SZENT ISTVÁN UNIVERSITY

PH.D. THESIS

ENVIRONMENTAL SAFETY EXAMINATION OF UBIQUITOUS BACTERIA

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1. INTRODUCTION AND AIMS

Biological safety is an important part of environmental risk management including the assessment of microorganisms used in biotechnology. Many opportunistic pathogen bacteria are ubiquitous in nature and are potential contributors in metabolic processes. Some of them are significant in clinical settings and cause nosocomial infections. It is a general hypothesis, that environmental strains of the same species possess lower health risk than clinical isolates. The possible biological risks of a bacterial strain are affected by numerous factors which are playing role in colonization, infection and the manifestation of pathogenic behaviour or the outcome of an infection.

The aim of my research was to examine ubiquitous opportunistic pathogen bacteria with clinical significance such as the ability to cause nosocomial infections or to develop antibiotic resistance. Therefore, species *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were chosen for further investigations.

My aims were as follows (Figure 1.):

- Isolation, identification and maintenance of environmental *Acinetobacter* sp. and *Pseudomonas aeruginosa* strains.
- Examination of their antibiotic resistance with quantitative phenotypic test (MIC test), and the detection of resistance genes with the PCR.
- Examination of virulence and virulence-related factors as follows
    - detection of phenotypic virulence with *Galleria mellonella* model
    - haemolytic activity (Columbia blood agar plates)
    - biofilm forming ability (microplate method, confocal laser scanning microscope)
    - detection of virulence genes (PCR)
    - serotyping (polyvalent and monovalent antisera)
    - motility (swimming, swarming, twitching)
- Determination of phylogenetic relationships of environmental and clinical strains with molecular methods in accordance with national and international databases

- MLST (Multilocus Sequence Typing)
- PFGE (Pulsed-filed Gel Electrophoresis).

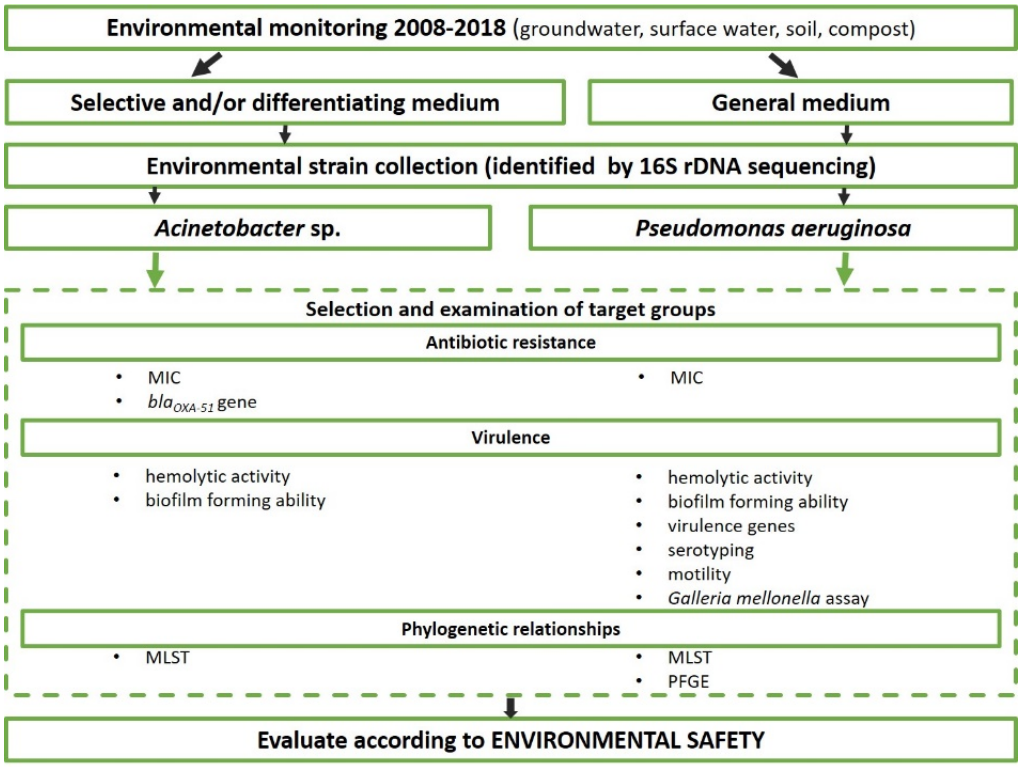


Figure 1. Flowchart of my research plan and methodology

2. MATERIALS AND METHODS

2.1 Sampling

Environmental samples were collected between 2008 and 2018 from groundwater, surface water, soil and other (agricultural sources as compost, vegetables, fish) sources according to Hungarian standards (MSZ 21464: 1998.; MSZ 5667-4:1995.; MSZ 21470-1:1998.). 405 samples were used for the isolation of *Acinetobacter* sp. and more than 300 samples for the isolation of *P. aeruginosa*.

2.2 Isolation and identification of environmental *Acinetobacter* sp. and *P. aeruginosa* strains

Tenfold dilution was prepared from each environmental sample. Acinetobacters were cultivated in *Acinetobacter* dilution broth and Leeds *Acinetobacter* Medium. Colonies with typical *Acinetobacter* morphology and mauve colour were isolated. In the case of *P. aeruginosa,* isolation and identification based on the Hungarian standard MSZ 21470-77:1988. Species level identification of individual strains was performed with 16S rDNA sequencing. *Acinetobacter* candidates were identified with universal 27f and 1492r (LANE, 1991) primers for 16S rDNA. Sequences were determined by capillary gel electrophoresis and were evaluated with MEGA7 program (TAMURA ET AL., 2013). Results were compared with EzTaxon database (YOON ET AL., 2017). Identification of *P. aeruginosa* strains were confirmed with PA-SS PCR (SPILKER ET AL., 2004), V2 and V8 specific subunits of 16S rDNA.

2.3 Antibiotic resistance assay

Antibiotic resistance was determined with a quantitative, phenotypic method (MIC test - Liofilchem, Italy) according to recommendation of CLSI (Clinical and Laboratory Standards Institute - CLSI, 2017). Antibiotic agents were chosen based on international recommendations.

In the case of *Acinetobacter baumannii* strains, the presence of a chromosomally located antibiotic resistance encoding gene (bla_{OXA-51}) was examined with PCR (MERKIER&CENTRON, 2006).

2.4 Haemolytic activity assay

Haemolytic activity, as a virulence factor, was determined on Columbia agar plates supplemented with sheep blood. After 22 hours of incubation, haemolysis was evaluated on a scale of one to five: - no haemolysis, +/- doubtful haemolysis, + slight haemolysis, ++ haemolysis, +++ intensive haemolysis.

