EFFECTS OF LYTIC BACTERIOPHAGES OF THE FAMILIES *HERELLEVIRIDAE* AND *ROUNTREEVIRIDAE* ON THE *STAPHYLOCOCCUS AUREUS* BIOFILMS

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Staphylococcus aureus causes a broad range of infections and is often characterized by multidrug resistance (MDR). Treatment of staphylococcal infections is further complicated by the ability of bacterium to form biofilms protecting it against antimicrobial agents and the immune system. The use of bacteriophages is one of the promising strategies for combating the bacteria showing MDR and biofilm formation activity. The study aimed to assess the effects of the lytic phages vB_SauM-515A1 (genus *Kayvirus*, family *Herelleviridae*) and vB_SauP-436A (genus *Rosenblumvirus*, family *Rountreeviridae*) on biofilms of the *S. aureus* clinical strains. The study involved 20 strains of eight sequence types, among which 45% (9/20) belonged to MRSA, and 35% (7/20) showed MDR. All the strains demonstrated the ability to form biofilms, and 65% (13/20) were strong biofilm producers. Genes of the *ica*ADBC operon responsible for synthesis of polysaccharide intercellular adhesin were found in genomes of all samples. The exposure of planktonic bacterial cells to bacteriophages showed that 70% (14/20) of strains were sensitive to phage vB_SauM-515A1 and 50% (10/20) were sensitive to phage vB_SauP-436A. Furthermore, the 24-h treatment of biofilms of sensitive strains with phage vB_SauM-515A1 led to the biofilm biomass increase in 64.3% (9/14) of cases, while phage vB_SauP-436A, on the contrary, significantly reduced the quantity of biofilm in 40% (4/10) of strains. The results obtained highlight the ambiguity of interaction between bacteriophages and S. aureus biofilms and suggest the need for further research aimed at optimizing phage therapy targeting the biofilm-forming strains.

Keywords: *Staphylococcus aureus*, bacteriophage, phage therapy, biofilm, *Herelleviridae*, *Rountreeviridae*

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ВОЗДЕЙСТВИЕ ЛИТИЧЕСКИХ БАКТЕРИОФАГОВ СЕМЕЙСТВ *HERELLEVIRIDAE* И *ROUNTREEVIRIDAE* НА БИОПЛЕНКИ *STAPHYLOCOCCUS AUREUS*

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Staphylococcus aureus вызывает широкий спектр инфекций и часто характеризуется множественной лекарственной устойчивостью (МЛУ). Лечение стафилококковых инфекций дополнительно осложнено способностью бактерии формировать биопленку, которая защищает ее от антимикробных агентов и иммунной системы. Одной из перспективных стратегий борьбы с бактериями, обладающими МЛУ и биопленкообразующей активностью, является применение бактериофагов. Целью исследования было оценить влияние литических фагов vB_SauM-515A1 (род *Kayvirus*, семейство *Herelleviridae*) и vB_SauP-436A (род *Rosenblumvirus*, семейство *Rountreeviridae*) на биопленки клинических штаммов *S. aureus*. Исследование включало 20 штаммов восьми сиквенс-типов, из которых 45% (9/20) относились к MRSA, а 35% (7/20) обладали МЛУ. Все штаммы продемонстрировали способность к биопленкообразованию, причем 65% (13/20) являлись сильными продуцентами биопленки. В геномах всех образцов обнаружены гены icaADBC-оперона, ответственного за синтез полисахаридного межклеточного адгезина. Воздействие бактериофагов на планктонные клетки бактерий показало, что 70% (14/20) штаммов были чувствительны к фагу vB_SauM-515A1, а 50% (10/20) — к фагу vB_SauP-436A. При этом 24-часовая обработка биопленок чувствительных штаммов фагом vB_SauM-515A1 в 64,3% (9/14) случаев приводила к увеличению биомассы биопленки, тогда как фаг vB_SauP-436A, напротив, достоверно снижал количество биопленки у 40% (4/10) штаммов. Полученные результаты подчеркивают неоднозначность взаимодействия бактериофагов с биопленками *S. aureus* и указывают на необходимость дальнейших исследований для оптимизации фаговой терапии в отношении биопленкообразующих штаммов.

