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Antibiotic resistance of bacteria and incidence of carbapenamase-coding genes *blaPER* and *blaGES* in isolates from wastewater treatment plants

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ABSTRACT

The increase in number of antibiotic resistant bacteria poses a serious environmental and health problem. The development of microbial resistance is intensified by the widespread use of antibiotics in medicine, veterinary, farming and aquaculture. The wastewater treatment plants receiving the wastewater from hospitals, slaughterhouses, farms, pharmaceutical industry and houses can be potential places of spreading of antibiotic resistant genes. The molecular mechanisms of the bacterial resistance, including the horizontal transfer of antibiotic resistant genes and the presence of mobile genetic elements can be responsible for the increase in antibiotic resistance during wastewater treatment. The aim of this study was to analyze the phenomenon of bacterial resistance to selected β-lactam antibiotics (ESBL) by detecting the genes that determine this resistance. The PCR method was used to analyze the occurrence of two genes: *bla*PER and *bla*GES in wastewater samples. It was revealed that the resistant/total bacteria ratio was significantly higher in the effluent compared with the influent wastewater. Genes *bla*PER and *bla*GES were isolated from several strains predominating in both aeration tank (5% and 20% respectively) and effluent wastewater (15% and 12% respectively).

***Keywords*:** antibiotics-resistant bacteria, wastewater, genes, pollution

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1. INTRODUCTION

The presence of antibiotic resistant bacteria (ARB) and their antibiotic resistance genes (ARGs) in the environment is one of the biggest global problems nowadays [1]. The contamination of the environment with ARB poses a serious health hazard. In USA each year 14 000 people die because of resistant pathogens and each year more than 2 million people are infected [2].

