MODERN TECHNOLOGIES OF BACTERIAL BIOFILM STUDY

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© **I.V. Chebotar,** PhD, Associate Professor, the Department of Microbiology and Immunology[1];
A.G. Pogorelov, D.Bio.Sc., Professor, Leading Research Worker[2];
V.A. Yashin, Head of the Optical Spectroscopy Laboratory[3];
E.L. Guryev, PhD, Research Worker, Scientific Research Institute of Applied and Fundamental Medicine[1];
G.G. Lominadze, Resident Medical Practitioner[4]

[1]Nizhny Novgorod State Medical Academy, Minin and Pozharsky Square, 10/1, Nizhny Novgorod, Russian Federation, 603005;
[2]Institute for Theoretical and Experimental Biophysics, Russian Academy of Sciences, Institutskaya St., 3, Pushchino, Moscow region, Russian Federation, 142290;
[3]Institute of Cell Biophysics, Russian Academy of Sciences, Institutskaya St., 3, Pushcino, Moscow region, Russian Federation, 142290;
[4]Scientific Children Health Centre of Russian Academy of Medical Sciences, Lomonosovsky Avenue, 2, Moscow, Russian Federation, 119991

The aim of the investigation was to estimate the availability of new biomedical technologies to identify bacterial biofilms and evaluate them on a staphylococcal biofilm model.

Materials and Methods. We studied staphylococcal biofilms by mass spectrometry, laser scanning (confocal) microscopy, scanning electron microscopy, enzymatic and oxidative destruction of extracellular biofilm matrix.

Results. We demonstrated the capabilities of new biomedical technologies in identification of generic specificity of biofilm-forming staphylococcus, and in detection of the necessary characteristics of staphylococcal biofilm. Mass spectrometry enabled to identify the type of biofilm-forming staphylococcus (*Staphylococcus aureus*). Microscopic study using laser scanning confocal microscopic technique revealed 3-demensional organization typical of *S. aureus* biofilms. Scanning electron microscopy enabled to visualize the structures of extracellular *S. aureus* biofilm matrix. The extracellular matrix of the test biofilm was found to be formed of DNA-protein complexes.

Key words: *Staphylococcus aureus*; biofilm; mass-spectrometry; laser scanning confocal microscopy; scanning electron microscopy.

The pathogenesis of many human bacterial infections is related to the biofilm formation. A bacterial biofilm is a layer of bacterial cells attached to the surface and to each other, and enclosed in biopolymer matrix [1]. Staphylococci are actual agents of pyoinflammatory diseases, and actively form biofilms in human body and on the surface of the adjacent devices (catheters, prostheses, grafts). It concerns *Staphylococcus aureus*, *Staphylococcus epidermidis, Staphylococcus lugdunensis*, *Staphylococcus haemoliticus* and other species [2–6]. Staphylococci as the part of biofilms acquire resistance to the action of antimicrobial agents, as well as the attacks from the human immune system [7, 8], therefore, staph biofilm infections are characterized by a lingering course, tendency to recurrences, and the respond failure to conventional methods of antimicrobial therapy [9]. However, even within one bacterial species not all the strains are able to form biofilms. For example, from 22 to 32% of *S. aureus* clinical isolates do not form biofilms [10, 11]. Hence, microbiological diagnostics is required to choose an optimal modality that should include the agent species identification, as well as contain the information on the presence or absence of biofilm process in each specific case. In other words, there is the necessity to differentiate biofilm and nonbiofilm strains of an isolated agent. Various techniques are used to prove the biofilm presence, and they are aimed at revealing: 1) the elements of biofilm extracellular matrix; 2) the genes controlling biofilm formation; 3) complex architectural structures specific to a biofilm [12]. Preliminary species identification of microbes forming a biofilm is performed.

