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Biofilms *Pseudomonas Aeruginosa*and the Prospects for Decontamination of Reservoirs of a Closed Water Purification System

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ABSTRACT

Objective: study of morphometric and densitometric parameters of biofilms bacteria Pseudomonas aeruginosa under the influence of a disinfectant.

Materials and methods: Bacteria Pseudomonas aeruginosa were cultivated at 37o C, 48 h - control; under the influence of various concentrations of the disinfectant "Abacteril" (LLC "Rudez", Russia) - experience. For morphometric and densitometric indices, 18-hour cultures of microorganisms, 105 CFU / ml were cultivated on coverslips placed in the wells of a microplate. For the detection of non-cultured microorganisms, we used media containing components for cell wall repair and L-form reversal. To obtain representative information, optical microscopy was performed by random selection of the field of view of a microscope model "BIOMED MS-1 Stereo" (Russia). The optical density (Optical Density - OD) of biofilms was determined by the degree of binding of crystal violet (Himedia, India) at a wavelength of 490 nm in anmicroplate photometric analyzer Immunochem-2100 (HTI, USA).

Results: The inhibitory effect of various concentrations of the disinfectant was expressed by the destruction of the extracellular matrix and a significant decrease in the frequency of occurrence of microcolonies and the frequency of occurrence of clusters - aggregation of microorganisms united by a layer of the extracellular matrix. When exposed to a 0,25 % solution of the drug, the optical density indicators significantly decreased: ODs – 0,374 ± 0,09, the intensity of formation - ID ≥ 0,2-0,3, respectively, pseudomonas - moderate producers of biofilms. The study of the effect of 0,50 and 0,75 % concentrations of the drug revealed a more intense effect on the processes of formation of the extracellular matrix. Thus, the decrease in optical density indices was ODs – 0,199 ± 0,09 – 0,245 ± 0,09, the intensity of biofilm formation - ID ≥ 0,1–0,2, accordingly, pseudomonads are weak producers of biofilms. For the detection of

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non-cultured microorganisms, we used media containing components for cell wall repair and L-form reversal.

Conclusion: Under the influence of various concentrations of the disinfectant, a correlation dependence (r = 0,93) of a significant decrease in morphometric and densitometric parameters of biofilms was established. The regularities of a decrease in the degree of formation of the extracellular matrix and the frequency of occurrence of clusters - the aggregation of microorganisms united by a layer of the extracellular matrix - have been established.

Keywords:water areas, aquatic organisms, ichtypathology, pseudomonads, biofilms, morphometry, densitometry.

INTRODUCTION

Spatio-temporal components of natural and anthropogenic ecological systems function and develop due to the diversity of species, including microorganisms, leading to the decomposition of organic substances and a decrease in the number of pathogenic and potentially pathogenic microorganisms [3]. In the presence of nitrites in water over 4 mg / l, the functional reserve of hemocytes decreases more than 6 times. When the pathogenic fungiinvades the Saprolegnia parasitica carp organism, there is a significant increase (1,64 ± 0,29 units) of the average cytochemical coefficient by 46,0 % [1, 6]. Culturing at a temperature of 40° Cand 5° C compared with 20 ° C, the properties of red cells and polymorphonuclear leucocytes Cyprinuscarpio,decreased, 5,0-64,0 %: elasticity, 5,0-8,0 %; adhesion, 24,0-49,0 % [2]. The activities of protease, lipase, amylase, Na+/ K+-ATPase and alkaline phosphatase in the proximal intestine of clinically healthy individuals significantly exceed the indicated indicators ($p<0.05$) with damage to the epithelial layer, therefore, the digestive and absorption function decreases, and the TJ barrier is impaired , and permeability of the intestinal mucosa of fish [20]. Epithelial cells with associated goblet glands of villi and crypts, as well as layers of loose fibrous connective tissue, were free from inflammatory and / or dystrophic changes with a potential hepatoprotective effect at an optimal dietary level [6]. In intestinal microbiocenoses Cyprinus carpio a positive correlation was established (r = 0,89) of the concentration of propionic, butyric acids, catalase, lysozyme activity, expression of the immune gene (IL-10,MyD-88) and the number of microorganisms Bacteroidetes, Cetobacterium, Bacteroidetes and Chitinophaga, negative correlation (r = -0,93) with the number of Fusobacteria, Proteobacteria and Aeromonas, the number of Pseudomonas spp. decreased, $P<0.05$ [14].