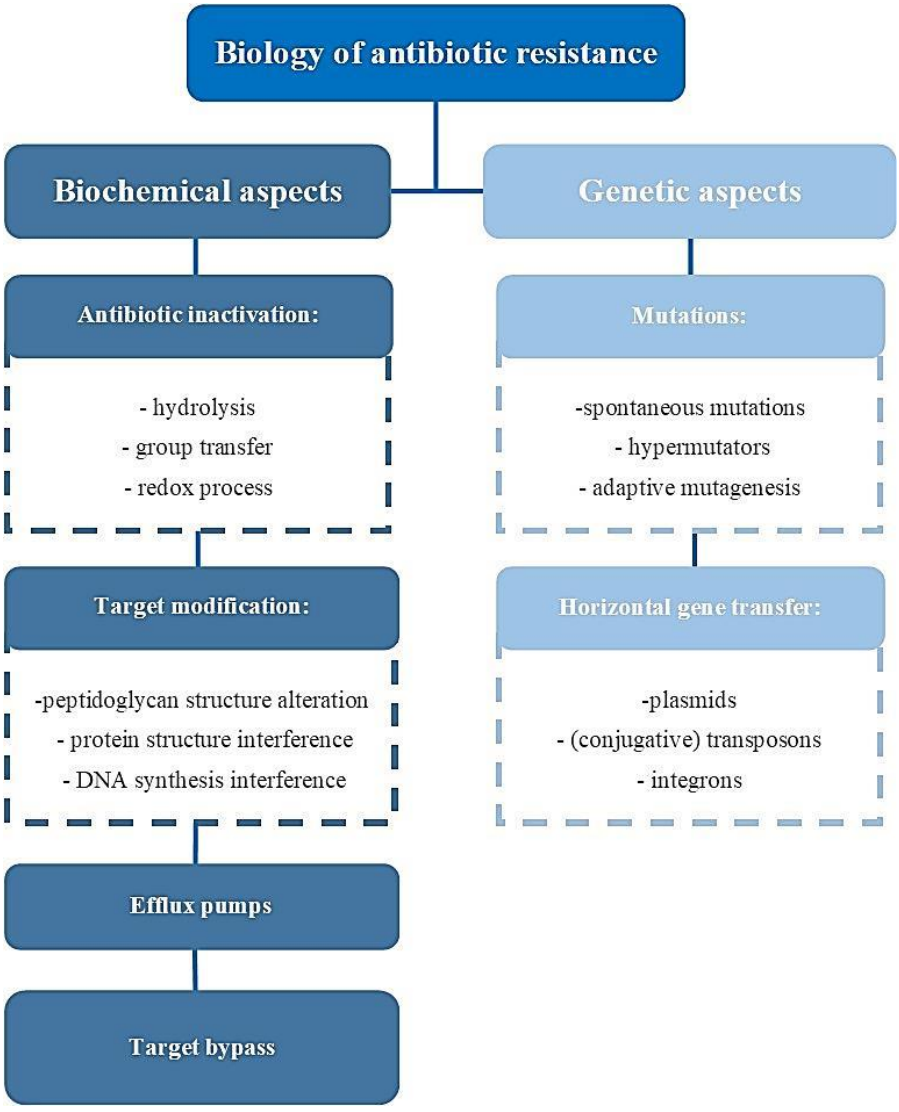

Figure 1. Types of mechanisms functioning in antibiotic resistant bacteria.

Antibiotics, the most common and important pharmaceuticals for the infectious diseases treatment, are widely used not only in medicine and veterinary but also in animal husbandry, household, aquaculture and agriculture [3, 15]. In consequence, the large amounts of

metabolized and partially metabolized antibiotics end up in wastewater. There is a lot of evidence that antibiotics are not fully biodegraded in the wastewater treatment plants (WWTPs) [4]. Moreover, wastewater treatment plants (WWTP) are the potential sources of expansion of ARB and ARGs [5]. The components of pharmaceuticals and some antibiotic resistant microorganisms can be released into the environment. Nowadays, ARB and ARGs can be found in surface waters, ground waters and soils. It was observed that the ARB can create the selection pressure on microorganisms in natural environment [2, 16].

Bacteria use different mechanisms – genetic and biochemical - to protect themselves against antibiotics (Fig. 1). The horizontal gene transfer (HGT) is the commonly observed phenomenon of antibiotic resistance in bacteria, responsible for the spreading of antibiotic resistant genes, based on three genetic mechanisms: transformation, conjugation and transduction [6].

In this study the samples of the wastewater were tested for the occurrence of antibiotic resistance genes *bla*PER and *bla*GES, responsible for the production of extended-spectrum-β-lactamases (ESBL).

2. MATERIALS AND METHODS

The wastewater samples (from different steps of wastewater treatment process, i.e. influent raw wastewater, wastewater taken from aeration tank of the biological wastewater treatment and the effluent from the wastewater treatment plant) were collected from municipal Wastewater Treatment Plant (WWTP) from April 2016 until October 2017. This WWTP receives wastewater from urban households, hospitals and industries. The analyzes were accomplished immediately after sampling.

2. 1. Quantitative microbiological analysis

The microbiological analyses covered the determination of the total number of bacteria and the number of bacteria resistant to imipenem (a broad spectrum β-lactam antibiotic used in case of severe bacterial infections). Bacteria were grown using spread-plate method on Mueller-Hinton agar (MH) without antibiotic and with an addition of 16 mg/L imipenem. The plates were incubated for 24 h at 37 °C. The results were presented as the number of colony forming units per milliliter (CFU/mL).

2. 2. DNA isolation

DNA was isolated from predominating strains of imipenem-resistant bacteria, prepared as pure cultures on solid media. For DNA isolation from bacteria the Extract Me DNA Bacteria Kit (from: DNAGDAŃSK) was used. The kit consisted of: BacL Buffer (Lysis Buffer), RNase (lyophilized), RNase Buffer, Proteinase K (lyophilized), Proteinase Buffer, BacB Buffer (Binding Buffer), BacW Buffer (Wash Buffer), Elution Buffer, DNA Purification Columns and Collection Tubes.