The aim of the investigation was to estimate the availability of new biomedical technologies to determine bacterial biofilms and evaluate them on a staphylococcal biofilm model. We identified the agent species, and proved the fact the staph strain under study forms a biofilm. The detection of two required attributes – bacterial cells and extracellular matrix served as the evidence of biofilm formation [12].

Materials and Methods. The object of the research was the bottom layer of broth culture of coagulase-positive staph clinical isolate (strain 5983/2) in plastic plates. We put in a plastic plate (40 mm in diameter) 3.5 ml of Tryptic Soy Broth (TSB) (Becton, Dickinson and Company, USA) containing 1% glucose, and added 0.5 ml of daily culture of the test staph in the same broth standardized by optical characteristics (0.5 units on DensiLaMeter II, ERBA Lachema Company, Czechia), 1 ml containing approximately 10^8 of staph colony-forming units, and incubated for 48 h at 37°C. After the incubation we racked supernatant nutrient broth, and washed the residual staph layer by Hanks' solution (4 ml).

For contacts: Chebotar Igor Victorovich, phone: 8(831)465-42-71, +7 903-607-70-54; e-mail: nizarnn@yandex.ru

We identified the species by MALDI-TOF MS technology (Germany) using direct profiling on mass-spectrometer Microflex (Bruker Daltonics, Germany) equipped by 337 nm nitrogen laser, using Maldi Biotiper automatic program (Germany). We took the microbial material from the plate bottom with the help of a wire inoculating loop, and put directly on the target. Then we mixed the material with 1 μl of matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in the mixture of 50% ethane nitrile and 2.5% trifluoroacetic acid), air-dried, and put the target into a mass-spectrometer and studied. The obtained mass-spectrum was compared with those of the reference library using flexControl and flexAnalysis software (Bruker Daltonics, Germany). The accuracy of the identification was supported by a scoring system developed on the basis of statistical algorithms: 2.300–3.000 scores corresponded to reliable species identification [13].

Microscopic investigation was performed using laser scanning (confocal) microscope LSM 510 Meta (Zeiss, Germany). The staph layer was stained in situ in the plate by fluorochrome stains using test-systems FilmTracer™ 1-43 Green Biofilm Cell Stain (Invitrogen, USA) according to a manufacturer's instruction. The images were reconstructed and analyzed by Zeiss LSM Image Browser program (Germany). A separate series of microscopic study of biofilm staph mobility (with the similar fluorochrome staining) was carried out using "resonance scanner" technology on the laser scanning (confocal) microscope TCS SP5 (Leica Microsystems, Switzerland); the images were reconstructed using LAS AF Lite software.

To study a biofilm ultrastructural pattern we used the scanning microscope JSM 6390A (JEOL, Japan). The preparation was fixed at 4°C in two stages. At first the sample was prefixed (30 min) in 2.5% glutaric dialdehyde (pH=7.2) (EM grade, Agar Scientific Inc., Great Britain) with the following three-time washing off by distilled water. Then the preparation was fixed (30 min) by 0.5% OsO_4 solution with the following three-time washing off by distilled water. After that the preparation was cryofixed in liquid nitrogen (–196°C) and exposed to low-temperature dehydration (–100°C) in high vacuum for 24 h. After lyophilization the preparation was mounted on the electron microscope holder, and covered by a platinum layer (10 nm) applying the technique of ionic sputtering in argon plasma using JFC-1600 (JEOL, Japan). We used scanning electron microscopy with magnification of 1000–10 000 times.

