was widespread among environmental isolates and the limitations of microtiter-plate test for detecting biofilms were revealed.

Regarding biofilm-forming ability, the detected rate of strong biofilm producer environmental isolates (22.7%) is closer to the previously reported rate for human isolates (23.5%) than for strains isolated from animals (9%) (Milivojevic et al. 2018). The fact that 34.8% of the positive microtiter-

plate results were not structured biofilms emphasize the importance of visual techniques in biofilm detection (Van Duuren et al. 2017).

Regarding the correlation between virulence and antibiotic resistance features, the connection was previously reported to be both positive (Neidig et al. 2013) and negative (Bartoli et al. 2015), but recent studies debated inverse correlation (Schroeder et al. 2017; Al Dawodeyah et al. 2018). Based on the results presented here, the negative relationship of antibiotic resistance and virulence is still possible in the environment.

Conclusions

A set of 44 environmental P. *aeruginosa* were examined isolated from various (e.g. hydrocarbon-contaminated) environmental sources. The majority of environmental *P. aeruginosa* was proved to be virulent with a verified ability for motility, haemolysis and biofilm-forming. Five examined environmental isolates were closely related to clinically detected PFGE types. The presence or absence of the revealed arsenal of virulence-related factors or genes did not correlate with phenotypic traits, which emphasize the complexity of virulence-related mechanisms. Despite the negative correlation between antibiotic resistance and wax moth killing, simultaneously resistant and virulent clones can be found among environmental isolates. The role of various environmental niches as a possible source of pathogen and resistant lineages is presumed: industrial wastewater effluents, composts and hydrocarbon-contaminated sites should be considered as hot spots of pathogen and resistant *P. aeruginosa*.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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