Ключевые слова: *Staphylococcus aureus*, бактериофаг, фаговая терапия, биопленки, Herelleviridae, *Rountreeviridae*, *Kayvirus, Rosenblumvirus*

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Staphylococcus aureus is one of the leading bacterial pathogens responsible for a broad range of infections: from superficial inflammation of the skin to severe life-threatening conditions, such as pneumonia, sepsis, and endocarditis [1]. The microorganism attracts special attention due to its resistance to a broad spectrum of antibiotics, and the most important are methicillin-resistant *S. aureus* strains (MRSA).

Treatment of staphylococcal infections is hampered not only by antibiotic resistance, but also by plenty of virulence factors, among which is the ability of staphylococci to form biofilms. Like biofilms of other bacteria, the *S. aureus* biofilm consists of two main components: water (about 97%) and organic matter represented by extracellular polymers and colonies of microorganisms. Extracellular polymers constitute 50–90% of all organic mass of the biofilm and include a combination of various compounds, such as extracellular DNA (eDNA), proteins, and polysaccharides. The remainder of biofilm is represented by bacterial cells [2, 3].

The biofilm ensures firm attachment of bacteria to various surfaces, including tissues of the body and medical equipment [2]. Furthermore, biofilms considerably increase resistance of bacteria to the immune system factors and antimicrobial drugs: the minimum inhibitory concentrations (MICs) of antibiotics necessary for disruption of bacteria in biofilms can 1000-fold exceed the concentrations that are enough to destroy planktonic cells [3, 4].

Bacteriophages increasingly considered as a promising remedy to treat bacterial infections caused by antibiotic-resistant strains. Only virulent bacteriophages that realize the lytic cycle only are used in medical practice. Such phages capable of infecting and destroying the *S. aureus* cells include members of the families *Herelleviridae* and *Rountreeviridae*. These demonstrate high efficacy in both *in vitro* experiments and *in vivo* models, are successfully used for therapy, which ensures reduction of bacterial load and improvement of clinical outcomes [5].

In this context, of special interest are the studies focused on assessing the effects of phages on biofilms. It has been shown that some S. aureus phages can effectively reduce the biofilm biomass [6, 7]. Nevertheless, a number of studies show that biofilm generation can be stimulated under exposure to phages, which can be associated with the features of interplay between phages and bacterial cells [8, 9]. Such effects are likely to depend on a number of factors: bacterial strain itself, bacteriophage used and its concentration, physiological state of cells, as well as morphological and structural characteristics of the biofilm.

The study aimed to assess the effects of the lytic bacteriophages vB_SauM-515A1 (family *Herelleviridae*) and vB_SauP-436A (family *Rountreeviridae*) on biofilms formed by the *S. aureus* clinical isolates.

METHODS

Bacterial strains and bacteriophages

In this study, we used 20 *S. aureus* strains, most different based on the origin and isolation locus, from the collection of the Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of the Federal Medical Biological Agency. The strains were collected in 2015–2020 from hospitals in various regions of Russia.

Bacterial culture was grown in the liquid LB (lysogeny broth) medium (Oxoid; UK) or Miller LB agar (Oxoid) at 37 °C for 18 h. Cells grown in the liquid medium were used to produce overnight cultures, which were later used for inoculation in both experiments on determining antibiotic resistance and all the tests related to assessment of biofilm formation and the effects of antibacterial agents on biofilm formation. Bacteria were cultured in LB agar in order to maintain the bacterial culture, as well as to enumerate cells in the experiments on assessing the effects of bacteriophages on the biofilms formed.