The RNase A and Proteinase K were suspended in respective buffers. The bacterial isolates were transferred into 300 µl of BacL Buffer in a sterile 1.5 ml Eppendorf tube and supplied with 4 µl of RNase A, mixed and incubated at 37 °C for 10 min. Then 10 µl of Proteinase K was added with subsequent mixing and incubation at 55 °C for 10 min. The next

step was an incubation with 350 µl of BacB Buffer for 5 min at 55 °C. After the incubation the sample was mixed vigorously for 15 seconds and centrifuged for 2 min at 11-15k × g. The obtained supernatant was transferred carefully into a purification minicolumn placed in a collection tube and centrifuged for 1 min at 11-15k × g. Then the purification minicolumn was put to a new collection tube and washed twice with BacW Buffer (600 µl and 400 µl respectively). The centrifugation was applied to remove the buffer's residues. After the transfer of the purification column into new sterile 1.5 ml Eppendorf, 80 µl of Elution Buffer (pre-heated to 70 °C) was added and the sample was centrifuged at 11-15k × g for 1 min. The isolated DNA was stored at -20 °C.

2. 3. PCR

The presence of *blaPER* and *blaGES* was tested by PCR (polymerase chain reaction) using specific primers (Table 1.) Two pairs of primers were designed to amplify the internal fragments by Syngen Biotech. All conditions, size of products and sequences are shown in Table 1. The composition of the reaction mixture for the detection of genes is presented in Table 2.

Table 1. Sequences of primers.

Gene	Primer sequence (5'> 3')	Size of product (bp)	Reference
BlaPER	F: TCGCCGCATACACTATTCTCAGAATGAC	422	[7]
	R: CAGCAATAAACCAGCCAGCCGGAAG		
blaGES	F: CTGGCAGGGATCGCTCACTC	604	
	R: GGTTTCCGATCAGCCACCTCTCA		

Table 2. Reaction mixture.

Reagent	Volume for 25 µl
RedTaq Master Mix (Sigma-aldrich)	12.5µl
Starter Forward (Syngen)	0,5 µl
Starter Reverse	0,5 µl
Sterilized Water (GenoPlast)	9,5 µl
DNA	2 µl

The amplification conditions were as follows:

- denaturation at 95 °C for 4 minutes
- 35 cycles at 94 °C for 25 seconds,
- 58 °C for 45 seconds
- 72 °C for one minute [7].

Amplification products were visualized after running the electrophoresis at 100 V for 50 minutes in 1.2 % agarose gel.

3. RESULTS

The results of quantitative analysis revealed that both the total number of bacteria and the number of imipenem-resistant bacteria decreased after the wastewater treatment (Table 3). Hovewer, the resistantbacteria/total bacteria ratio was the highest in the effluent (Figure 2).

The analysis of the occurrence of β-lactamase resistance genes revealed that in the strains isolated from influent wastewater *bla*PER and *bla*GES genes were not detected. In the wastewater taken from the aeration tank gen *bla*PER was detected in 1 strain of 25 (5%) and gen *bla*GES in 5 strains (20%). In the effluent gen *bla*PER was detected in 5 strains of 33 isolates (15%) and gen *bla*GES in 4 isolates (12%) (Table 4)

