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The Effect of Azithromycin on Biofilms Formation by Pathogens of Implant-Associated Infection in Large Joints

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We studied the effect of subbacteriostatic azithromycin concentrations on the formation of microbial biofilms by *Pseudomonas aeruginosa* strains that caused implant-associated infection of large joints. Azithromycin in subinhibitory for planktonic cells concentrations 0.01-0.02 μg/ml stimulated biofilm formation by both clinical and reference *P. aeruginosa* strains, while in concentrations of 1 μg/ml and higher completely inhibited the growth of both reference and clinical plankton *P. aeruginosa* strains, but stimulated biofilm formation. Increasing azithromycin concentration to 10 μg/ml led to inhibition of *P. aeruginosa* biofilm growth.

Key Words: *azithromycin; microbic biofilms; Pseudomonas aeruginosa; replacement arthroplasty*

Biofilm formation is the mechanism responsible for the resistance of pathogens of implant-associated infection to antimicrobial treatments and immune factors as well as the mechanism underlying pathogenicity and persistence of gram-negative bacteria [1,5]. Inefficiency of etiotropic antibiotic treatment in the presence of biofilm infection is due to barrier function of biopolymer matrix providing selective diffusion of antibiotic macromolecules and due to enzyme destruction or modification of antibiotic molecules [1]. It was shown that some antibiotics in low concentrations stimulate the growth of bacterial biofilms, which can be related to expression of genes responsible for biofilm phenotype [6,10]. Some authors hypothesized that adhesion of bacteria to biotic and antibiotic surfaces can by stimulated by carbapenems [2,9].

The principles of rational etiotropic antibacterial chemotherapy do not consider peculiarities of antibiotic resistance of implant-associated infectious agents in the biofilm form.

Here we studied the effect of subbacteriostatic azithromycin concentrations on the formation of microbial biofilm by *Pseudomonas aeruginosa* strains that caused implant-associated infection of large joints.

MATERIALS AND METHODS

The study objects were 20 *P. aeruginosa* strains isolated from various biomaterials of patients with inflammatory complications following large joint replacements in 2016-2019 and a reference *P. aeruginosa* ATCC 27853 strain. The identification was performed using Crystal Autoreader (Becton Dickinson) identification system.

For preparing azithromycin stock solution with a concentration of 1 mg/ml, commercial azithromycin for intravenous infusions containing 500 mg active substance was dissolved in 500 ml of sterile water. Further dilutions were prepared using fish-flour hydrolysate broth with glucose relying on maximum concentration in the series of dilutions and considering drug dilution factor for further inoculation simultaneously for testing both clinical and reference strains.

The bacterial suspension with a concentration of 5×10^6 CFU/ml was prepared using Densi-La-Meter densitometer (PLIVA-Lachema Diagnostika). To study the sensitivity of the plankton form of bacteria to azithromycin, the suspension was diluted with fish-flour hydrolysate broth with glucose to 5×10^3 CFU/ml,

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then 1 ml suspension and 1 ml azithromycin in required dilution in fish-flour hydrolysate broth with glucose were added to sterile test tubes (final azithromycin concentrations were 0.01, 0.02, 0.05, 1.0, 3.0, and 5.0 μg/ml). Bacterial suspension without antibiotic was used as the positive control.

To study azithromycin effect on plankton forms of microorganisms, the minimum inhibitory concentration (MIC) ensuring total inhibition of visible growth of the strain under study and the inhibiting doses of antibiotic suppressing culture growth rate by 50 and 90% (ID_{50} and ID_{90}) were determined. All experiments were performed in 5 repetitions for each strain.

The effect of azithromycin on biofilm formation was assessed by the degree of gentian violet binding [12]. The calculated azithromycin concentrations were mixed with bacterial suspension in sterile tubes as described above. Then, 150 ml suspension was transferred to wells of a microtitration plate (in 5 repetitions) and incubated for 24 h followed by separation of plankton cells, biofilm staining with 0.1% water solution of gentian violet for 20 min, and triple washing with 0.9% NaCl to remove unbound dye. The intensity of biofilm formation was estimated by gentian violet extraction with 96% ethanol after 20-min incubation measured at λ=620 nm using an Epoch Microplate spectrophotometer (BioTek) (in optical density units).