We performed biochemical identification of intercellular biofilm matrix elements treating the biofilms by the substances providing specific destruction of different matrix types [14–16]. To confirm the structural role of proteins in matrix formation we used proteinase K (Amresco, USA) and trypsin (Thermo Scientific, Finland), and to detect extracellular DNA (eDNA) in matrix we used deoxyribonuclease (Fermentas, Lithuania). To reveal polysaccharides the biofilms were treated by sodium periodate. Proteinase K was dissolved in 100 mmol of Tris-Cl-buffer (pH=7.5) in concentration of 1000 μg/ml. Deoxyribonuclease was dissolved in 10 mM Tris-Cl-buffer (pH=8.0) containing 2 mmol of $MgCl_2$, in concentration of 1000 μg/ml. We added these enzymes to biofilms (in 4 ml of Hanks' solution) in the ratio of 1:9 to Hanks' solution; the biofilms with enzymes were incubated for 60 min at 37°C. Deoxyribonuclease (final concentration of 100 μg/ml, 60 min incubation at 37°C) and proteinase K (final concentration of 100 μg/ml, 60 min incubation at 37°C) were added successively to the sample series. Each set of experiments had control samples, in which we added appropriate buffer solutions without enzymes. From the samples with biofilms we removed Hanks' solution and added 4 ml of distilled water containing 40 mmol of sodium periodate, and incubated for 23 h at 4°C. We used the following biofilms as control adding one of the systems: 1) 4 ml of distilled water containing 40 mmol of sodium periodate and 40 mmol of glucose; 2) 4 ml of distilled water containing 40 mmol of glucose; 3) 4 ml of distilled water. After incubation the biofilms were washed off twice by distilled water, stained by 1% crystal violet solution (4 min at room temperature), then rinsed by distilled water, and eluted the stain by the ethanol-isopropanol (4 ml) mixture (1:1). The biofilm integrity was evaluated by the eluate color degree, and color density (light absorbance) was assessed spectrophotometrically (610 nm). The findings were expressed in percent in relation to control samples.

All experiments were carried out thrice, and the sets of experiments were repeated four times; the findings were analyzed using both standard statistical methods (calculation of mean values, standard deviations, Mann–Whitney criterion), and the modules included in software package of the devices used in the investigation (a mass-spectrometer Microflex, a scanning electron microscope JSM).

Results and Discussion. The findings of species identification of biofilm bacteria using MALDI-TOF MS technology (Germany) (Fig. 1) reliably confirmed (score — 2.420) that they belong to *S. aureus*.

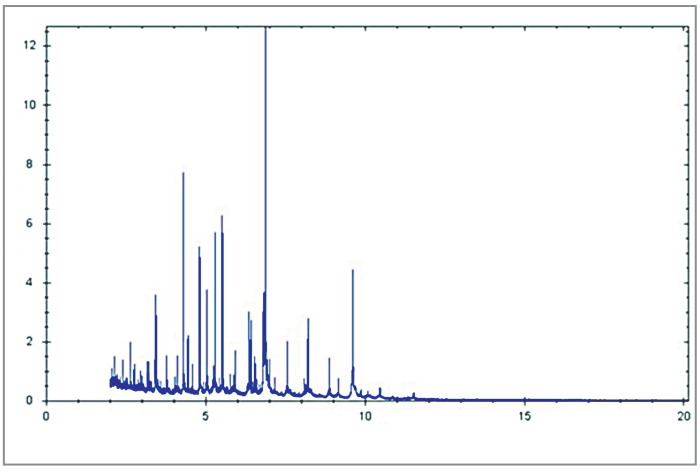

Fig. 1. Mass-spectrogram of the biofilm-forming strain showing the spectrum of the correlation between the mass and charge of ionized peptides — derivatives of bacterial proteins. X-direction — peaks, each of which corresponds to an ionized peptide (a protein or a part of protein) with unique mass/charge ratio, Y-direction — peak intensity (in relative units) that is equivalent to the amount of protein (peptide). Statistical analysis of spectra enables to suggest that the test isolate belongs to *S. aureus* (score — 2.420)

The biofilm imaging by means of confocal microscopy and LSM Image Browser software revealed three-dimensional organization typical of biofilms (Fig. 2). The thickness of two-day *S. aureus* biofilm varied from 20 to 47 μm.

