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Resistance to Antibiotics in Plankton and Biofilm Cultures of *Pseudomonas aeruginosa* Clinical Strains

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Biofilms formed by *Pseudomonas aeruginosa* strains isolated from biomaterial of patients with implant-associated infection are characterized by much higher resistance to antibiotics of various classes than plankton cultures of these strains. The concentrations of antibiotics causing the death of 90% of *P. aeruginosa* biofilm (MIC_{90}) was 2-6 μg/ml for fluoroquinolones, 267-356 μg/ml for cephalosporins, and 92-215 μg/ml for amikacin, which significantly ($p<0.05$) differed from MIC_{90} for plankton cultures that did not exceed 0.8 μg/ml for fluoroquinolones, 19 μg/ml for cephalosporins, and 3 μg/ml for amikacin. The degree of the microbial biofilm maturity also affected antibiotic resistance.

Key Words: *implant-associated infection; preformed biofilms; antibiotics; Pseudomonas aeruginosa*

Treatment of infectious inflammatory complications after total replacements of large joints remains a challenging issue due to the severity of state in these patients, complexity of bacteria diagnosis in this pathology, and empiric etiotropic treatment [4]. Implant-associated infections (IAI) caused by gram-negative bacteria including *Pseudomonas aeruginosa* that exhibits high resistance to antibiotics and liability to biofilm formation are still a problem in orthopedic surgery [5]. In a biofilm formed by gram-negative bacteria, virulence factors can be activated due to quorum sensing [7]. *P. aeruginosa* can form biofilms within 24-48 h, which makes etiotropic treatment inefficient and often leads to the development of chronic IAI [5,7]. It was established that some antibiotics in concentrations used in clinical practice can stimulate biofilm growth [1,2]. Some features of IAI pathogenesis require new approaches to its diagnostics, changes in antibiotic treatment tactics, search for alternative methods of destruction, and inactivation of microbic biofilms [1,2,8].

Here we studied the effect of antibiotics with different mechanisms of action on plankton and sessile *P. aeruginosa* strains isolated from patients with IAI.

MATERIALS AND METHODS

We analyzed antibiotic resistance of plankton and sessile forms of 10 *P. aeruginosa* strains harvested from various biological materials of patients with infectious complications after primary replacements of large joints.

The minimum inhibition concentrations inducing death of 90% bacterial cells (MIC_{90}) of cephalosporins, fluoroquinolones, and amikacin were compared. For plankton cultures, antibiotic MIC_{90} was determined according to clinical recommendations using the method of serial dilutions: a suspension of 24-h

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bacterial culture with optical density of 1 McFarland standard corresponding to 3×10^8 CFU/ml was prepared using Densi-La-Meter densitometer (Lachema) and then diluted with fish hydrolysate to a concentration of 10^3 CFU/ml. Different concentrations of antibiotics in sterile distilled water and bacterial suspension were mixed 1:1 in wells of cultural plates and incubated for 24 h at 37°C. The culture fluid with the plankton form of bacteria was collected in a sterile plate. MIC_{90} corresponded to the concentration of antibiotic ensuring the absence of visible bacterial growth in a well. Inoculation on solid culture media resulted in the growth of single colonies in 10% isolates.

The biofilms were formed in sterile 96-well plates: 200 µl bacterial suspension (10^3 CFU/ml) was added to each well and the plates were incubated for 24 and 48 h at 37°C. The culture fluid was then aspirated and 200 µl antibiotic of a certain concentration was added to fish hydrolysate. The plates were incubated for 24 h at 37°C and then rinsed with 0.9% NaCl. The biofilm was destructed by ultrasound (37 kHz for 30 min). After resuspending, 100 µl cultures were inoculated on Endo agar and the bacterial growth was evaluated. The plates with bacterial biofilms preformed over 24 and 48 h without antibiotics served as the control.

The data were processed with using Statistica 12.0 software (StatSoft, Inc.). The median (Me), and quartiles (Q1; Q3) were calculated using nonparametric methods; for multiple comparisons, the Kruskal—Wallis test was applied. The differences were significant at $p<0.05$.