A general pattern in the development of biocenotic systems is the formation of heterogeneous biofilms, which are a multicellular community of cells adhered to biotic and abiotic surfaces and

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united by an exocellular polysaccharide matrix when the growth parameters change as compared to single microorganisms [3, 5]. A promising direction of scientific research is the identification of general patterns of cyclical growth of biofilms of microorganisms under the influence of chemotherapeutic and disinfecting drugs. This will allow solving applied problems in the biological assessment of critical points of technologies, as well as reducing the level of contamination water areas to improve the complex of diagnostic, antiepidemic, antiepizootic measures during the joint cultivation of aquatic organisms with other representatives of the animal and plant kingdoms.

The aim of the work is to study of morphometric and densitometric parameters of biofilms of bacteria Pseudomonas aeruginosa under the influence of a disinfectant.

MATERIALS AND METHODS

This work with certified Pseudomonas aeruginosa ATCC 9027 does not require approval from the Ethics Committee.

The study of biofilms and phenotypic traits was carried out using a certified strain (ATCC): Pseudomonas aeruginosa ATCC 9027 [7].

The experiments used meat-peptone broth - "MPB", "Nutient Broth" ("HiMedia", India); disinfectant drug "Abacteril" (LLC "Rudez", Russia) in various concentrations. Organisms were cultured at 37 °C, 48 hours - control; when exposed to the drug - experience. For morphometric and densitometric indices, 18-hour cultures of microorganisms, 105 CFU / ml were cultivated on coverslips placed in the wells of a microplate [3]. The samples under study were stained with a 0,1 % gentian violet solution, 0,5 % methylene blue, 0,5 % trypan blue, 0,1 % acridine orange, 0,1 % congo red aqueous solution, and an aqueous crystal violet solution at a dilution of 1:2000, according to Gram, "Gram-color-stain set for the Gram staining method" (BioVitrum, Russia).

To obtain representative information, optical microscopy was performed by random selection of the field of view of a microscope model "BIOMED MS-1 Stereo" (Russia). The optical density (Optical Density - OD) of biofilms was determined by the degree of binding of crystal violet (Himedia, India) at a wavelength of 490 nm in anmicroplate photometric analyzer Immunochem-2100 (HTI, USA).

For the detection of uncultured microorganisms, we used media containing components for cell wall repair and reversal of L-forms [4].

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The experimental data were subjected to statistical processing using the "Statistika" program for PC Microsoft Excel 2007.

RESULTS AND DISCUSSION

Morphometric parameters. After 48 h of cultivation, 37 °C, optical microscopy (≥90% of the field of view) revealed general patterns of the formation of the heterogeneous structure of biofilms of the studied microorganisms: adhesion; fixation; maturation; height; dispersion.

Adhesion and fixation of bacterial cells of irregular, slightly elongated shape to the substrate occurred through the production of exocellular matrix of varying color intensity. Coaggregation was a sign of the early stage of biofilm formation, characterized by the interaction of adhesion receptors between complementary molecules on the surface of microbial cells. With the accumulation of exocellular structures, areas of formation of short or long chains and a diffuse layer of bacteria with a typical shape and size for the species were revealed. The formation of a three-dimensional structure of biofilms in the form of a dense network consisting of gram-negative bacteria surrounded by an intercellular polymer matrix proceeded gradually. Densely packed and intercellular matrix groups of cells attached to the surface formed closed structures of various sizes, due to the cells and extracellular matrix attached to the substrate, microcolonies were formed (Fig. 1).