2.5 Biofilm forming ability assay

Biofilm forming ability was tested with microplate method (STEPANOVIC ET AL., 2000; KUMARI ET AL., 2013). Cells were solubilized with phosphate buffer and biofilm was fixed by methanol. After crystal violet staining, cells were resolubilized with 33% glacial acetic acid and the optical density (OD) was measured at 550 nm. Results were compared with a control and strains were classified into four categories: $OD \leq OD_c$[1] - no biofilm producer (0); $OD_c < OD \leq 2 \times OD_c$ - weak biofilm producer (+); $2 \times OD_c < OD \leq 4 \times OD_c$ - moderate biofilm producer (++); $4 \times OD_c < OD$ – strong biofilm producer (+++).

Some *P. aeruginosa* strains were chosen to examine their biofilm structures with SP8 system Leica type confocal laser scanning microscope (CLSM) at TWINCORE Research Institute in Germany. 48 h old, live/dead stained, static biofilm formations were visualised with the method of van Duuren and his colleagues (2017).

2.6 Complex study with *P. aeruginosa* strains

Determination of virulence genes: Based on the recommendation of scientific literature, alginate (*algD*), elastase (*lasB*), haemolytic phospholipase (*plcH*), alkaline protease (*aprA*) and exoenzyme/exotoxin (*exoS* and *exoU*) encoding genes were examined as virulence factors of *P. aeruginosa* strains, and were detected by PCR (AJAYI ET AL., 2003., LANOTTE ET AL., 2004; BADAMCHI ET AL., 2017).

[1] OD_c value: three times of standard deviation of the negative control plus average OD value of negative control.

Serotyping of *P. aeruginosa* strains was performed with polyvalent and monovalent antisera kit in the National Center for Epidemiology, according to the manufacturer's recommendation.

Motility (swimming, swarming and twitching) of *P. aeruginosa* strains was examined in Germany at TWINCORE Research Institute. Bacterial suspensions with a density of 1.5-2.5 (OD_{600}) were used in the motility assay. PA14 strain was used as a positive control. After 15 hours of incubation, the zone of motility was calculated by ImageJ program. Results of motility test were categorized into three classes: non-motile, motile, hypermotile.

Virulence examinations with *Galleria mellonella* model were made in Germany at TWINCORE's laboratory as well. 300 µl liquid overnight cultures of *P. aeruginosa* strains (with 0.5 OD600) were diluted to 10^5 and were used for infection of *Galleria mellonella*'s larvae (10 larvae/strain). Negative control was uninoculated phosphate solution and PA14 reference strain was used as positive control. Vitality of larvae was determined after 24 and 48 hours. Strains were classified into four groups according to the rate of surviving larvae as avirulent (100-75%), middle avirulent (74-50%), middle virulent (49-25%), virulent (24-0%).

Phylogenetic analysis: Sequences of 7 housekeeping genes were determined for of all *A. baumannii* (BARTUAL ET AL., 2005) and some chosen *P. aeruginosa* strains (CURRAN ET AL., 2004) by Multilocus Sequence Typing method (MLST). Sequences of housekeeping genes were compared with an international database (www.pubmlst.org) and the allelic numbers of a given locus were determined. The sequence type number (ST) of a strain can be determined according to the combination of the allelic numbers of all the seven housekeeping genes. Based on STs, phylogenetic connections can be determined among strains with various geographical origin.

For environmental *P. aeruginosa* strains, phylogenetic connections were examined with Pulsed-filed Gel Electrophoresis (PFGE) too in National Center for Epidemiology. Strains were classified according to their DNA fingerprints

(restriction patterns): indistinguishable (>95% homology), closely related (85-95% homology), possibly related (>85% homology, but Tenover's criteria are not fulfilled) and different (<85% homology) (TENOVER ET AL., 1995). Strains with >95% homology are classified as the members of the same clone, while isolates with <85% homology are considered as sporadic strains.

3. RESULTS

3.1 Results of isolation and identification of *Acinetobacter* strains

According to my data obtained with the examination of 403 samples, the detection rate of cultivable acinetobacters in Hungary was 12.2% in the period between 2008 and 2018 (Table 1.). Two more *Acinetobacter* strains were identified from Tanzanian soil samples. In total, 51 *Acinetobacter* strain were collected.

Table 1. Detection frequency of *Acinetobacter* species in Hungary

	Number of examined samples from Hungary	Number of samples, with cultivable *Acinetobacter spp.*	Number of isolated *Acinetobacter* strains	Rate of detection (%)
groundwater	341	27	31	9.1
surface water	28	8	11	39.3
soil	15	3	3	20
other	19	3	4	21
Total number	**403**	**41**	**49**	**12.2**

Distribution of *Acinetobacter* species among Hungarian samples is summarized in Figure 2. Strains belonging to the clinically important species *Acinetobacter baumannii* were detectable in ~1% of the examined samples.

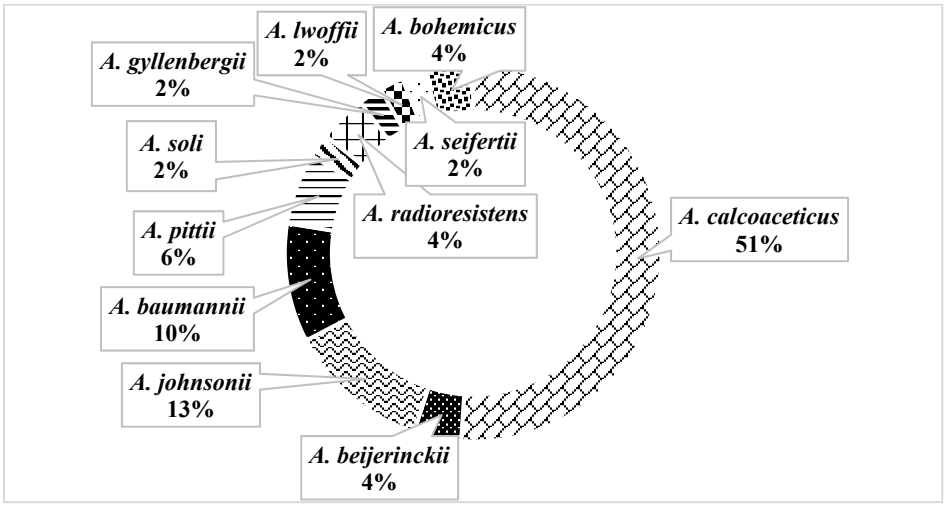


Figure 2. Distribution of *Acinetobacter* species isolated and identified from Hungarian environmental samples (%) (n=49)

NEW SCIENTIFIC RESULT (based on the results of chapter 3.1):

(Thesis 1) Based on the examination of 403 samples it was concluded, that the detection rate of cultivable *Acinetobacter* species in Hungarian environmental samples is 12.2%. *Acinetobacter* spp. were primarily detected in groundwater and surface water samples and the most frequent species was *A. calcoaceticus*. Results were published in an international scientific journal (RADÓ ET AL., 2019).

3.2 Results of antibiotic resistance investigations among *Acinetobacter* strains

52 *Acinetobacter* strains (51 environmental and the reference strain ATCC 19606) were examined with the phenotypic MIC test using 19 antimicrobial agents (Figure 3.).