The results were statistically processed using Statistica 10.0 software (StatSoft, Inc.). Shapiro—Wilk test was used to check normality of data distribution. The nonparametric Kruskal—Wallis test was used to compare several samples and Mann—Whitney *U* test was applied to compare two independent groups; the mean (*M*), standard deviation (*SD*), median (Me), and the upper and lower quartiles (Q25, Q75) were calculated. The differences were considered significant at $p<0.05$.

RESULTS

It has been found that ID_{50} for plankton reference strains and clinical strains was 0.02 and 0.05 mg/ml, which was higher than for the corresponding controls without antibiotic. ID_{90} of azithromycin for the reference and clinical strains was 0.05 and 0.1 μg/ml, respectively. Azithromycin in concentrations 1 μg/ml and higher completely inhibited the growth of both reference and clinical *P. aeruginosa* strains.

The susceptibility of the reference and clinical *P. aeruginosa* strains to various azithromycin concentrations at 24 h incubation at 37°C are presented in Figure 1.

Analysis of susceptibility of plankton strains to azithromycin allowed determining the subinhibiting and inhibiting concentrations for *P. aeruginosa* clinical and reference strains for further research of their effect on the rate of biofilm formation by these strains. The effects of various azithromycin concentrations on biofilm formation in polystyrene plates during 24 h incubation are presented in Table 1.

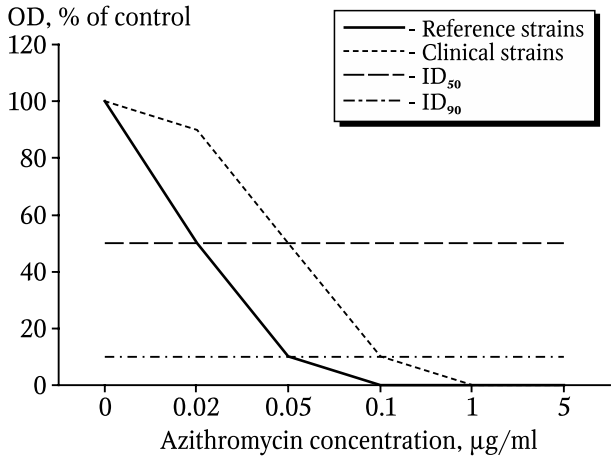

Fig. 1. The effect of azithromycin on the growth of plankton forms of clinical and reference *P. aeruginosa* strains during 24-h incubation

The investigation of biofilm formation by clinical strains in presence of azithromycin concentration of 0.01 μg/ml revealed tendency for the increase in optical density of extracts in comparison with the control without the antibiotic by 78% (Table 1, Fig. 2). Azithromycin in a concentration of 0.02 μg/ml significantly ($p<0.05$) stimulated biofilm formation in comparison with the control (Table 1, Fig. 2). After increasing azithromycin concentration to 0.5-1.0 μg/ml, the stimulating effect of the antibiotic on biofilm formation significantly ($p<0.05$) decreased in comparison with its maximum level, but remained above the control level. Azithromycin in a concentration of 10 μg/ml significantly ($p<0.05$) reduced the intensity of biofilm formation in comparison with the control (Table 1, Fig. 2).

Azithromycin in lower subinhibiting concentrations (0.01 and 0.02 μg/ml) significantly ($p<0.05$) stimulated biofilm formation by the reference *P. aeruginosa* strains. The intensity of biofilm formation was significantly ($p<0.05$) reduced by azithromycin in a concentration of 0.05 μg/ml; the optical density of stain extracts decreased to the control level at azithromycin concentration of 5 μg/ml and was below the control level at azithromycin concentration of 10 μg/ml.