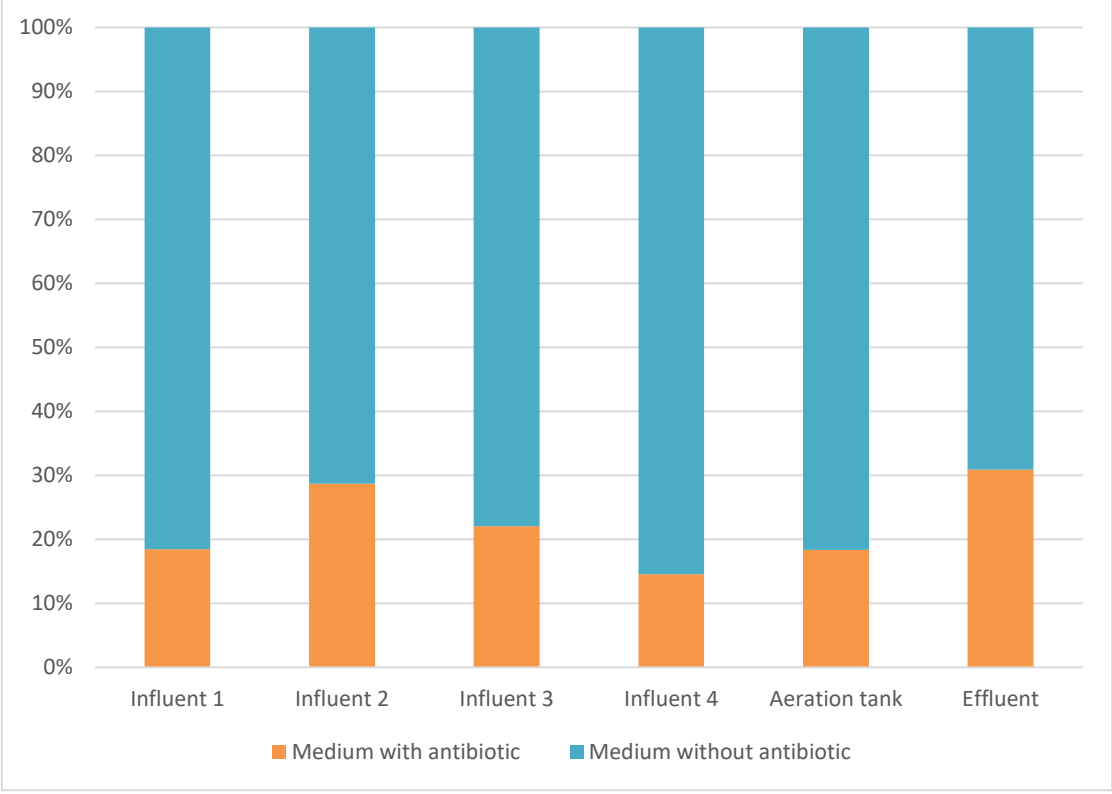

Figure 2. The number of resistant bacteria to the total number of bacteria.

Table 3. Numbers of bacteria in different samples from the wastewater treatment plant

Cultivation medium	1 Influent	2 Influent	3 Influent	Aeration tank	Effluent
MH with imipenem	$2{,}08\times10^4$	$1{,}65\times10^4$	$3{,}31\times10^4$	$1{,}10\times10^4$	$8{,}5\times10^3$
MH without antibiotic	$9{,}2\times10^4$	$4{,}1\times10^4$	$1{,}17\times10^5$	$4{,}9\times10^4$	$1{,}9\times10^4$

Table 4. Presence of resistant genes *bla*PER and *bla*GES.

Gene	1 Influent		2 Influent		3 Influent		Aeration tank		Effluent	
	Total number of isolates	Number of isolates with the gene	Total number of isolates	Number of isolates with the gene	Total number of isolates	Number of isolates with the gene	Total number of isolates	Number of isolates with the gene	Total number of isolates	Number of isolates with the gene
blaPER	9	0	9	0	8	0	25	1	33	5
blaGES	9	0	9	0	8	0	25	5	33	4