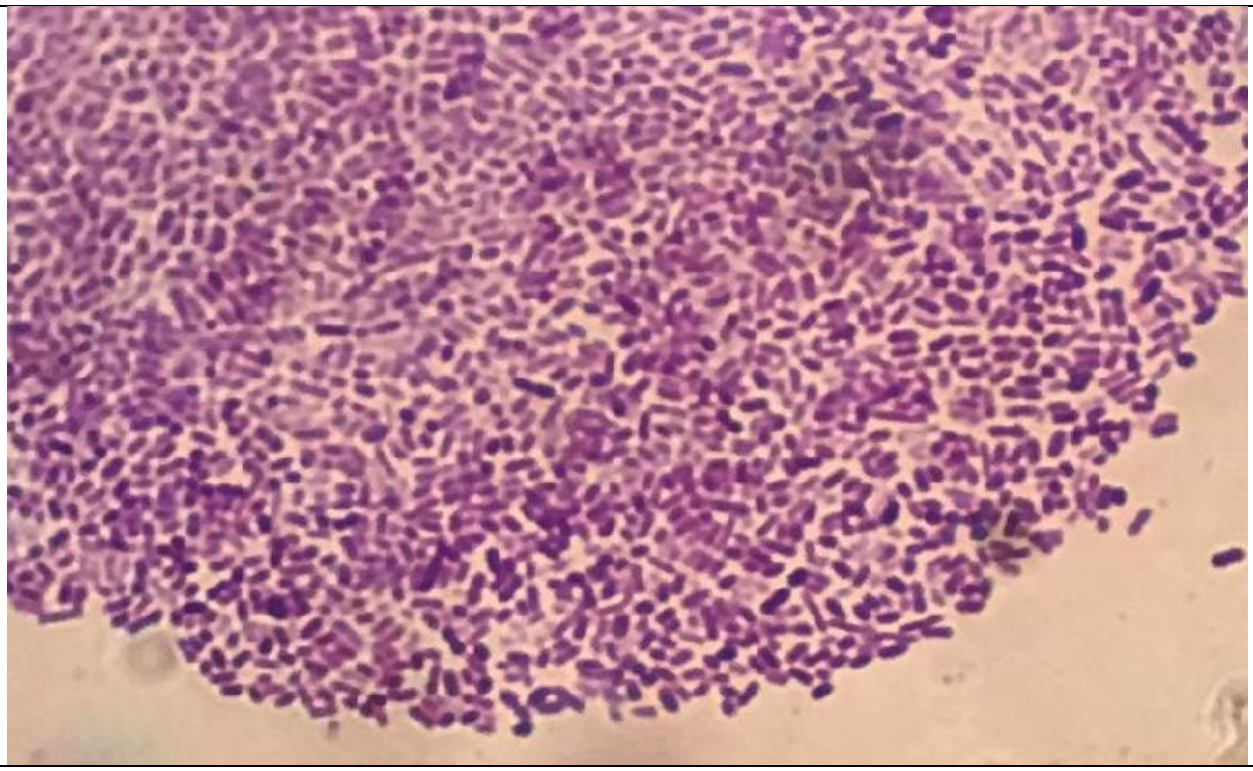

Fig. 1. Intensity of biofilm *P. aeruginosa* formation, 37° C, 48 h (control): biofilm architectonics in the form of a dense network of rod-shaped cells surrounded by an intercellular polymer matrix. Gentian violet. Ey. 10, le. 100, immersion

The inhibitory effect of various concentrations of the disinfectant was expressed by the

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destruction of the extracellular matrix and the separation of bacterial cells from microcolonies. There was a significant decrease in the frequency of occurrence of microcolonies and isolated branched heteromorphic structures colonizing areas of the substrate free of microorganisms. Depending on the concentration of the drug, general patterns of an increase in the degree of dispersion were established, hence a significant decrease in the extracellular matrix, and the frequency of occurrence of clusters - the aggregation of microorganisms united by a layer of the extracellular matrix (Fig. 2).