Microscopic real-time biofilm viewing enabled to detect a specific type of chaotic (heat) motion of staphylococci forming a part of a biofilm (Fig. 3). Some staphylococci being microscopic particles carried out chaotic oscillations within the limited volume comparable with their dimensions. In addition, staphs could not have direct contacts with the neighboring cells. In contrast to classical Brownian motion, the movements of staphs were limited. It indicated the staphs to be mechanically linked, and was an indirect proof that any biofilm should have a required attribute — extracellular matrix binding biofilm microbes among themselves and with the underlying surface. Matrix structural elements could not be seen by confocal microscopic technique due to their small size.

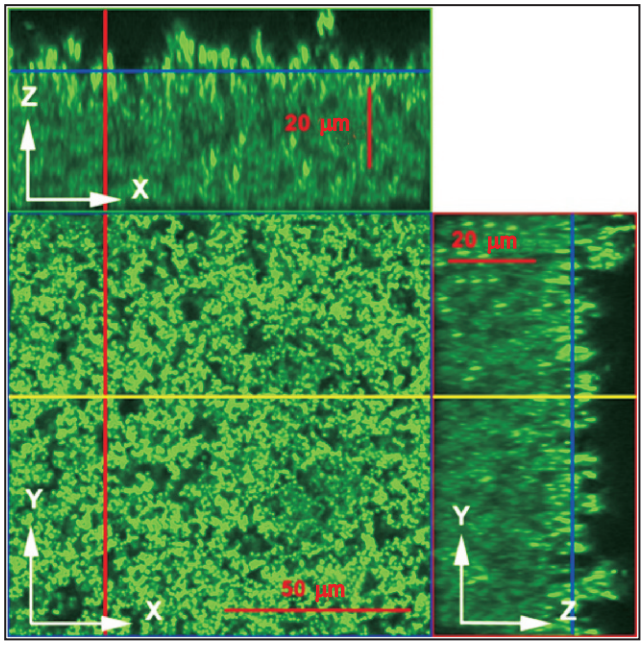

Fig. 2. The two-day biofilm formed by *S. aureus* (strain 5983/2) is reconstructed by laser scanning (confocal) microscopy. *In vivo* biofilm study. Staphylococci are stained by FilmTracer™ 1-43 Green Biofilm Cell Stain (Invitrogen). Extinction/emission ratio — 488 nm/543 nm. Objective x63, water immersion. Marking on X-direction — 50 μm, Z-direction — 20 μm

Ultramicroscopic investigation of biofilms and direct matrix imaging were carried out using scanning electron microscopy (Fig. 4). The spatial arrangement of staphs had two organization levels. The first level consisted in the staphs forming clusters including from 9 to 55 cells. At higher level staph clusters formed a unified supracluster structure consisting of many layers — a biofilm. The body of a biofilm is penetrated by numerous channels, the openings of which entered the external (test) biofilm surface. The substrate on which a biofilm formed was not seen through channel lumens. The average 4–6 channels opened on 100 μm^2 biofilm surface. From 9 to 27 staphylococci participated in channel wall formation in the opening area. There were observed two contact types between the staphs: direct contact by means of which clusters form, and matrix-mediated bonds. The latter was found at 3000–15 000 magnification: between some staphs there were unusual "connection elements" — the biofilm matrix structures connecting bacteria with each other. The cell surface of the most staphylococci had smooth round shape, except for the cell surface areas connected to extracellular matrix, their surface was irregular, and matrix was like non-structural unshaped substance. It should be noted that biofilm matrix *in vitro* can be formed by both actively secreted bacterial polymers, and cells derivates resulted from autolysis [17].