Figure 3. Number of resistant, intermediate resistant and sensitive *Acinetobacter* strains against the examined antimicrobial agents (n=52); CTX- cefotaxime, CAZ-ceftazidime, CRO-ceftriaxone, FEP-cefepime, IMI-imipenem, MRP-meropenem, PIP-piperacillin, TZP-piperacillin/tazobactam, AMS-ampicillin/sulbactam, CN-gentamicin, TOB-tobramycin, AK-amikacin, NET-netilmicin, CI-ciprofloxacin, LEV-levofloxacin, CS-colistin, MN-minocycline, TGC-tigecycline, SXT-trimethoprim/sulfamethoxazole; n.d.-no data

Environmental strains T3N6 (*A. beijerinckii)*, 6/1 and J6 (*A. johnsonii)* were resistant or intermediate against agents of first line therapy (Carbapenems) and alternative treatment (Penicillins, Fluoroquinolones, Cephalosporins, Polymyxins)

of *A. baumannii* (Figure 4.), therefore these three environmental strains met the criteria of multidrug resistance (MAGIORAKOS ET AL., 2011).

Minimal Inhibitory Concentration (µg/ml)																			
Strain	Species	Cephalosporin				Carbapenem		Penicillin			Aminoglycoside				Fluoroquinolones		Polymixin	Tetracycline		Other
		CTX	CAZ	CRO	FEP	IMI	MRP	PIP	TZP	AMS	CN	TOB	AK	NET	CI	LEV	CS	MN	TGC	SXT
T3N6	*A. beijerinckii*	>256	>256	>256	48	>32	>32	24	64	32	0.38	1	3	0.5	0.75	0.25	3	0.032	0.023	0.064
J6	*A. johnsonii*	>256	>256	128	>256	0.5	0.19	6	8	>256	0.19	0.5	0.064	0.032	2	0.75	1	0.19	0.094	0.032
6/1	*A. johnsonii*	>256	>256	>256	24	0.5	0.38	>256	3	6	0.75	0.125	0.19	0.064	6	0.032	1,5	<0.016	0.023	0.064
		resistant				intermediate resistant					sensitive									

Figure 4. Antimicrobial resistance results of T3N6, J6 and 6/1 *Acinetobacter* strains

The presence of *bla*$_{OXA-51}$, which is responsible for carbapenem-hydrolysing enzyme(s) production was verified in the case of all examined *A. baumannii* strains.

3.3 Results of haemolytic activity of *Acinetobacter* strains

Haemolytic activity test revealed that the majority of *Acinetobacter* strains (48 of 52) can grow on blood agar plate, but clear haemolytic activity was detected in 9 cases only [*A. beijerinckii* (T3N6, Z4N3); *A. johnsonii* (Z4SZ2, MT-6, FK-3/1); *A. calcoaceticus* (PT2/2, 22, FK-3/0); *A. soli* (II H-4/2)].

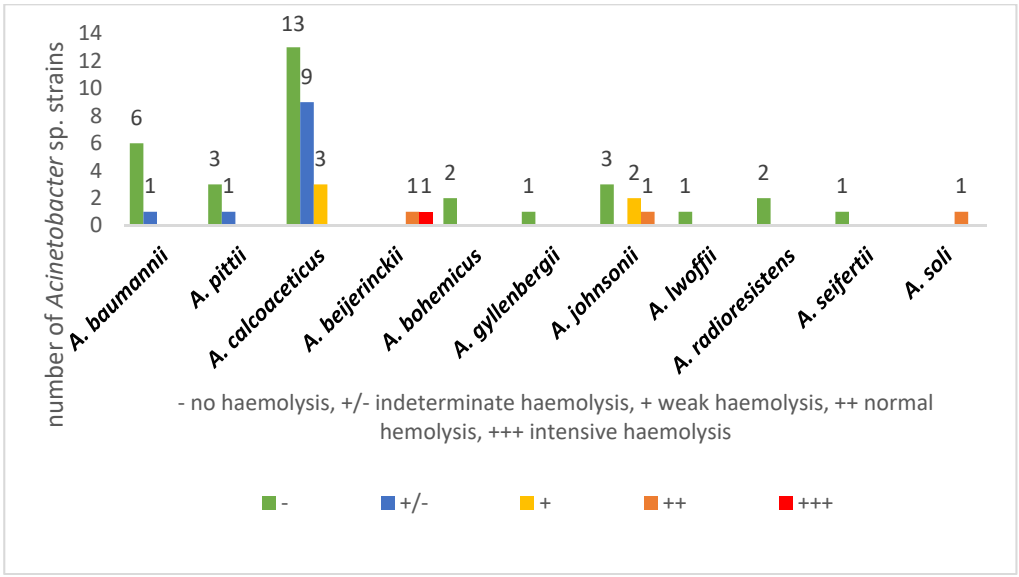


Figure 5. Results of haemolytic activity test of *Acinetobacter* strains (n=52)

Results are summarized in Figure 5. Weak haemolytic activity was detected in the case of 11 isolates. The remaining 61.5% of strains did not show any haemolytic activity.

NEW SCIENTIFIC RESULT (based on the results of chapter 3.2 and 3.3): (Thesis 2) It is the first report of an environmental, multidrug resistant *Acinetobacter beijerinckii* strain (T3N6) with intensive beta-haemolytic activity, and the first verification of haemolytic activity for an *Acinetobacter soli* strain. The new scientific results were published in an international journal (RADÓ ET AL., 2019).

3.4 Results of biofilm forming ability among *Acinetobacter* strains

According to the results of microtiter plate tests, 3 of 52 *Acinetobacter* strains showed a strong biofilm forming ability after 48 hours of incubation. TZCO3 *A. pittii* and TZSO1 *A. baumannii* isolates were strong biofilm producers with 24, 72 and 96 hours of incubation, too. J4 *A. calcoaceticus* strain was strong biofilm producer after 24 and 48 hours, but weak and intermediate biofilm former after 72 and 96 hours. The strongest biofilm producer species were *A. baumannii* and *A. pittii*.

3.5 MLST results of *Acinetobacter* strains

Six environmental *A. baumannii* and the type strain of this species were examined with Multilocus Sequence Typing method. According to the database of pubmlst.org, three of these strains related to an already existing Sequence Type (Sk-V/3, II HT-3/1 and ATCC 19606). The remaining 4 strains got newly generated ST numbers (HT-4/1, HT-4/3, JBBV and TZSO1). The phylogenetic tree of the examined strains is shown in Figure 6.

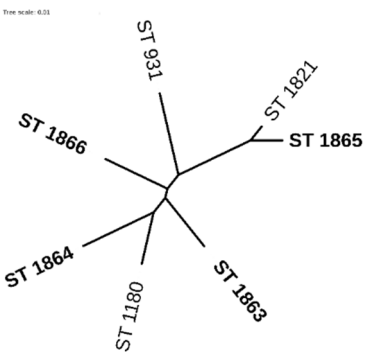


Figure 6. Phylogenetic tree of *Acinetobacter baumannii* strains (iTOL v3.6, unrooted display mode); bold font: new ST numbers

3.6 Results of isolation and identification of *P. aeruginosa* strains

Between 2008 and 2018, 73 *P. aeruginosa* strains were isolated and identified of which 64 strains originated from groundwater, soil or compost and 9 strains were isolated from surface water. According to the examination of 92 surface water samples, the detection rate of *P. aeruginosa* in surface waters was only 5.4%.