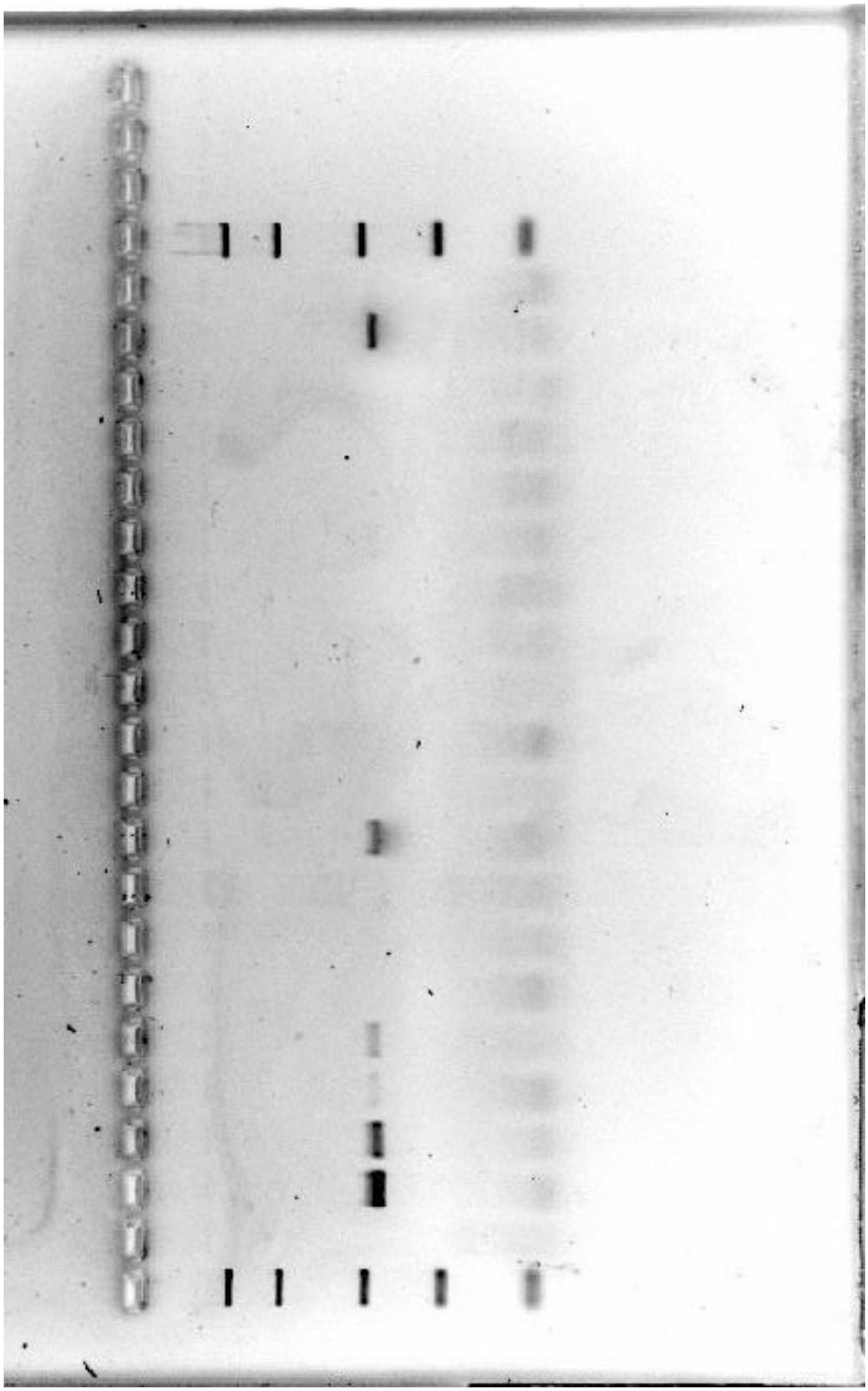

Figure 3. Detection of the *bla*PER genes.