Sensitivity to oxacillin (beta-lactam antibiotic), vancomycin (glycopeptide), gentamicin (aminoglycoside), tetracycline (tetracycline antibiotic), levofloxacin (fluoroquinolone), and erythromycin (macrolide) (all drugs manufactured by Sigma-Aldrich, USA) was determined by the serial dilution method in accordance with the EUCAST guidelines (v.14.0) [10]. The strains showing multidrug resistance (MDR) were determined as resistant to three or more antibiotics of different classes.

The study involved lytic bacteriophages vB_SauM-515A1 (genus *Kayvirus,* family *Herelleviridae*) and vB_SauP-436A (genus *Rosenblumvirus,* family *Rountreeviridae*) used in the form of sterile filtrates of phage lysates in the LB medium. Bacteriophages were earlier isolated from the commercially available combination phage product “Staphylococcal Bacteriophage” of the series P332 (Microgen; Russia) in the *S. aureus* SA515 (ST8 (ST, sequence type)) and SA436 (ST1) strains and characterized in detail [11]. Phages were grown in appropriate host strains.

Molecular genetic characteristics of bacterial strains

DNA was extracted using the DNA-Express kit (Lytech; Russia) following the manufacturer's instructions. DNA samples were stored at –20°C.

Strain typing was performed by multilocus sequence typing (MLST) in accordance with the standard scheme [12]. The *icaA, icaB, icaC,* and *icaD* genes responsible for biofilm formation were detected by polymerase chain reaction (PCR) using the previously reported primers [13]. The PCR reaction mixture (25 μL) contained 66 mM Tris-HCl (pH = 9), 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 250 μM of each dNTP, 1 unit of Taq DNA polymerase (Lytech; Russia) and 10 pmol of each appropriate primer. Amplification was performed using the DNA Engine Tetrad 2 kit (MJ Research; USA) in accordance with the previously proposed regimes [13]. The amplification products were assessed by horizontal 2% agarose gel electrophoresis with ethidium bromide visualization.

Assessment of biofilm formation

Assessment was performed by the previously reported method [14] with certain modifications. For that the suspension of bacterial cells being in their exponential growth phase (optical density (OD) at 620 nm was 0.12) was inoculated into the wells of the uncoated 96-well flat bottom ventilated plate (Thermo Scientific; USA) containing TSBg (tryptic soy broth supplemented with 1% glucose) (Himedia; India) to the final concentration of 10^4 cells per well and incubated at 37 °C for 24 h without shaking. The final volume in each well was 200 μL. The sterile medium was used as a negative control. After incubation the wells were carefully triple washed with the sterile phosphate buffer (PBS, pH = 7.4) to remove planktonic cells, then stained with the 0.1% crystal violet (CV) aqueous-alcohol solution (Sigma-Aldrich; USA) for 30 min at room temperature. After incubation the dye was triple washed with the sterile PBS. To perform further analysis, the dye bound in each well was eluted by adding 200 μL of 96% ethanol, and optical density of the solution was measured using the Microplate Reader