Microscopic investigations showed the presence of attributes required for biofilms — bacteria (*S. aureus*) and extracellular matrix. It indicates that the object of our study was a biofilm. The fact a biofilm is formed by *S. aureus* was not unexpected, since approximately 74% of invasive and

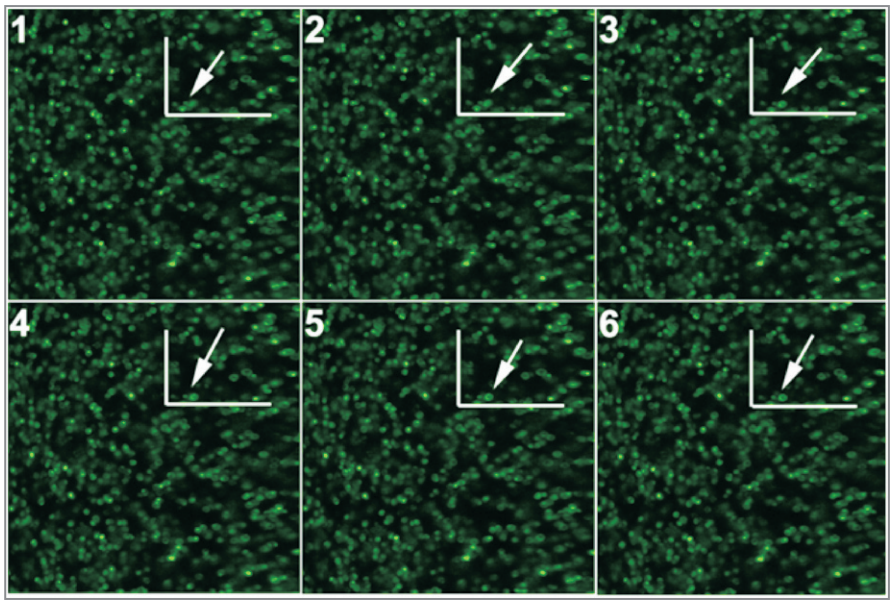

Fig. 3. Microphotograph series demonstrating the restriction of biofilm staphylococci in chaotic (heat) movements. Photographs *1–6* show the motion of green-stained staphylococci. The arrow indicates a group of staphylococci changing their position in relation to coordinate grid (crossed white segments) with invariable coordinates. Laser scanning (confocal) microscopy. *In vivo* staining by fluorochrome stain FilmTracer™ 1-43 Green Biofilm Cell Stain (Invitrogen). Extinction/emission ratio — 488 nm/543 nm. Objective x40. Image rate — 0.075 s