It was found that azithromycin concentrations of 0.01-0.1 μg/ml are subinhibiting for plankton *P. aeruginosa* cells, but increase the rate of biofilm formation by 1.38-2.45 times. Further increase in azithromycin concentration to 1.0-10 μg/ml produced an antibacterial effect on plankton cells and reduced the rate of biofilm formation by pseudomonades (Fig. 2).

TABLE 1. The Effect of Various Azithromycin Concentrations on Optical Density of Gentian Violet Extracts from *P. aeruginosa* Strains (Me (Q25;Q75)

Azithromycin concentration	Reference strain	Clinical strain
Control	0.28 (0.26; 0.34)	1.11 (0.98; 1.26)
0.01 μg/ml	0.51 (0.49; 0.59) *p=0.03 #p=0.000001	2.10 (1.98; 2.57) °p=0.01 ˣp=0.00001 #p=0.00001
0.02 μg/ml	0.67 (0.62;0.73) *p=0.001 #p=0.000001	2.727 (2.35; 2.85) *p=0.02 #p=0.000001
0.5 μg/ml	0.35 (0.32; 0.45) +p=0.03 #p=0.02	1.54 (1.45; 1.79) ˣp=0.02 #p=0.0003
1 μg/ml	0.35 (0.32; 0.39) +p=0.01 #p=0.05	0.96 (0.84; 1.12) +p=0.001
5 μg/ml	0.29 (0.26; 0.34) +p=0.002	0.58 (0.567; 0.76) +p=0.000003
10 μg/ml	0.16 (0.15; 0.20)	0.33 (0.26; 0.38) *p=0.05

Note. Significant differences from *control, +0.02 μg/ml, #10 μg/ml, ˣ5 μg/ml, °1 μg/ml

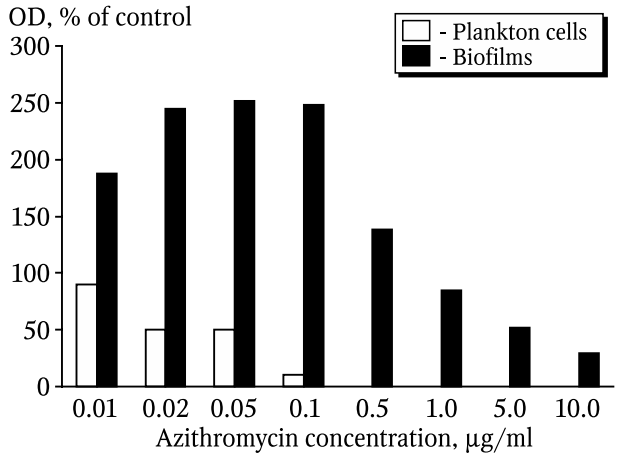

Fig. 2. The growth of plankton and sessile *P. aeruginosa* clinical strains in presence of various azithromycin concentrations.

Activation of biofilm formation by subinhibitory concentrations of various antibiotics has been described in a number of studies for many saprotrophic and pathogenic microorganisms and should be considered when prescribing etiotropic therapy [10]. Different hypotheses were put forward to this phenomenon. The effect of low doses of antibiotics on the adhesive properties of gram-negative bacteria has been proven [11]. In another report, the activating effect of tobramycin on biofilm formation was explained by its influence on the level of cyclodiguanosine monophosphate that regulates the synthesis of matrix exopolysaccharides [13]. The role of acylhomoserinlactone-dependent "quorum sensing" system (that promotes the formation of a massive matrix) in the stimulation biofilm formation by *P. chlororaphis* in the presence of azithromycin was demonstrated [3]. This effect is typical not only of pseudomonads, but also of gram-positive cocci (their matrix also contains polysaccharides) [4].

The diversity of discovered mechanisms providing induction of biofilm formation does not allow us to identify a single pattern of the stimulating effect of subinhibitory concentrations of different groups of antibiotics on biofilm formation. This necessitates further studies in this field and the development of alternative antibacterial drugs capable of destructing biofilms, *e.g.* metal nanoparticles with high antibacterial, antiviral, and antimycotic activity [7,8].

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