3.7 Results of antibiotic resistance assay of *P. aeruginosa* strains

A phenotypic test was performed using 74 *P. aeruginosa* strains (73 environmental and a clinical strain) and 13 clinically relevant antibiotics (Figure 7.). Based on my results, 28 strains were intermediate, and 6 strains were resistant to imipenem, while 24 strains were intermediate, and 19 strains were resistant to colistin.

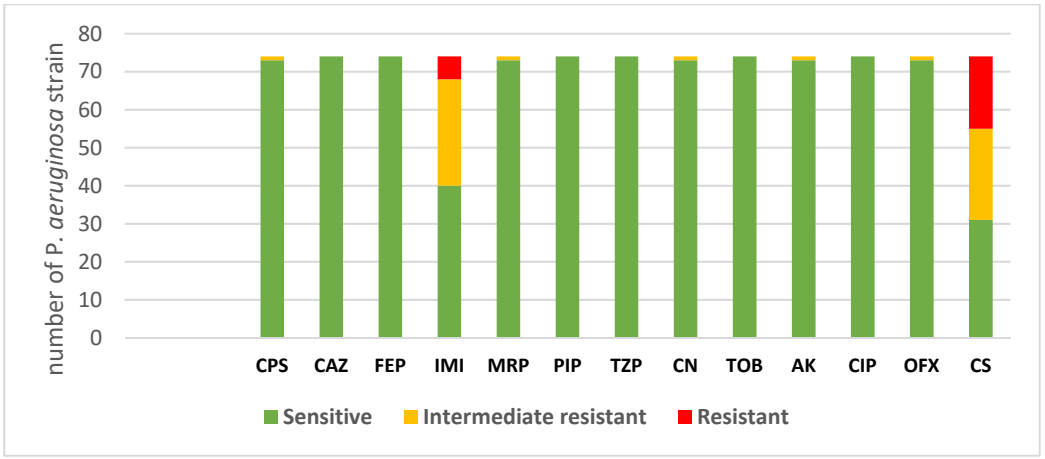


Figure 7. Number of resistant, intermediate and sensitive *P. aeruginosa* strains (n=74); CPS-cefoperazone/sulbactam, CAZ-ceftazidime, FEP-cefepime, IMI-imipenem, MRP-meropenem, PIP-piperacillin, TZP-piperacillin/tazobactam, CN-gentamicin, TOB-tobramycin, AK-amikacin, CIP-ciprofloxacin, OFX-ofloxacin, CS-colistin

3.8 Results of haemolytic activity test among *P. aeruginosa* strains

All examined *P. aeruginosa* strains showed haemolytic activity except P156, but the haemolytic activity has greatly varied (Figure 8.)

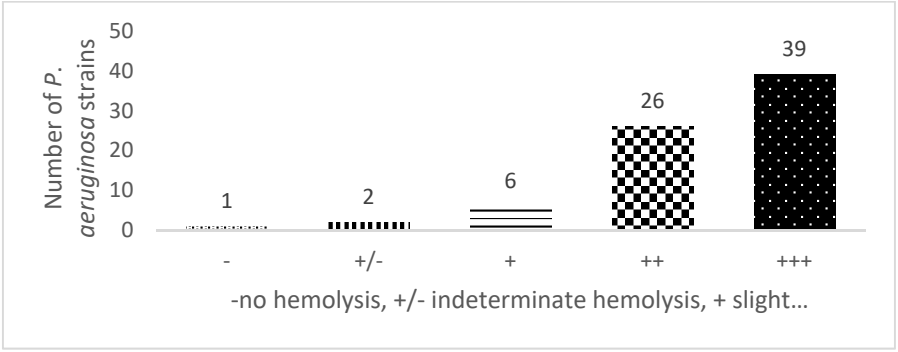


Figure 8. Result of haemolytic activity test among the examined 74 *P. aeruginosa* strain

3.9 Result of biofilm forming assay among *P. aeruginosa* strains

Based on the microplate test of 74 *P. aeruginosa* strains after 48 hours of incubation, the rate of strong biofilm producers was 10.8% (Figure 9.).

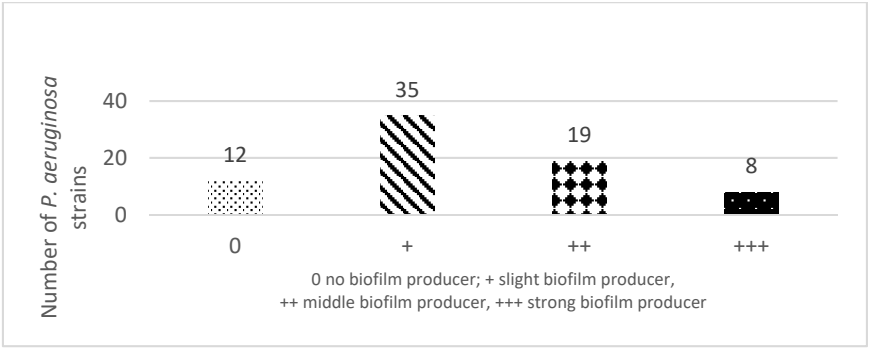


Figure 9. Results of biofilm forming ability in microplate test after 48 hours of incubation

3.10 Results of the complex examination of *P. aeruginosa* strains

Based on their antibiotic resistance profiles and their diverse origin, 46 *P. aeruginosa* strains of the 200-member strain collection of our Department were chosen for further examinations (44 environmental, 2 clinical: ATCC 27853, KPS-3)

Biofilm forming ability – comparison of microplate method and confocal laser scanning microscopy (CLSM):

Density of adhered and resolubilized cells was measured with the above-mentioned microplate method, while images of confocal laser scanning microscopy with live/dead staining revealed the structure of the formed biofilm. According to my comparative study, the accumulated cells of microtiter plates were not

necessarily three-dimensional biofilm structures. In some cases, the massive accumulation of dead planktonic cells was detected as biofilm formation, as it is was represented by strains P14 and P28 (Figure 10). Microtiter test suggested the strong biofilm forming abilities of both strains, but CLSM revealed that only P28 had a real 3-D biofilm structure, while P14 showed the accumulation of dead planktonic cells. The one-way ANOVA variance analysis revealed a significant correlation between microplate and CLSM results, however, the standard deviation was very high. My detailed analysis clarified that microplate method does not give false negative results however, the rate of false positive results was 31.8%, mainly due to the accumulation of dead planktonic cells.