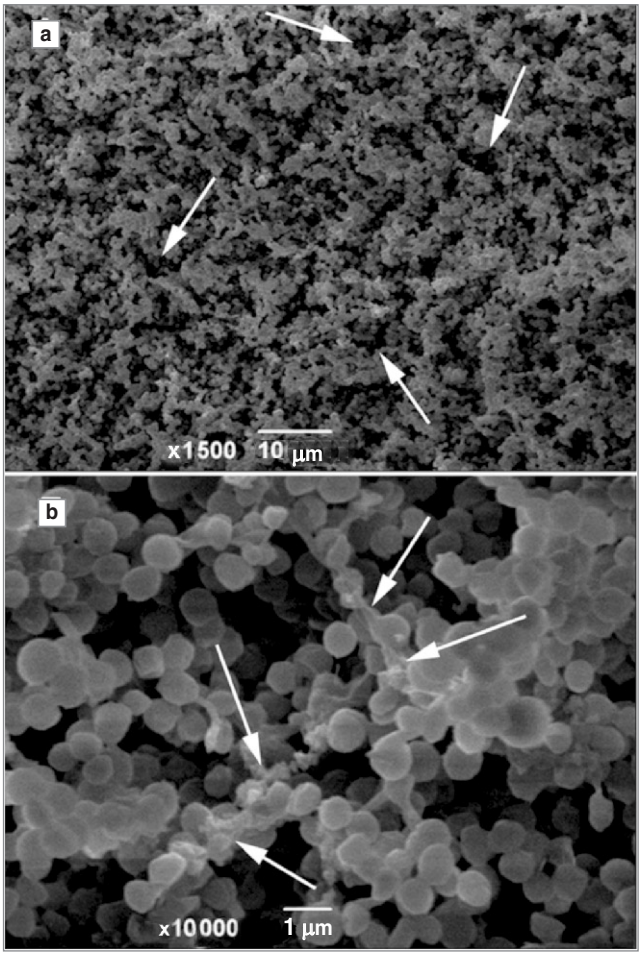

Fig. 4. Scanning electron microscopy of two-day biofilm surface formed by *S. aureus* (strain 5983/2): *a* — magnification 1500, mark — 10 μm; arrows indicate biofilm channels; *b* — magnification 10 000, marking — 1 μm; arrows indicate the elements for extracellular biofilm matrix

68% of noninvasive clinical staph isolate are known to be biofilm-forming [18].

The study of the effect of the substances destroying extracellular matrix (Fig. 5) on a biofilm showed the proteinase treatment to result in statistically significant decrease of biofilm staining indices: trypsin treatment causes the index decrease of 57% and was 43.3±5.8% compared to negative control values ($p<0.01$), proteinase K treatment causes the index decrease of 45% and was 55.4±4.3% compared to control ($p<0.01$). It indicates the biofilm mass decrease due to extracellular matrix protein degradation. Deoxyribonuclease caused less expressed biofilm destruction: the values decreased approximately of 15%, but statistically significantly differed from control — 84.9±7.1% ($p<0.05$). It indicates the possible DNA participation in extracellular matrix formation. The results confirming DNA role in matrix formation were obtained in a set of experiments, when the biofilms were successively treated by deoxyribonuclease and proteinase K: the biofilm staining indices decreased of 83% and were 16.9±2.0% compared to control values ($p<0.001$). Biofilm matrix was resistant to periodate oxidation: the biofilms treated by sodium periodate did not differ from control ($p>0.05$). The periodate oxidation resistance indicates the absence of polysaccharide structures as part of extracellular biofilm matrix.

The findings carry inference of biochemical composition of extracellular matrix of the test biofilm: it consists of DNA-protein complexes, and biofilm mechanical integrity does not depend on polysaccharide components. It is consistent with the data reported in literature [17, 19–22], which confirm that there are *S. aureus* strains with various matrix types. Matrix destruction always results in biofilm rejection, therefore, the information of its biochemical structure is crucial to control biofilm processes. It will enable to optimize the choice of anti-biofilm agents, the action of which can be