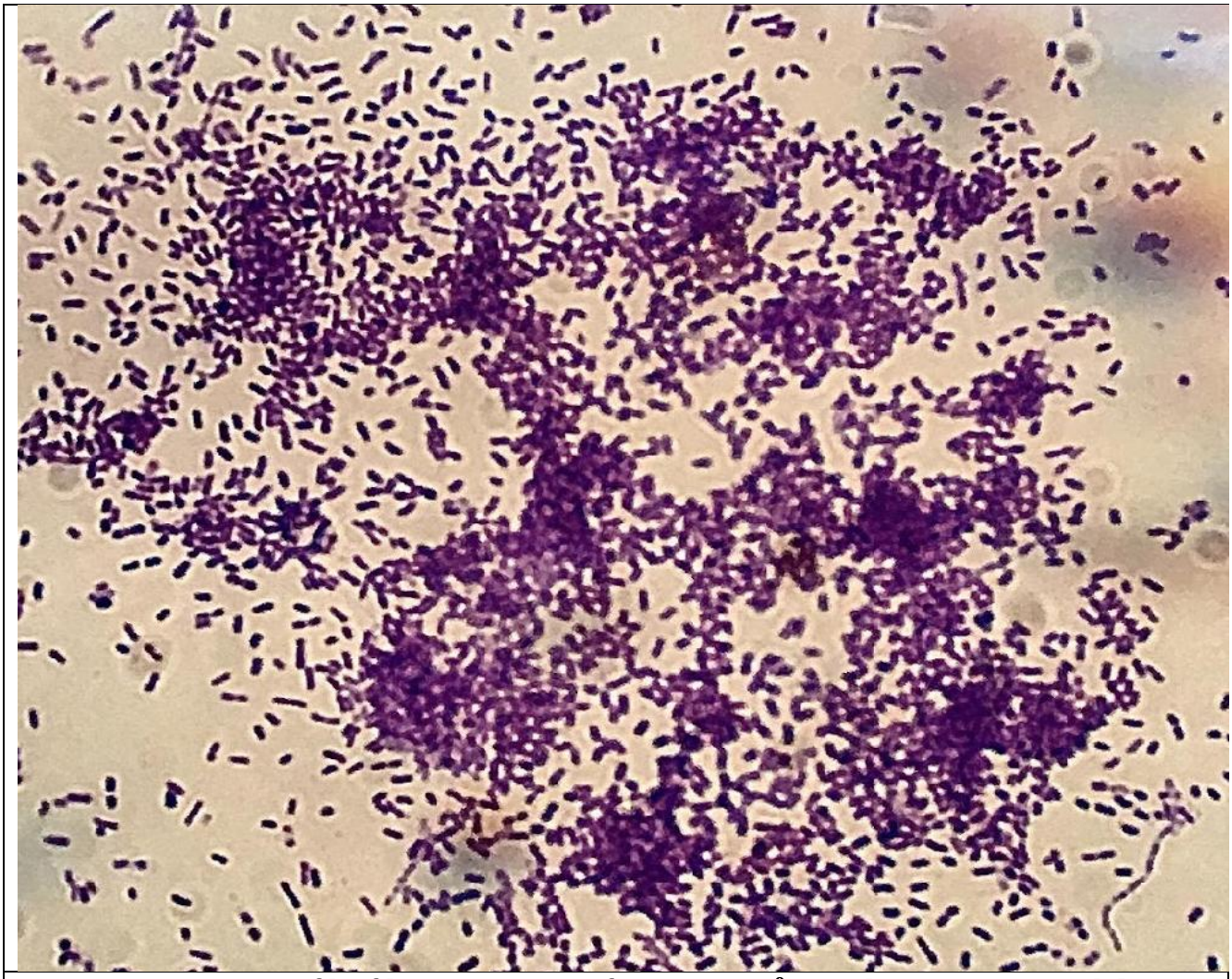

Fig. 2. Intensity of biofilm *P. aeruginosa* formation, 37^{o} C, 48 h (experiment): inhibitory effect of 0.25 solution of the disinfectant "Abacteril". Gentian violet Ey. 10, le. 100, immersion

Cells *P. aeruginosa* (control) were gram-negative, rod-shaped with rounded ends, located singly, in pairs, or in chains. When exposed to various concentrations of the drug (experiment), along with typical gram-negative bacteria, heteromorphic structures were revealed with different intensity of color of cells and intercellular matrix. S-shaped colonies were formed by rod-shaped bacteria with rounded ends (≥94.4%), united in short and long chains. R-forms of colonies were represented by ovoid and globular cells located singly (≥45.6%). The M-forms of the colonies were pale-colored; most of the cells (≥94.4%) were not stained and fused with each other.***Densitometric indicators.*** After 48 h of

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cultivation, the values of the absolute values of the optical density of microorganisms were: OD_s –0,621 ± 0,06, intensity of formation - *ID* biofilm ≥ 0,4, respectively, pseudomonads are strong producers of biofilms (Table 1).

Table 1

Densitometric parameters of biofilms of microorganisms, 48 h

Culture of microorganisms	**Optical density (OD)**			
	Control (OD_c)	**Experiment (OD_s)**	**Δ(OD_s-OD_c)**	**Intensity (*ID*)**
P. aeruginosa	0,099 ± 0,04	0,621 ± 0,06	0,522 ± 0,10	*ID* ≥ 0,4
Note. *OD is the optical density; OD_C - OD control; OD_S - OD of the investigated sample; ID - intensity: the difference between the OD of the test sample (OD_S) and the control (OD_C)*				

When exposed to a 0,25 % solution of the drug, the optical density indicators significantly decreased: OD_s – 0,374 ± 0,09, formation intensity - *ID* ≥ 0,2–0,3, respectively, pseudomonads-moderate producers of biofilms. The study of the effect of 0,50 and 0,75 % concentrations of the drug revealed a more intense effect on the processes of formation of the extracellular matrix. Thus, the decrease in optical density indices was OD_s – 0,199 ± 0,09 – 0,245 ± 0,09, the intensity of biofilm formation - *ID* ≥ 0,1–0,2, accordingly, pseudomonads are weak producers of biofilms (Table 2).