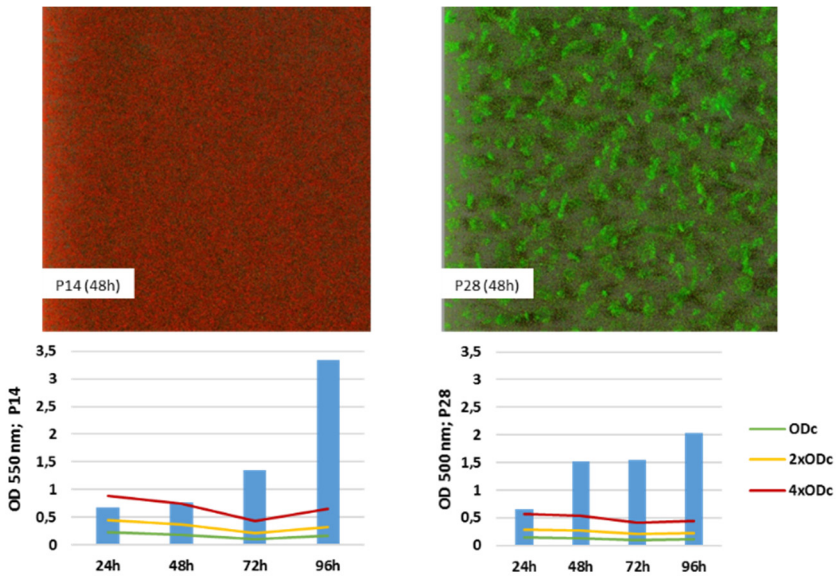


Figure 10. Planktonic cell accumulation (P14) and biofilm-forming ability (P28) of environmental *P. aeruginosa* strains by confocal laser scanning microscope (CLSM) and microplate method

NEW SCIENTIFIC RESULT (based on the results of chapter 3.10):

(Thesis 3) The comparison of microplate method and confocal laser scanning microscopy (CLSM) revealed that biofilm forming ability of environmental *P. aeruginosa* strains detected in microplate method is not always a structured biofilm. 31.8% of positive microplate results were only the accumulation of dead planktonic cells.

Results of virulence genes detection:

The presence of virulence associated genes *exoS*, *exoU, lasB*, *algD*, *aprA* and *plcH* was investigated in the case of 45 *P. aeruginosa* strains. The detection rate of these genes among environmental strains was compared to clinical data described in scientific literature, as it can be seen in Figure 11.

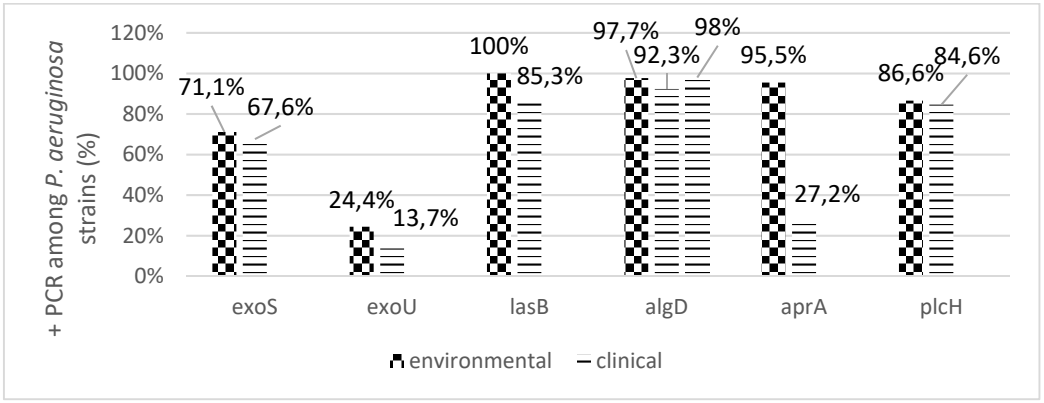


Figure 11. Detection rate of the examined virulence genes among our environmental *P. aeruginosa* strains (n=45) and in clinical environment (FAZELI&MOMTAZ, 2014; ROSHANI-ASL ET AL., 2018; GEORGESCU ET AL., 2016; TAEE ET AL., 2014; HASSUNA, 2016)

Results of serotyping:

The examined 46 *P. aeruginosa* strains were classified into 10 different serotypes (Figure 12.).

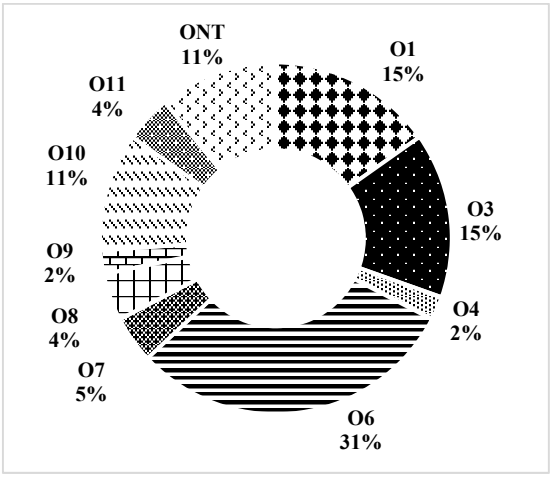


Figure 12. Distribution of serotypes among the examined environmental *P. aeruginosa* strains (%) (n=46)

Results of motility examinations:

The examined 44 environmental *P. aeruginosa* isolates showed different levels of swimming, swarming and twitching motility (Figure 13.).

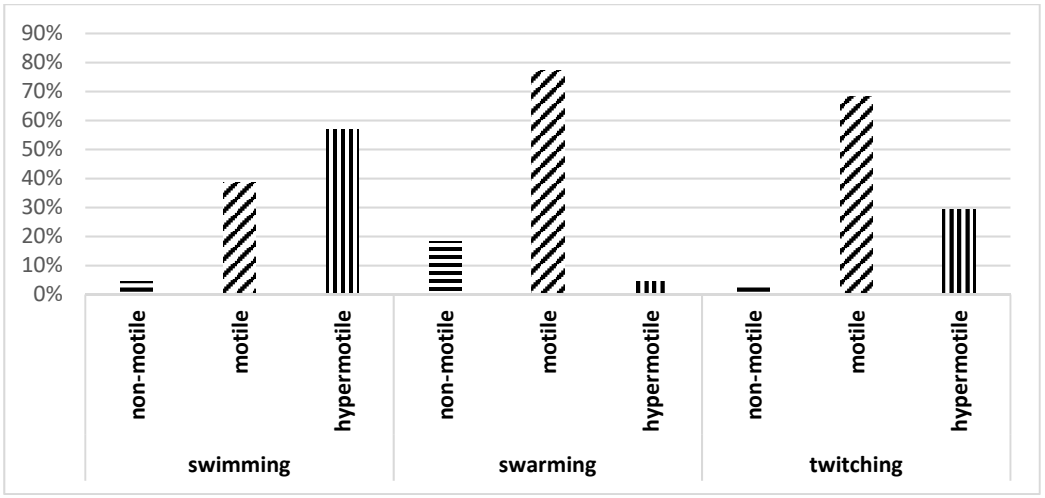


Figure 13. Distribution of motility categories among environmental *P. aeruginosa* strains (n=44)

Results of *Galleria mellonella* test:

After the evaluation of 48-hour results, 11% of examined environmental strains was avirulent (0-25% mortality), 11% was middle avirulent (25-50% mortality), 13% was middle virulent (50-75% mortality), while 65% of the strains was classified as virulent (75-100% mortality) (Figure 14.).