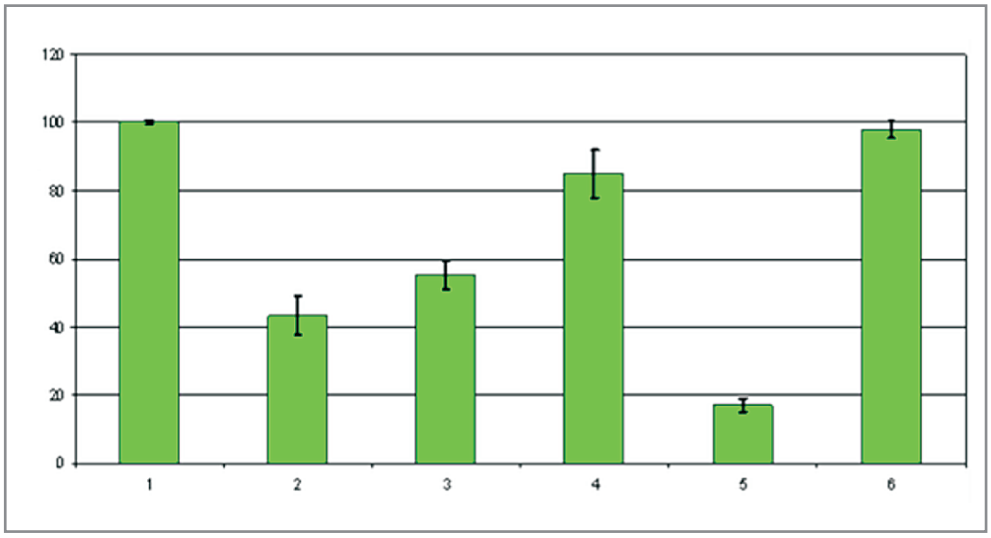

Fig. 5. The effect of the substances destroying various extracellular biofilm matrix structures on the two-day biofilm formed by *S. aureus* (strain 5983/2). X-direction: *1* — control (the samples with no substances destroying matrix added); *2* — trypsin; *3* — proteinase K; *4* — deoxyribonuclease; *5* — consecutive deoxyribonuclease and proteinase K treatment; *6* — sodium periodate. Y-direction — biofilm staining intensity reflecting its integrity, in percentage points relative to control

aimed at DNA-matrix degradation (the products based on deoxyribonucleases of streptococcal origin), protein matrix (trypsin) destruction, disintegration of the poly-β-(1,6)-N-acetylglucosamine-based matrix (dispersin B).

It should be mentioned that the authors deliberately did not used in the study some techniques employed in medical practice, it is referred to the tests with Congo red, and some genetic findings, the use of which can be not only less informative, but also incorrect [12].

The techniques used in the present investigation to study staphylococcal biofilm enable to draw the following conclusions: 1) the test staph strain growing (48 h) in the broth forms a biofilm on the polymer surface; 2) the test biofilm-forming isolate belongs to *S. aureus*; 3) the extracellular matrix of a biofilm is formed by DNA-protein complexes, and its mechanical integrity does not depend on polysaccharide components.

Conclusion. New biomedical modalities enable to succeed in identifying species assignment of biofilm-forming staphylococcus, and detecting essential attributes of staph biofilm. Mass-spectrometry helps to identify the species of biofilm-forming staphylococcus (*Staphylococcus aureus*). Microscopic investigation performed by laser scanning confocal microscopy reveals three-dimensional organization typical of *S. aureus* biofilms. Scanning microscopy visualizes the structures of extracellular *S. aureus* biofilm matrix.

References

1. Romanova Yu.M., Gintsburg A.L. Bakterial'naya bioplenka kak estestvennaya forma sushchestvovaniya bakteriy v okruzhayushchey srede i organizme khozyaina [Bacterial biofilms as a natural form of existence of bacteria in the environment and host organism]. *Zhurnal mikrobiologii, epidemiologii i immunologii — Journal of Microbiology, Epidemiology and Immunobiology* 2011; 3: 99–109.

2. Mayanskiy A.N., Chebotar I.V. Stafilokokkovye bioplenki: struktura, regulyatsiya, ottorzhenie [Staphylococcal biofilms: structure, regulation, rejection]. *Zhurnal mikrobiologii, epidemiologii i immunologii — Journal of Microbiology, Epidemiology and Immunobiology* 2011; 1: 101–108.

3. Gotz F. Staphylococcus and biofilms. *Mol Microbiol* 2002; 43: 1467–1378.

4. Vuong C., Otto M. Staphylococcus epidermidis infections. *Microbes Infect* 2002; 4: 481–489.

5. Frank K.L., del Pozo J.L., Patel R. From clinical microbiology to infection pathogenesis: how daring to be different works for Staphylococcus lugdunensis. *Clin Microbiol Rev* 2008; 1: 111–133.

6. Fredheim E.G., Klingenberg C., Rohde H., Frankenberger S., Gaustad P., Flaegstad T., Sollid J.E. Biofilm formation by Staphylococcus haemoliticus. *J Clin Microbiol* 2009; 47(4): 1172–1180.

7. Bekhalo V.A., Bondarenko V.M., Sysolyatina E.V., Nagurskaya E.V. Immunobiologicheskie osobennosti bakterial'nykh kletok meditsinskikh bioplenok [Immunobiological features of bacterial cells of medical biofilms]. *Zhurnal mikrobiologii, epidemiologii i immunologii — Journal of Microbiology, Epidemiology and Immunobiology* 2010; 4: 97–105.