Table 2

Densitometric parameters of biofilms of microorganismswhen exposed to the drug "Abacteril", 48 hours

Concentration of the drug,%	**Optical density (OD)**			
	Control (OD_c)	**Experiment (OD_s)**	**Δ(OD_s-OD_c)**	**Intensity (*ID*)**
0,25	0,098 ± 0,02	0,374 ± 0,09	0,276 ± 0,11	*ID* ≥ 0,2-0,3
0,50	0,099 ± 0,07	0,245 ± 0,09	0,146 ± 0,16	*ID* ≥ 0,1-0,2
0,75	0,097 ± 0,03	0,199 ± 0,09	0,102 ± 0,12	*ID* ≥ 0,1-0,2
Note. *OD is the optical density; OD_C - OD control; OD_S - OD of the investigated sample; ID - intensity: the difference between the OD of the test sample (ODS) and control (OD_C)*				

CONCLUSION

Under the influence of various concentrations of the disinfectant "Abacteril", a correlation was established (r = 0,93) of a significant decrease in morphometric and densitometric parameters of biofilms. The regularities of a decrease in the degree of formation of the extracellular matrix and the frequency of occurrence of clusters - the aggregation of microorganisms united by a layer of the extracellular matrix - have been established.

Analysis of literature data indicates that in the structure of infectious pathology of mammals, birds, as well as fish and amphibians, a significant proportion is chronically occurring diseases caused by pathogenic microorganisms, a component of the life cycle of which is the formation of biofilms [13]. The formation of a biofilm is accompanied by an increase in

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resistance to chemotherapeutic and disinfectant drugs, the effect on the single-species biofilm, yeast-like fungi, is less pronounced than on the two-species biofilm of Candida spp. and Pseudomonas spp. [10, 11].

The absolute values of the optical density of the biofilm P. aeruginosa, S. aureus, Mycobacterium B5 ranged within 0,699–1,510 (OD), that is, gram-negative pseudomonads, like gram-positive staphylococci and mycobacteria, were strong biofilm producers [4, 9].

In multi-species biofilms, the densitometric parameters of P. aeruginosa decreased in the presence of C. albicans (32,0 %), C. krusei (48,0 %), and C. glabrata (78,0 %). however, the number of viable pseudomonads increased significantly in the presence of C. tropicalis (72,0 %) [8]. With confocal microscopy, three-dimensional images of biofilms make it possible to reveal the biofilm structure, dynamics and speed of the cell sedimentation process. Confocal images also make it possible to determine the roughness coefficient of the biofilm surface [19].

Dynamic and static methods of cultivation of microorganisms in vitro and in vivo allowed it is possible to evaluate the components of the exocellular matrix and heteromorphic structures during the formation of biofilms of microorganisms without disturbing the natural architectonics of microbial populations [3]. Coaggregation was a sign of the early stage of biofilm formation, characterized by the interaction of adhesion receptors between complementary molecules on the surface of microbial cells. In co-cultivation after 4 h, the coaggregation indices (%) of the referencestrain L. reuteri ATCC PTA 5289andisolates C. albicans were 9,0–22,0 % ($p < 0.05$) [12].

The highest rates of coaggregation (%) were established during co-cultivation for 4 h of mixed cultures of L. acidophilus and C. albicans – 64,0 %; L. plantarum and C. albicans – 63,0 %. The antagonistic effect (100,0 %) ofrevealed L. plantarum and L. acidophilus on the cultures ofMicroorganisms was Candida spp.. at a concentration of 1x1010 and 1x 108 CFU / ml, respectively [16].

To optimize the scheme of microbiological studies, which are extremely long-term and retrospective, a priority direction is the expediency of developing differential diagnostic media with the addition of growth factors for the reversal of cells with a defective cell wall, spheroplasts, needle-like and giant structures, as well as L-forms of bacteria to the initial cultured state. [3]. Probiotic strains are promising due to production of diffuse extracellular exopolysaccharides and effector molecules, in particular - lactic acid, Lactobacillus spp., Inhibited the growth of the isolates C. albicans (2,23 ± 1,44 - 14,40 cfu /lg), extracted with systemic pathologies [17, 18]. The use of several medicinal substances with different mechanisms of action prevented the transition of the yeast phase of development to mycelial growth, which causes the effect of reducing the triggering mechanisms of virulence factors - the adhesion of pathogens both to cells of susceptible species and to abiotic surfaces [15].

Competing Interests

The authors declare that they have no competing interests.

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