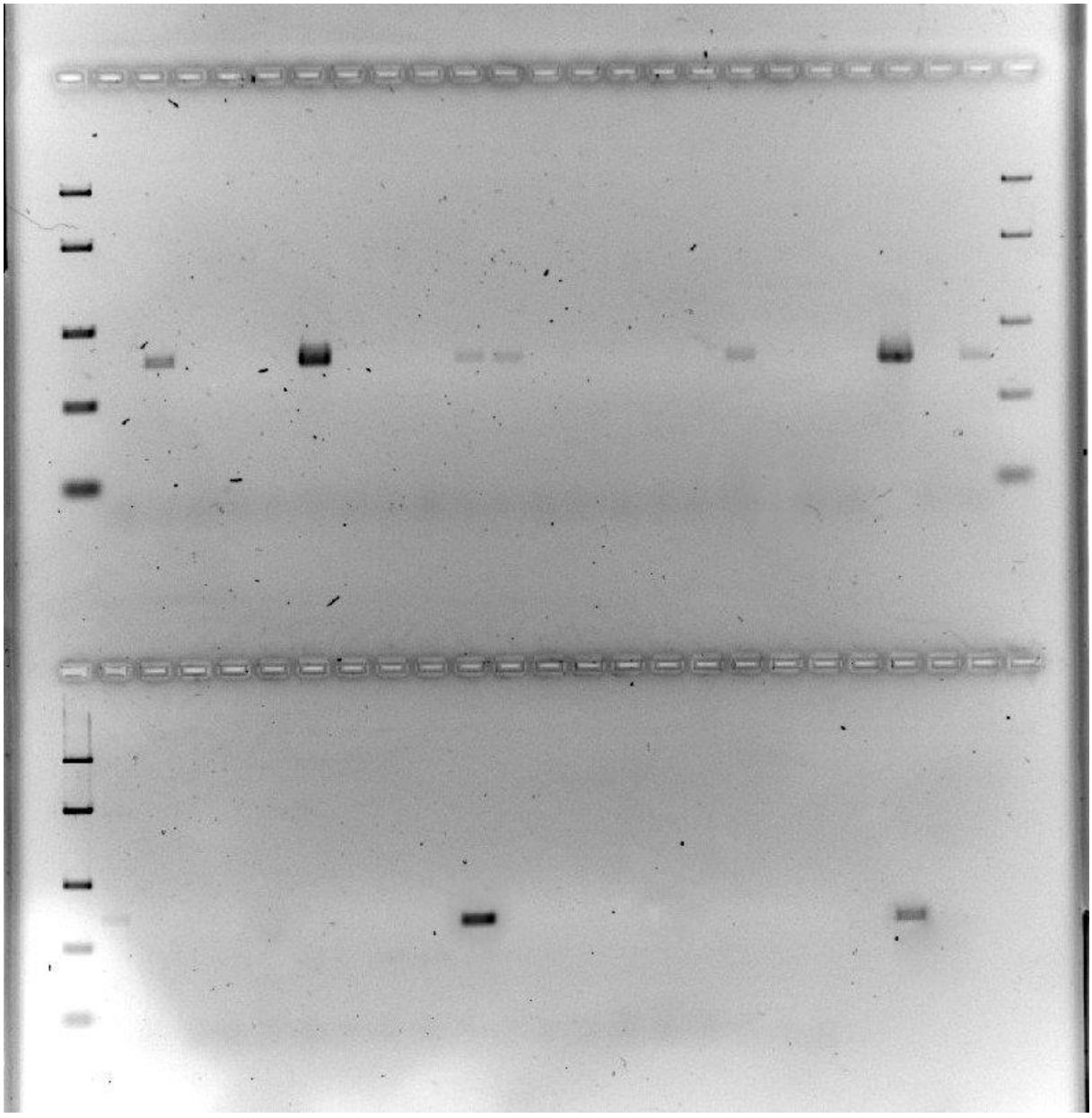

Figure 4. Detection of the *bla*GES genes.

4. DISCUSSION AND CONCLUSIONS

The molecular analysis of the antibiotic resistance genes can be a useful tool for the evaluation of the microbial potential in drug resistance. In this research two resistance genes were considered. The *bla*GES (Guiana extended spectrum) gene was detected for the first time in French Guiana in clinical strains of *Pseudomonas aeruginosa* [8]. This gene has been also reported in Asia, South America, Europe and South Africa. It was detected in various strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* [9, 17]. Most of strains carried this gene on the mobile genetic elements, usually integrons.