RESULTS

The sensitivity to analyzed antibiotics in the plankton forms of *P. aeruginosa* clinical strains was significantly ($p<0.05$) higher than in biofilms. Levofloxacin was most efficient against *P. aeruginosa* biofilms; its MIC_{90} did not exceed the concentrations used in clinical practice with significant ($p<0.05$) differences in concentrations for plankton and biofilm forms.

For another fluoroquinolone, ciprofloxacin, MIC_{90} was 25-74 µg/ml with significant ($p<0.05$) differences in the concentrations for plankton and biofilm forms, which considerably surpassed the permissible therapeutic concentrations. The use of this drug in clinical practice is therefore limited.

The comparison of MIC_{90} for cephalosporins (ceftriaxone, cefoperazone) for *P. aeruginosa* biofilm showed that resistance biofilm to this group of antibiotics increased by 19 and 14 times in 24-h preformed biofilm and by 23 and 18 times in 48 h biofilm, respectively, in comparison to bacteria in their plankton form turning the use of cephalosporins inefficient in infections associated with biofilms.

Aminoglycoside representative amikacin commonly used for infectious complications caused by *P. aeruginosa* showed high efficiency for plankton forms, but the resistance of 24- and 48-h biofilms was higher than that of the plankton form by 30.6 and 71.6 times, respectively, which also suggests that amikacin is inefficient in inflammatory complications associated with biofilm formation.

Comparative analysis of the efficiency of antibiotics against biofilms depending on the time of their formation revealed significant ($p<0.05$) increase in the resistance with increasing the time of biofilm formation for all analyzed antibiotics, except ceftriaxone (Fig. 1).

Thus, the analyzed *P. aeruginosa* strains isolated from patients with infectious complications after large joint replacements were resistant to high

TABLE 1. Susceptibility of Clinical *P. aeruginosa* Strains (MIC_{90}, µg/ml) in Biofilm and Plankton Form (Me (Q1; Q3))

Drug	Plankton forms (n=10)	Biofilms	
		24-h incubation (n=10)	48-h incubation (n=10)
Levofloxacin	0.3 (0.2; 0.4)	2 (1; 3)	6 (5; 6)
		*p=0.003	^+p=0.012566
Ciprofloxacin	0.8 (0.7; 1.0)	25 (23; 27)	74 (67; 85)
		*p=0.005	^+p=0.00001
Ceftriaxone	14 (11; 15)	293 (256; 315)	327 (295; 378)
		*p=0.0002	^+p=0.42
Cefoperazone	19 (11; 27)	267 (185; 314)	356 (312; 396)
		*p=0.0016	^+p=0.04
Amikacin	3 (1; 3)	92 (78; 110)	215 (185; 283)
		*p=0.0003	^+p=0.01

Note. Significance of differences from *plankton forms, $^+$24-h incubation. *n* is number of strains.

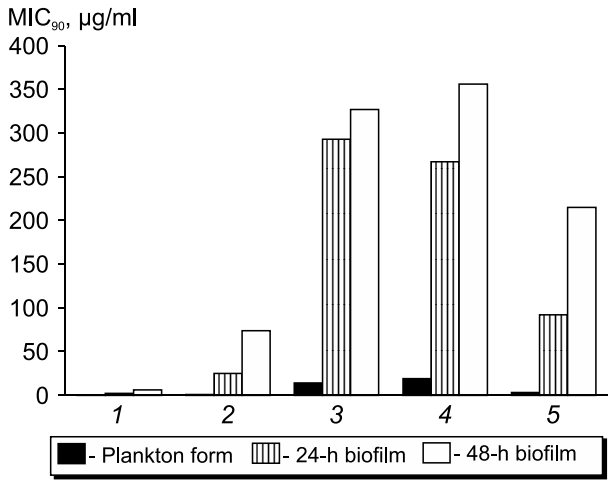

Fig. 1. Antibiotic resistance in plankton and sessile *P. aeruginosa* strains to levofloxacin (*1*), ciprofloxacin (*2*), ceftriaxone (*3*), cefoperazone (*4*), and amikacin (*5*).

concentrations of antibiotics if they form a biofilm, which confirms high protective functions of biofilm matrix [5,9]. It was established that the resistance of sessile *P. aeruginosa* strains was higher than that of their plankton form by 20-920 times for fluoroquinolones, 18-23 times for cephalosporins, and 71 times for amikacin.