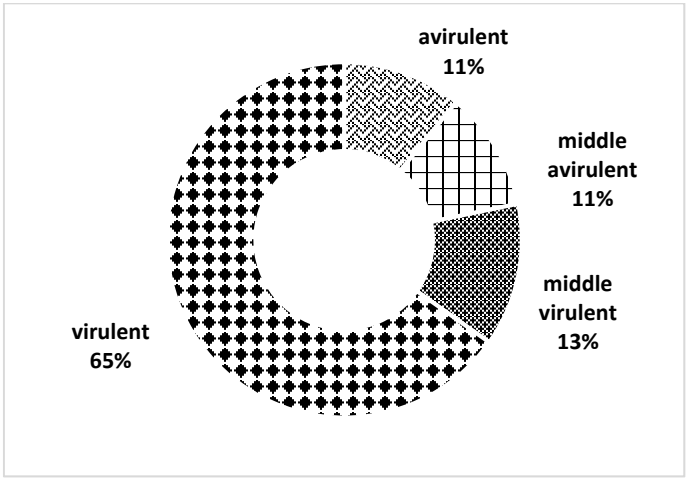


Figure 14. Distribution virulence categories among environmental *P. aeruginosa* strains in *G. mellonella* virulence test (n=46)

Results of MLST examination:

According to their allelic profile, 14 environmental and 2 clinical *P. aeruginosa* strains had a matching sequence type with an existing ST of pubmlst.org, while 4 isolates (P14, P32, P39, P46) got a new ST number. Strains P28, P53, P69, P30, P62, P42, P77, P9, P43, ATCC27853 and KPS-3 shared their STs with strains, previously isolated from patients who suffered from cystic fibroses (CF). The phylogenetic tree of the examined strains can be seen in Figure 12.

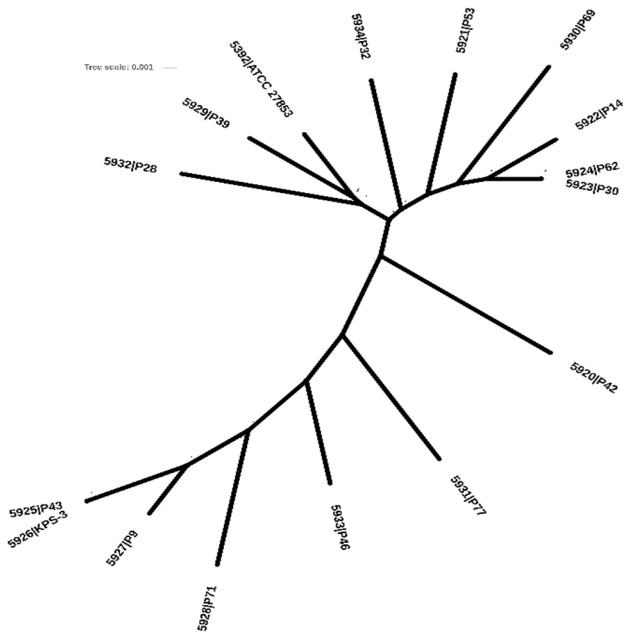


Figure 15. Dendrogram generated according to the phylogenetic relations of the examined *P. aeruginosa* strains (iTOL v3.6, unrooted display mode)

Results of PFGE examination:

Phylogenetic relationships verified that some groups of environmental strains (P66-P78-P77-P106, P79-P46, P42-P60, P113-P169, P14-P18, P62-P30, P119-P125) share the same PFGE type. Comparison with the database of the National Center for Epidemiology showed that PFGE profiles of P16, P80 and P174 have 84-88% homology with clinical records, while P59, P119, P124, P125 and P172 showed an even higher (85-95%) homology with clinical strains and met Tenover's criteria. Therefore, the common origin of several clinical and environmental strains can be

assumed. At the same time, 40.9% of environmental PFGE types was not related to any other environmental or clinical isolates of the database. These isolates are considered as sporadic strains.

NEW SCIENTIFIC RESULT (based on the results of chapter 3.10):

(Thesis 4) According to MLST and PFGE examinations of environmental *P. aeruginosa* strains, it was the first time to reveal phylogenetic relationship between Hungarian environmental isolates and strains from cystic fibrosis patients all around the world, furthermore with nosocomial isolates originated from Hungarian hospitals. Results were published in an international journal (RADÓ ET AL., 2017).

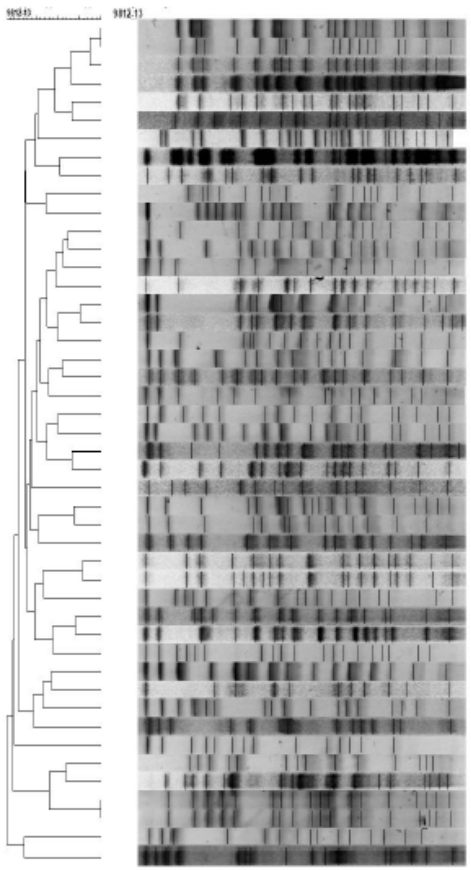
3.11 Comparative evaluation of different features of *P. aeruginosa* strains

Relationship between antibiotic resistance and virulence in *Galleria mellonella* test (n=46):

Based on a statistical evaluation of environmental strains, significant, negative (inverse) correlation was detected between antibiotic resistance to five different antibiotics (gentamicin, imipenem, cefepime, ceftazidime, cefoperazone-sulbactam) and the rate of mortality in the *G. mellonella* test. Thus, higher minimum inhibitory concentrations led to lower lethality in the *in vivo* experiment.

Relationship of motility and virulence in *Galleria mellonella* test (n=46):

Significant, positive correlation was detected between twitching motility and virulence in *G. mellonella* model, which means that mortality of hypermotile strains was higher. The higher capacity for any form of motility (swimming, swarming, twitching) increased the mortality rate, which means that the role of twitching in infection can be partly replaced by motility/hypermotility in swimming/swarming. This finding is verified by the fact that hypermotility was completely absent among avirulent strains with only one exception (P14).