8. Chebotar I.V., Mayanskiy A.N., Konchakova E.D., Lazareva A.V., Chistyakova V.P. Antibiotikorezistentnost' bioplenochnykh bakteriy [Antibiotic resistance of biofilm bacteria]. *Klinicheskaya mikrobiologiya i antimikrobnaya khimioterapiya — Clinical Microbiology and Antimicrobial Chemotherapy* 2012; 14(1): 51–58.

9. Archer N.K., Mazaitis M.J., Costerton J.W., Leid J.G., Powers M.E., Shirtliff M.E. Staphylococcus aureus biofilms: properties, regulation, and roles in human disease. *Virulence* 2011; 2(5): 445–459.

10. Jain A., Agarwal A. Biofilm production, a marker of pathogenic potential of colonizing and commensal staphylococci. *J Microbiol Methods* 2009; 76(1): 88–92.

11. Taj Y., Essa F., Aziz F., Kazmi S.U. Study on biofilm-forming properties of clinical isolates of Staphylococcus aureus. *J Infect Dev Ctries* 2012; 6(5): 403–409.

12. Chebotar I.V., Gurev E.L. Laboratornaya diagnostika klinicheski znachimykh bioplenochnykh protsessov [Laboratory diagnostics of clinically significant biofilm processes]. *Voprosy diagnostiki v pediatrii — Diagnostic Issues in Pediatrics* 2012; 4: 15–20.

13. Mayanskiy N.A., Kalakutskaya A.N., Motuzova O.V., Lominadze G.G., Kryzhanovskaya O.A., Katosova L.K. MALDI-TOF mass-spektrometriya v rutinnoy rabote mikrobiologicheskoy laboratorii [MALDI-TOF mass spectrometry in microbiological laboratory routine]. *Voprosy diagnostiki v pediatrii — Diagnostic Issues in Pediatrics* 2011; 3 (5): 20–25.

14. Frank K.L., Patel R. Poly-N-acetylglucosamine is not a major component of the extracellular matrix in biofilms formed by icaADBC-positive Staphylococcus lugdunensis isolates. *Infect Immun* 2007; 75(10): 4728–4742.

15. Izano E.A., Amarante M.A., Kher W.B., Kaplan J.B. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis. *Appl Environ Microdiol Biofilms* 2008; 74(2): 470–476.

16. Wang X., Preston J.F. III, Romeo T. The pgaABCD locus of Escherichia coli promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J Bacteriol* 2004; 186(9): 2724–2734.

17. Flemming H.C., Wingender J. The biofilm matrix. *Nat Rev Microbiol* 2010; 8(9): 623–633.

18. Jain A., Agarwal A. Biofilm production, a marker of pathogenic potential of colonizing and commensal staphylococci. *J Microbiol Methods* 2009; 76(1): 88–92.

19. Chaignon P., Sadovskaya I., Ragunah Ch., Ramasubbu N., Kaplan J.B., Jabbouri S. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Appl Microbiol Biotechnol* 2007; 75(1): 125–132.

20. Mack D., Fischer W., Krokotsch A., Leopold K., Hartmann R., Egge H., Laufs R. The intercellular adhesin involved in biofilm accumulation of Staphylococcus epidermidis is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J Bacteriol* 1996; 178(1): 175–183.

21. Hansen U., Hussain M., Villone D., Herrmann M., Robenek H., Peters G., Sinha B., Bruckner P. The anchorless adhesin Eap (extracellular adherence protein) from Staphylococcus aureus selectively recognizes extracellular matrix aggregates but binds promiscuously to monomeric matrix macromolecules. *Matrix Biol* 2006; 25: 252–260.

22. Montanaro L., Poggi A., Visai L., Ravaioli S., Campoccia D., Speziale P., Arciola C.R. Extracellular DNA in biofilms. *Int J Artif Organs* 2011; 34(9): 824–831.