Table. Biofilm formation patterns of the *S. aureus* strains

Strain	Origin	Locus	ST	Antibiotic resistance						Biofilm production type	EOP (vB_SauM-515A1),%	EOP (vB_SauP-436A),%
				OXA	VAN	GEN	TET	LFX	ERY			
SA515	Novosibirsk	Wound discharge	8	R	I	R	R	S	S	strong	100	–
SA64	Saint Petersburg	Blood	8	R	S	S	R	R	S	strong	267	400
SA412	Lipetsk	Skin and soft tissues	8	R	S	R	I	S	R	strong	–	150
SA2242	Novosibirsk	Bones and joints	239	R	S	R	S	S	S	strong	300	–
SA191	Moscow	Cerebrospinal fluid	239	R	S	R	R	R	R	strong	–	–
SA364	Saint Petersburg	Skin and soft tissues	764	R	S	R	R	n/d	R	strong	–	–
SA436	Nizhny Novgorod	Nasopharyngeal discharge	1	R	S	R	R	S	S	moderate	–	100
SA402	Lipetsk	Bones and joints	5	R	S	R	R	R	R	moderate	183	83
SA88	Krasnodar	Wound discharge	25	R	S	S	S	S	S	moderate	183	167
SA103	Krasnodar	Blood	1	S	S	S	S	S	S	strong	250	133
SA172	Moscow	Wound discharge	1	S	S	S	S	S	S	strong	–	200
SA2153	Smolensk	Sputum	5	S	S	S	S	S	R	strong	250	–
SA2464	Yakutsk	Skin and soft tissues	5	S	S	S	S	S	S	strong	190	–
SA54	Irkutsk	Conjunctival discharge	25	S	n/d	S	S	S	S	strong	500	90
SA2003	Voronezh	Skin and soft tissues	25	S	S	S	S	S	S	strong	245	–
SA837	Smolensk	Skin and soft tissues	8	S	S	S	S	S	S	strong	200	–
SA117	Vologda	Eyelid discharge	121	S	n/d	S	S	S	S	moderate	90	75
SA156	Smolensk	Conjunctival discharge	121	S	n/d	S	S	S	S	moderate	383	–
Sa226	Moscow	Eyelid discharge	121	S	n/d	n/d	n/d	n/d	n/d	moderate	150	200
SA606	Oryol	Skin and soft tissues	398	S	S	S	S	S	S	moderate	–	–

Note: «–» — the strain is resistant to the bacteriophage; S — the strain is sensitive to the antibiotic; R — the strain is resistant to the antibiotic; OXA — oxacillin; VAN — vancomycin; GEN — gentamicin; TET — tetracycline; LFX — levofloxacin; ERY — erythromycin.

Flex-A (Allsheng; China) at 570 nm. All the experiments were conducted in three biological replicates.

The ability of the bacterium to form biofilms was determined in accordance with the earlier proposed criteria: OD ≤ ODc — the strain does not produce biofilms; ODc < OD ≤ 2 × ODc — the strain is a weak biofilm producer; 2 × ODc < OD ≤ 4 × ODc — the strain is a moderate biofilm producer; 4 × ODc < OD — the strain is a strong biofilm producer, where ODc — is the average OD of the negative control + 3 × SD (standard deviation). The sterile medium was used as a negative control [15].

Sensitivity of planktonic forms of strains to bacteriophages

The strains' sensitivity to bacteriophages was determined when assessing the efficiency of plating (EOP), as previously reported [11]. EOP is a ratio of the bacteriophage titer in the test strain to the bacteriophage titer in the host strain (*S. aureus* SA515 for phage vB_SauM-515A1; *S. aureus* SA436 for phage vB_SauP-436A) expressed as a percentage. Bacteriophage titer in the test strain was determined by the Gracia titration method, as previously reported [16]. For that aliquots (5 µL) of the 10-fold serial dilutions of each bacteriophage product (stock of 2×10^9 plaque-forming units/mL or PFU/mL) were applied to the surface of the plates with the semi-solid LB agar (0.6% agar) containing 0.1 mL of the test strain overnight culture (10^6 CFU/mL) and incubated at 37 °C for 24 h. Concentration of phage particles in PFU/mL was estimated for each strain. The efficacy of plating was assessed in three replicates.

Assessment of the impact of bacteriophages on the biofilms formed

The experiments involved the 24-h biofilms produced and washed in accordance with the above method. After washing, phage lysate in TSBg was added to the well to the final concentration of 10^8 PFU/mL, the volume in each well was 200 µL. Sterile PBS was added to the control samples instead of phage lysate. Incubation was carried out for 24 h at 37 °C. Then CV staining was performed as described above, with subsequent OD measurement at 570 nm. All the experiments were conducted in three biological replicates.