Designation of strains	Serotype	Motility			Virulence in *G. mellonella* test (48h)		Virulence						Hemolytic activity	Biofilm forming (48h)
		Swimming (cm^2)	Swarming (cm^2)	Twitching (cm^2)	Mortality (%)	Category	*exo S*	*exo U*	*lasB*	*algD*	*aprA*	*plc H*		
P66	O11+	HM	M	HM	95	virulent	+	-	+	+	+	+	+++	+++
P78	O7	HM	M	M	90	virulent	+	-	+	+	+	+	+++	+
P77	O8	M	M	M	85	virulent	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	++
P106	O8	HM	NM	M	100	virulent	+	-	+	+	+	+	+	+++
P28	O1	M	M	M	5	avirulent	+	-	+	+	+	+	+	+++
P2	ONT	M	NM	M	65	middle virulent	+	-	+	+	+	-	-	+++
P164	O11+	M	M	M	100	virulent	+	-	+	+	+	-	+++	0
P9	ONT	NM	NM	M	25	avirulent	-	+	+	+	+	+	-	++
P10	O3	M	NM	M	40	middle avirulent	+	-	+	+	+	+	-	+
P32	O7	M	NM	M	55	middle virulent	+	-	+	+	+	+	++	+++
P170	O1+	HM	NM	M	100	virulent	+	-	+	+	+	-	-	++
P172	O6+	HM	M	M	100	virulent	+	-	+	+	+	-	+	+
P177	O6+	HM	M	HM	70	middle virulent	+	-	+	+	+	+	++	+
P80	O6+	NM	M	M	100	virulent	+	-	+	+	+	+	+++	++
P3	ONT	HM	M	HM	80	virulent	-	+	+	+	+	+	+	+
P79	O6+	HM	M	M	80	virulent	-	+	+	+	+	+	++	+++
P46	O6	M	M	M	70	middle virulent	+	-	+	+	+	+	+	+++
P59	O6+	HM	M	M	80	virulent	+	-	+	+	+	+	+++	+++
ATCC 27853*	O6	n.d.	n.d.	n.d.	85	virulent	+	-	+	+	+	+	+	++
P71	O1	M	M	HM	95	virulent	-	+	+	+	+	+	++	+
P104	O4+	HM	M	M	100	virulent	+	-	+	+	+	+	+++	++
P16	O9+	HM	NM	M	50	middle avirulent	+	-	+	+	+	-	+++	+++
P124	ONT	HM	HM	M	100	virulent	+	-	+	+	+	+	+++	+
P60	O6	M	M	HM	95	virulent	+	-	+	+	+	+	+++	+++
P42	O6	HM	M	HM	90	virulent	+	-	+	+	+	+	+	++
P114	O1	HM	HM	HM	95	virulent	+	-	+	+	+	+	+++	+
P113	O10+	HM	M	M	80	virulent	+	-	+	+	+	+	+	+
P169	O10+	HM	M	M	95	virulent	+	-	+	+	+	+	+	++
P69	O6	M	M	M	50	middle avirulent	-	+	+	+	+	+	++	0
P14	O3	HM	NM	M	5	avirulent	-	+	+	+	+	+	+/-	+++
P18	O3	M	M	M	10	avirulent	-	+	+	+	+	+	+/-	+
P144	O1+	HM	M	HM	100	virulent	-	+	+	-	-	-	+	0
P62	O3	M	M	HM	100	virulent	-	-	+	+	+	+	-	+
P30	O3	M	M	M	75	middle virulent	-	-	+	+	+	+	-	++
P152	O1+	HM	M	M	100	virulent	+	-	+	+	+	+	0	+
P183	O6+	M	M	HM	95	virulent	+	-	+	+	+	+	+++	+++
P39	O6	M	M	M	90	virulent	+	-	+	+	+	+	+++	++
P174	O1	HM	M	HM	100	virulent	+	-	+	+	+	+	+++	+
P53	O6	M	M	HM	90	virulent	+	-	+	+	+	+	+	0
P160	O6+	HM	M	M	100	virulent	+	-	+	+	+	+	+	+
KPS-3*	O10	n.d.	n.d.	n.d.	65	middle virulent	-	+	+	+	+	+	+	+++
P43	ONT	M	M	M	10	avirulent	-	+	+	+	+	+	+++	+
P119	O10+	HM	M	M	100	virulent	-	+	+	+	+	+	+	+++
P125	O10+	HM	M	M	100	virulent	-	+	+	-	-	-	+	++
P135	O3+	HM	M	HM	30	middle avirulent	-	-	+	+	+	+	+++	+
P108	O3	HM	M	NM	40	middle avirulent	-	-	+	+	+	+	++	+++

Figure 16. Phylogenetic tree based on PFGE examination and results of 46 *P. aeruginosa* strains in different examinations (serotype, motility, virulence in *Galleria mellonella* test, virulence genes, haemolytic activity, biofilm forming ability in microplate method)

NEW SCIENTIFIC RESULT (based on the results of chapter 3.10.1):

(Thesis 5) Motility of environmental *P. aeruginosa* influences the outcome (lethality) in the *G. mellonella* infection. Hypermotility and twitching is in positive correlation with results of the applied phenotypic virulence test.

Correlation between serotyping and virulence in *Galleria mellonella* test (n=46), and connection between serotypes and results of MLST (n=16):

Environmental strains belonging to clinically relevant serotypes (O1, O6, O10, O11) caused higher rate of lethality in average than non-clinical (O3, O4, O7, O8, O9) or non-typable (ONT) strains. However, due to the very high standard deviation values, it is not a significant difference. According to scientific resources, infections caused by O1 serotype are more severe (LU ET AL., 2014), as it was confirmed by the high rate of mortality in the applied *G. mellonella* test.

Correlation between the presence of virulence genes, haemolytic activity, phenotypic virulence and serotypes (n=46):

According to statistical analysis, there is no significant correlation between the presence of the examined virulence genes (*lasB, algD, aprA, plcH, exoS, exoU*), their haemolytic activity and their mortality in *G. mellonella* test. The only exception was the simultaneous lack of *exoS* and *exoU* genes (strains P108 and P135). A new profile was reported among environmental *P. aeruginosa*: as far as we know P71 strain is the first detection of an O1 serotype strain carrying *exoU* gene.

According to my simultaneous examination of phenotypic virulence and virulence factors, the hypothesis to determine the pathogenicity of a given strain based on the presence or absence of virulence genes (DIVYA ET AL., 2018; RADHAPRIYA ET AL., 2015) was disproved.

NEW SCIENTIFIC RESULT (based on the results of chapter 3.10.1):

(Thesis 6) It was the first report of an O1 serotype *P. aeruginosa* strain carrying *exoU* gene. P71 strain originated from a hydrocarbon contaminated

groundwater sample of Debrecen (Hungary). Results were published in an international journal (RADÓ ET AL., 2017).