The *bla*PER (Pseudomonas extended resistance) was also found in *Pseudomonas aeruginosa* strain in Turkey [10], and then it was isolated in all European countries and in Asia (Korea, China, Japan and India) [11]. The *bla*PER genes are transferred by mobile genetic elements [12]. The enzyme coded by the gene is able to hydrolyze penicillin, oxyimino-cephalosporins and aztreonam [13]. In this research the quantitative analysis of bacteria in wastewater revealed that antibiotc resistant bacteria as well as antibiotic resistance genes can be present at different stages of the wastewater treatment. The percentage of antibiotic resistant bacteria in effluent was comparatively higher than in influent wastewater, so the effluent from the wastewater treatment plant can still contain some antibiotic resistant bacteria, which can end up in the environment. Moreover, the increase in the frequency of occurrence of the tested resistance genes *bla*PER and *bla*GES was observed during the process of wastewater treatment, although they were absent in influent wastewater samples. The *bla*PER gene was more common in the effluent even compared to the wastewater in biological treatment aeration tank. It may suggest the potential transfer of antibiotic resistance genes between bacteria in wastewater treatment process. According to several literature data, such genes as *bla*GES are related to mobile genetic elements like integrons [14]. This study confirmed that the WWTP is not able to remove all bacterial pollutants. The presence of high numbers of *bla*PER and *bla*GES genes in the effluent can be the reservoir of genes of a clinical relevance.

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References

[1] T. Schwartz , W. Kohnen, B. Jansen, U. Obst, FEMS *Microbiology Ecology* 43(3) (2003) 325-35.

[2] A. Pruden, R. Pei, H. Storteboom, K.H. Carlson, *Environmental Science & Technology* 40 (2006) 7445-7450.

[3] Rodriguez-Mozaz S, Chamorro S, Marti E, Martia E, Huertaa B, Grosa M, Sànchez-Melsióa A, Borregoa CM, Barceló a D, Balcázar JL, *Water Research* 69 (2015) 234-242.

[4] K. Kümmerer, A. al-Ahmad, V. Mersch-Sundermann, *Chemosphere* 40 (7) (2000) 701-10.

[5] Y.Yang, B. Li, F. Ju, T. Zhang, *Environmental Science & Technology* 47 (18) (2013) 10197-10205.

[6] A. R. Burmeister, *Evolution, Medicine, and Public Health* (2015) 193-194.

[7] NT. Trung, TT. Hien, TT. Huyen, DT. Quyen, MT Binh , PQ Hoan, CG. Meyer, TP. Velavan, H. Song le, *Annals of Clinical Microbiology and Antimicrobials* (2015) 14 23.

[8] K. Wolska, B. Kot, M. Piechota, A. Frankowska. *Advances in Hygiene and Experimental Medicine* 67 (2013) 1300-1311.

[9] P. Bogaerts, T. Naas, F. El Garch, G. Cuzon, A. Deplano, T. Delaire, T. Huang, B. Lissoir, P. Nordmann, Y. Glupczynski, *Antimicrobial Agents and Chemotherapy* 54 (11) (2010) 4872-4878.

[10] G. Celenza, C. Pellegrini, M. Caccamo, B. Segatore, G. Amicosante, M. Perilli, *Journal of Antimicrobial Chemotherapy* 57 (2006) 975-978.

[11] IK. Bae, SJ. Jang, J. Kim, SH. Jeong, B. Cho, K. Lee, *Antimicrobial Agents and Chemotherapy* 55 (3) (2011) 1305-1307.

[12] A. Opuş, T. Yıldırım, A. Birinci, B. Durupınar, *Biomedical Research* 28 (6) (2017) 2689-2695.

[13] A. Namysłowska, A.E. Laudy, S. Tyski, *Postepy Mikrobiologii* 54 (4) (2015) 392-406.

[14] L. Poirel, A. Carrër, JD. Pitout, P. Nordmann, *Antimicrobial Agents and Chemotherapy* (2009) Jun, 53(6) 2492-2498.

[15] I. Bueno, J. Williams-Nguyen, H. Hwang, JM. Sargeant, AJ. Nault, RS. Singer R, *Zoonoses Public Health* 65(1) (2018)162-184.

[16] LK. Zou, LW. Li, X. Pan, GB. Tian, Y. Luo, Q. Wu, B. Li, L. Cheng, JJ. Xiao, S. Hu, Y. Zhou, YJ. Pang, *World Journal of Microbiology and Biotechnology* 28 (5) (2012) 1891-1899.

[17] RA. Bonnin, P. Nordmann, A. Potron, H. Lecuyer, JR. Zahar, L. Poirel, *Antimicrobial Agents and Chemotherapy* 55 (1) (2011) 349-354