In preliminary experiments, we determined the number of cells in the biofilm by adding 200 µL of sterile PBS to the pre-washed biofilm and destroying the biofilm by active pipetting. Serial dilutions of the cell suspension were sown on the LB agar. Colonies were enumerated after the 24-h incubation at 37 °C.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism v. 8.0.1 software (GraphPad Software Inc.; USA) based on the *t*-test data. During the analysis we compared optical density values obtained after the 24-h incubation of the biofilms treated and not treated with the bacteriophage. The differences were considered significant at $p < 0.05$. The Shapiro–Wilk test was used to confirm normal distribution of data, the data on the samples were considered to be almost normally distributed at $p > 0.05$.

Fisher's exact test was used to reveal significant correlations based on two nominal traits in small samples. The correlations were considered significant at $p < 0.05$.

RESULTS

Characteristics of strains and their ability to form biofilms

According to the MLST results, strains of the collection belonged to eight sequence types, among which the most abundant was ST8 (4/20, 20%) (Table). ST1, ST121, ST5, and ST25 accounted for three strains each (15%). As for

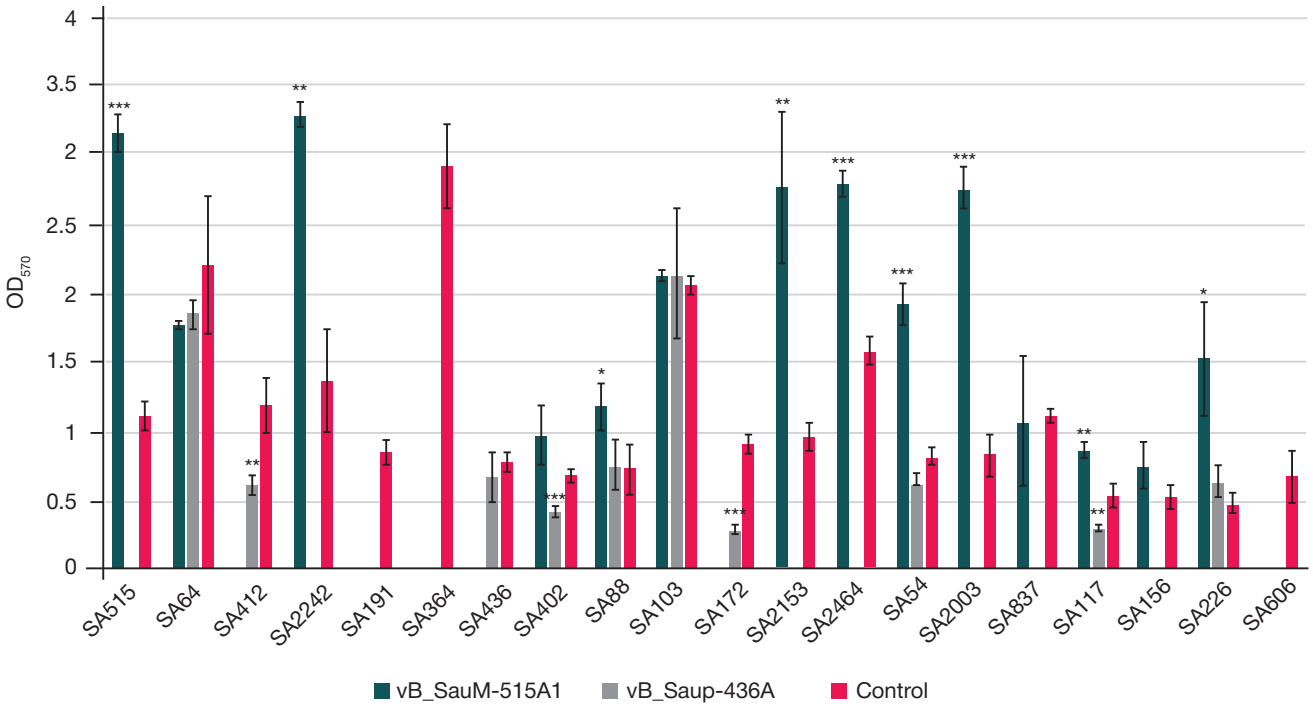

Fig. Effects of bacteriophages vB_SauM-515A1 and vB_SauP-436A on the biofilm biomass. Biofilms treated with bacteriophage vB_SauM-515A1 are highlighted in *green*; those treated with bacteriophage vB_SauP-436A are highlighted in *blue*; the untreated control is highlighted in *orange*. The lack of data in the column indicates that the strain is resistant to the effects of appropriate bacteriophage. The ranges show standard deviation. * — $p \leq 0.05$; ** — $p \leq 0.01$; *** — $p \leq 0.001$