Characterization of environmental *P. aeruginosa* strains according to their pulse-field types and their virulence, antibiotic resistance profiles (n=46):

Strains with different virulence and antibiotic resistance profiles were randomly located on phylogenetic tree generated by PFGE fingerprinting (Figure 16.). Significant correlation was not detected among the possession of virulence genes, biofilm forming ability, motility. According to PFGE, environmental strains belonging to the same clone may have different *exoS/exoU* profile, biofilm forming ability, haemolytic activity or motility properties, furthermore they can belong to different serotypes.

Characterization of environmental and clinical *P. aeruginosa* strains of the same PFGE types (n=5):

According to the database of the National Center for Epidemiology, several environmental strains were closely related to clinical isolates (P59, P119, P124, P125, P172), moreover they shared several additional properties. Most of them belonged to the clinically relevant O6 and O10 serotypes. Clinically related environmental strains showed hypermotility or motility in swimming, swarming, and/or twitching and their pathogenicity was verified with genotypic and phenotypic methods such as the presence of *lasB* and *exoS*/*exoU* genes. At the same time, their virulence arsenal, haemolytic activity, biofilm forming ability and geographical origin were different.

Comparison of PFGE and MLST assay (n=16):

14 environmental and 2 clinical strains were examined with both genotyping methods. It was shown that a matching ST number indicates the close relationship of PFGE genotypes, too. At the same time, the reverse connection between PFGE and MLST types is not verified. In the future, MLST can be suggested to explore the clonal relationship of strains from geographically diverse origin and the more

discriminatory PFGE method can be proposed to monitor genetic variations of environmental strains isolated from the same site.

NEW SCIENTIFIC RESULT (based on the results of chapter 3):

(Thesis 7) Based on my complex, statistical analysis of virulence, antibiotic resistance, biofilm forming and phylogenetic properties among environmental *P. aeruginosa* strains it was verified, that none of the examined virulence, resistance or biofilm forming factors can be used to clearly determine the human health concerns of a given strain. According to my results, the currently available phenotypic or genotypic methods are not applicable to ensure the biological safety of a given *P. aeruginosa* strain.

4. CONCLUSIONS AND PROPOSALS

Based on examination of more than 400 environmental samples of Hungary, the detection rate of *Acinetobacter baumannii* in natural environment was only 1%. The primary source of *A. baumannii* is surface water. The *bla*$_{OXA-51}$ gene, associated with carbapenem-hydrolysing enzyme production, was detected in all examined *A. baumannii* strains, however their carbapenem resistance was not expressed phenotypically. The examined *A. baumannii* strains showed slight or moderate biofilm forming ability and had no haemolytic activity. According to Multilocus Sequence Typing, only two matches were found with previously determined STs of the international database (a Russian sample of unknown origin and an American sample of animal origin) which means inadequate information for exploring the phylogenetic relationship of my strains.

Regarding other species of genus *Acinetobacter*, 11 different species were isolated from Hungarian environmental samples with the dominance of *A. calcoaceticus*. Based on the phenotypic antibiotic resistance examination, three environmental strains of two species (*A. beijerinckii*, *A. johnsonii*) were resistant to at least three classes of antibiotic agents thus fulfilled the criteria of multidrug resistance (MAGIORAKOS ET AL., 2011). According to our current knowledge, it was the first time to report a multidrug-resistant and haemolytic representative of *A. beijerinckii*, which means that natural environment can be a reservoir of pathogenic microorganisms with increasing human health concerns.

As a result of my work, the current knowledge of the role of *P. aeruginosa* in surface water was increased, and a detection rate of 5.4% was determined. Altogether, 73 new strains were isolated from groundwater, surface water, soil and compost samples and further examinations were done with 46 members of the departmental strain collection.

Our environmental strains were classified into four, clinically important (O1-15%, O6-31%, O10-11%, O11-4%) and further six, non-clinical serotypes (O3, O4, O7, O8, O9, ONT) with the dominance of O6. Environmental antibiotic resistance was notable to imipenem (Carbapenem agent) and outstanding to colistin with 8.3%

and 25.6% of resistant and 38.8% and 32.4% of intermediate categories, respectively. Biofilm forming ability of environmental *P. aeruginosa* strains determined by microplate method was checked by confocal laser scanning microscopy and the reliability of microplate assay was revised. Regarding virulence genes, the presence of *lasB*, *algD* and *aprA* was proved to be general among environmental *P. aeruginosa,* with a higher frequency than in clinical settings. The invasive *exoS* gene was detected in 32 strains (71.1%), while *exoU* associated with cytotoxicity was present in 11 isolates (24.4%). The simultaneous lack of *exoS* and *exoU* was determined as a new environmental genotype and was attributed with a lower rate of lethality in the applied virulence test. No other virulence genotypes were correlated with phenotypic virulence results in the *G. mellonella* test. The detection rate of *plcH* which is responsible for phospholipase C production and play a role in haemolytic activity was 86.6%, but the presence or absence of this gene did not correlate with phenotypically experienced beta-haemolysis on Columbia blood agar.

In summary, my results verified that the widely accepted practice to determine pathogenicity based on the presence or absence of virulence genes (DIVYA ET AL., 2018; RADHAPRIYA ET AL., 2015) is not correct. The sequential monitoring of virulence factors should be a priority in the future, since gene pools and the expression of virulence genes are in a continuous adaptation and are greatly influenced by climatic and environmental changes. Phylogenetic investigations revealed that, based on their MLST types, environmental *P. aeruginosa* strains are related to isolates originated from cystic fibroses. Based on PFGE examinations, 5 of 44 environmental strains were closely related to clinical strains. In summary, it can be assumed that clinical and environmental strains do not form completely different phylogenetic groups and presumably use several pathways to be in connection.

46 environmental *P. aeruginosa* strains were examined in detail with the result, that different properties such as antibiotic resistance, virulence, biofilm forming ability, serotype and motility (which play a role in pathogenicity or the outcome of

the infection) are not in a significant correlation with genetic fingerprints of MLST and PFGE methods. Most of the examined traits occur independently, which emphasizes the role of the known and widespread mobile genetic elements of species *P. aeruginosa*.

The detection of phenotypically expressed virulence with *G. mellonella* has verified, that the overwhelming majority (78%) of the examined environmental *P. aeruginosa* strains were middle virulent/virulent for this test organism. Strains belonging to O1 serotype, which is attributed to severe clinical infections, have expressed a particularly high rate of virulence in *G. mellonella* test. The positive correlation between phenotypic virulence and twitching motility or hypermotility of environmental strains was observed, which emphasize the role of motility in the clinical outcome (lethality) of an infection. I proved that antibiotic resistance to clinically important agents (gentamicin, imipenem, cefepime, ceftazidime, cefoperazone-sulbactam) significantly reduces the mortality in the *G. mellonella* test.

My results confirm the necessity of the strict legal regulation of *P. aeruginosa* in the Hungarian law, but similar requirements are recommended in an international front. Hungarian law currently does not have any regulations or limitations for genus *Acinetobacter,* but in the future, acinetobacters and especially *A. baumannii* should be incorporated into the legislation to ensure the safety of microbial formulas and the quality of drinking water.

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