Analysis of the effect of antibiotic on preformed biofilms brings the experimental conditions closer to the conditions of IAI in the body, because gram-negative nonfermentable bacteria including *P. aeruginosa* form biofilm within 24-48 h, and antibiotics are prescribed to the patients, when the biofilm has already formed in the body [5,10].

The analyzed antibiotics in therapeutic concentrations, except levofloxacin, produced no antibacterial effect on preformed biofilms. Therefore, new drugs are needed to treat infections associated with biofilms. These drugs should have various mechanisms of action and be able to destroy the biofilm matrix and affect quorum sensing and virulence factors associated with it [3,6].

The methodical errors in evaluation of antibiotics sensitivity by common bacteriological methods are related to the formation of microbial biofilms in IAI that protects microorganisms from the effect of antibacterial drugs and immune system factors due to the selective permeability of the matrix, the presence of enzyme systems that inactivate antibiotics, and the exchange of resistance determinants within the biofilm [2,4,5].

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REFERENCES

1. Babushkina IV, Mamonova IA, Ulyanov VY, Shpinyak SP, Bondarenko AS. Effects of Ciprofloxacin on the Dynamics of Biofilm Formation by Staphylococcus Epidermidis Strains Isolated from Implant-Associated Infection. Vestn. Eksper. Klin. Khir. 2020;13(3):241-247. doi: 10.18499/2070-478X-2020-13-3-241-247. Russian.
2. Babushkina IV, Ulyanov VY, Mamonova IA, Shpinyak SP. The Effect of Azithromycin on Biofilms Formation by Pathogens of Implant-Associated Infection in Large Joints. Bull. Exp. Biol. Med. 2020;169(6):798-801. doi: 10.1007/s10517-020-04982-8
3. Borodulin VB, Durnova NA, Vasiliadis RA, Losev OE, Chesovskih YS, Goroshinskaya IA, Kachesova PS, Babushkina IV, Polozhentsev OE. Study of the biological effect of iron nanoparticles. Nanotechnologies in Russia. 2015;10(3-4):268-277.
4. Murylev V, Kukovenko G, Elizarov P, Rukin Ya, Tslgln N. Periprosthetic infection during hip arthroplasty. Vrach. 2018; 29(3):17-22. doi: 10.29296/25877305-2018-03-04. Russian.
5. Babushkina IV, Bondarenko AS, Ulyanov VY, Mamonova IA. Biofilm formation by gram-negative bacteria during implant-associated infection. Bull. Exp. Biol. Med. 2020;169(3):365-368. doi: 10.1007/s10517-020-04888-5
6. Babushkina IV, Gladkova EV, Belova SV, Norkin IA. Application of preparations containing copper nanoparticles for the treatment of experimental septic wounds. Bull. Exp. Biol. Med. 2017;164(2):162-164. doi: 10.1007/s10517-017-3948-y
7. Chatterjee M, D’Morris S, Paul V, Warrier S, Vasudevan AK, Vanuopadath M, Nair SS, Paul-Prasanth B, Mohan CG, Biswas R. Mechanistic understanding of Phenyllactic acid mediated inhibition of quorum sensing and biofilm development in Pseudomonas aeruginosa. Appl. Microbiol. Biotechnol. 2017;101(22):8223-8236. doi: 10.1007/s00253-017-8546-4
8. Guitor AK, Wright GD. Antimicrobial resistance and respiratory infections. Chest. 2018;154(5):1202-1212. doi: 10.1016/j.chest.2018.06.019
9. Li S, Chen S, Fan J, Cao Z, Ouyang W, Tong N, Hu X, Hu J, Li P, Feng Z, Huang X, Li Y, Xie M, He R, Jian J, Wu B, Xu C, Wu W, Guo J, Lin J, Sun P. Anti-biofilm effect of novel thiazole acid analogs against Pseudomonas aeruginosa through IQS pathways. Eur. J. Med. Chem. 2018;145:64-73. doi: 10.1016/j.ejmech.2017.12.076
10. Müh U, Schuster M, Heim R, Singh A, Olson ER, Greenberg EP. Novel Pseudomonas aeruginosa quorum-sensing inhibitors identified in an ultra-high-throughput screen. Antimicrob. Agents Chemother. 2006;50(11):3674-3679. doi: 10.1128/AAC.00665-06