sensitivity to antibiotics, a large number of strains belonged to MRSA (9/20, 45%). Furthermore, seven strains (35%) were characterized by MDR.

All the tested strains were able to form biofilms, and more than a half were strong biofilm producers (13/20, 65%). In the remaining cases (7/20, 35%), the strains were moderate biofilm producers. CFU enumeration showed that the number of cells in the 24-h biofilms reached 10^8 CFU/mL for all the studied strains. The amplification results demonstrated that all the isolates contained a complete set of the *icaADBC* operon genes.

Effects of bacteriophages of various taxonomic groups on the *S. aureus* planktonic cells and biofilms

Based on the results of assessing the effects of bacteriophages vB_SauM-515A1 and vB_SauP-436A on the planktonic cells of strains of the collection it was determined that 14 strains (70%) were sensitive to phage vB_SauM-515A1, while 10 strains (50%) were sensitive to phage vB_SauP-436A. Three strains of the collection (15%) turned out to be resistant to both phages. The phage vB_SauM-515A1 efficiency of plating in sensitive strains varied between 90 and 500%, while that of phage vB_SauP-436A varied between 75 and 400%.

The effect of bacteriophages on biofilm was assessed only for strains sensitive to the corresponding bacteriophage, based on data from experiments with planktonic cells. Biofilms were treated with the bacteriophage titer of 10^8 PFU/mL, which, based on CFU enumeration in the untreated biofilms, corresponds to the multiplicity of infection (MOI) value of 1.

According to the experimental results (Figure), in the majority of cases (9/14, 64.3%), the 24-h treatment with the vB_SauM-515A1 bacteriophage did not cause reduction of the biofilm biomass but, on the contrary, stimulated biomass production. As for remaining five strains (5/14, 35.7%), the exposure to the vB_SauM-515A1 bacteriophage had no significant effect on the biofilm. The exposure to bacteriophage vB_SauP-436A, on the contrary, significantly reduced the biofilm biomass in four strains (4/10, 40%). This bacteriophage had no effect on the biofilms formed by other strains.

It should be noted that statistical analysis has revealed no significant correlations between the fact that the strain belongs to MRSA or certain sequence type and the ability of bacteriophage to stimulate biofilm formation (in the case of vB_SauM-515A1) or destroy biofilms (in the case of vB_SauP-436A). These effects also did not depend on the baseline strains' ability to form biofilms.

DISCUSSION

In this study, the strains were considered isolated from the heterogeneous clinical material that belonged to epidemiologically significant sequence types (Table). Among them the most abundant was ST8, one of the most common sequence types in Russia and all over the world among hospital strains [17, 18]. Strains of this sequence type include the pandemic clones, such as USA300, causing multiple infection outbreaks and often belonging to MRSA [19]. Along with ST8, the ST1, ST5, and ST121 strains were identified, which, according to the literature data, are characterized by antibiotic resistance and are capable of causing severe infections [20–22].

The findings showed that all the studied samples could form biofilms, regardless of the origin and sequence type. Furthermore, all strains were characterized by the presence of the *icaADBC* operon responsible for biosynthesis of polysaccharide intercellular adhesin, the major and best studied component of the *S. aureus* clinical strains' biofilm matrix, in the genome [23, 24]. It should also be noted that the majority of strains turned out to be strong biofilm producers. In combination with antibiotic resistance, this once again emphasizes severity of the problem of treating the infections caused by these strains.

Members of the families *Herelleviridae* and *Rountreeviridae* were used to assess the efficacy of bacteriophages. Phages were selected based on the differences in their morphological

(myoviruses and podoviruses), microbiological (host spectrum, parameters of infection based on the single-step growth curve), and genetic (genome size and the number of genes encoded) characteristics [11]. According to the findings, vB_SauM-515A1 (genus *Kayvirus*, family *Herelleviridae*) showed higher efficacy against planktonic cells compared to vB_SauP-436A (genus *Rosenblumvirus*, family *Rountreeviridae*), which is consistent with the earlier reported data on these bacteriophages [11]. As for other lytic bacteriophages of the family *Herelleviridae* (earlier referred to as *Myoviridae*), it has been also shown that their lytic spectrum varies between 85.3 and 99.2% depending on the collection, while in members of the family *Rountreeviridae* (earlier referred to as *Podoviridae*) this indicator is 64–68% [11, 25, 26].

As for biofilms, it was shown that bacteriophage vB_SauM-515A1 stimulated the biofilm biomass increase, while phage vB_SauP-436A reduced its quantity (Figure). It was earlier reported that myovirus phiIPLA-RODI belonging to the same genus, as vB_SauM-515A1, could stimulate biofilm formation in S. aureus [9]. The authors explain the findings by the increased eDNA content in the matrix, which, in turn, is associated with high lytic activity of bacteriophages of this family. The increased eDNA content contributes to the biofilm structural integrity and stability, as well as modulates formation of amyloid fibers essential for the biofilm architecture maintenance [27]. In another study, the researchers have shown that the use of phages of the genera *Kayvirus и Rosenblumvirus* (separately or in combination) in initial phases of biofilm formation or in mature biofilms does not result in the decrease in biofilm quantity [8]. The authors observed such an effect when using phages at both low (0.1) and high (10) MOI values. Furthermore, the increase in biofilm quantity was observed after the 24-h incubation, except for the case of treating the mature biofilm with the mixture of phages at MOI 10.

It should be noted that in the studies discussed the authors confined themselves to testing one and two bacterial isolates, which limits comprehensive comparison of the results. Nevertheless, the available data suggest the trend towards stimulation of biofilm formation in the *S. aureus* strains shown by phages of the genus *Kayvirus*. According to our data, reduction of the biomass quantity or the lack of significant effect was observed, when treating biofilms with bacteriophage of the genus *Rosenblumvirus*. Perhaps, vB_SauP-436A causes less intense cell lysis and lower eDNA release, which hampers stimulation of biofilm formation. In this regard, the combination use of bacteriophages, either in the form of the cocktail comprising several phages, or in combination with the agents destroying the matrix, such as depolymerases, seems to be a promising approach to combating biofilms [28].

It should also be noted that in this study phage efficacy was assessed by staining the biofilm biomass consisting of matrix and cells (both live and dead) with crystal violet. This method makes it impossible to assess the share of viable cells after treatment with antibacterial agents, so it is necessary to conduct further research for better understanding of the effects of phages on biofilms.

CONCLUSIONS

The study emphasizes complexity and ambiguity of the effects of bacteriophages on the *S. aureus* biofilm, especially in the case of clinical isolates showing high antibiotic resistance. Despite the ability of phages of the families vB_SauM-515A1 and vB_SauP-436A to inhibit growth of planktonic cells, their efficacy against biofilms of the *S. aureus* strains turned out to be low. The findings emphasized the importance of selecting phages based on their specific characteristics and efficacy against biofilms, as well as possible need for combination approaches involving bacteriophages and the agents destroying the biofilm matrix. At the same time, it should be considered that the results of this study have been obtained in the *in vitro* experiments by the limited number of methods. Further research is required to translate the findings into clinical practice, including the experiments involving *in vivo* models, which will make it possible to more accurately assess the efficacy and safety of the proposed approaches under conditions close to reality.

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