COMBINED EFFECTS OF BACTERIOPHAGE VB_SAUM-515A1 AND ANTIBIOTICS ON THE *STAPHYLOCOCCUS AUREUS* CLINICAL ISOLATES

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Currently, the search for new therapy options for infectious diseases caused by multidrug-resistant *Staphylococcus aureus* is a priority. Combining antibiotics with virulent (lytic) bacteriophages may be considered a viable alternative to conventional antibiotic therapy. The study was aimed to assess the combined effects of the lytic bacteriophage vB_SauM-515A1 of *Herelleviridae* family and antibiotics of various classes on the *Staphylococcus aureus* clinical strains. Strains (n = 4) belong to the clinically significant sequence types ST1, ST8, ST121 and are characterized by multidrug resistance. Efficiency of the combination use of two antibacterial agents was assessed by comparison of optical densities of the test samples and controls after 24 hrs. of incubation. Mutually enhancing activities of bacteriophage used in combination with oxacillin, tetracycline and linezolid were revealed, in contrast to the separate use of each agent. Efficiency generally increased with the selected optimum multiplicity of infection values. No antagonism was revealed when combining the phage with antibiotics. Thus, virulent bacteriophage vB_SauM-515A1 can be considered as a possible auxiliary therapeutic agent for antimicrobial-resistant strains of *Staphylococcus aureus*.

Keywords: bacteriophage therapy, *Staphylococcus aureus*, *Herelleviridae*, combined effects, gentamicin, tetracycline, vancomycin, oxacillin, linezolid, levofloxacin

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КОМБИНИРОВАННОЕ ВОЗДЕЙСТВИЕ БАКТЕРИОФАГА VB_SAUM-515A1 И АНТИБИОТИКОВ НА КЛИНИЧЕСКИЕ ИЗОЛЯТЫ *STAPHYLOCOCCUS AUREUS*

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Поиск новых вариантов терапии инфекционных заболеваний, вызванных *Staphylococcus aureus* с множественной лекарственной устойчивостью, на сегодняшний день является приоритетной задачей. В качестве одной из перспективных альтернатив классической антибиотикотерапии может быть рассмотрена комбинация антибиотиков с вирулентными (литическими) бактериофагами. Целью работы было оценить результат совместного воздействия литического бактериофага vB_SauM-515A1 семейства *Herelleviridae* и антибиотиков различных классов на клинические штаммы *Staphylococcus aureus*. Штаммы (n = 4) относятся к клинически значимым сиквенс-типам ST1, ST8, ST121 и характеризуются множественной лекарственной устойчивостью. Эффективность комбинированного воздействия двух антибактериальных агентов оценивали при сравнении значений оптической плотности опытных и контрольных образцов после 24 ч инкубации. Наличие взаимодополняющих эффектов было показано при совместном использовании бактериофага с оксациллином, тетрациклином и линезолидом, по сравнению с использованием каждого из агентов по отдельности. Эффективность повышалась в основном в рамках подобранных оптимальных значений множественности инфекции. Антагонистические эффекты комбинации фага и антибиотиков не были выявлены. Таким образом, вирулентный бактериофаг vB_SauM-515A1 можно рассматривать в качестве возможного вспомогательного терапевтического агента против устойчивых к антибактериальным препаратам штаммов *Staphylococcus aureus*.

Ключевые слова: бактериофаговая терапия, *Staphylococcus aureus*, *Herelleviridae*, комбинированное воздействие, гентамицин, тетрациклин, ванкомицин, оксациллин, линезолид, левофлоксацин

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Staphylococcus aureus is a pathogenic microorganism causing severe inflammatory disorders of the skin and soft tissues, as well as invasive infections, such as pneumonia, endocarditis, osteomyelitis, etc. [1]. It is difficult to treat such diseases due to wide spread of the multidrug-resistant (MDR) strains, among which methicillin-resistant *Staphylococcus aureus* (MRSA) is the most clinically significant. About 4.95 million people died due to antibiotic-resistant infections in 2019. Staphylococcal infections were the major cause of deaths, and more than 100,000 deaths were caused by methicillin-resistant strains [2]. In 2020 in Russia, the share of bacteria of genus *Staphylococcus* resistant to such antibiotics as tetracycline, gentamicin, erythromycin and oxacillin was 15–25%. The vast majority of strains showed intermediate resistance to levofloxacin and ciprofloxacin [3]. More recently isolated cases of acquired resistance to vancomycin and linezolid used as drugs of choice in treatment of MRSA infections have been reported [4, 5]. These statistics highlight the need to search for alternative antimicrobial agents. Bacteriophage preparations might be considered as such agents [6, 7].

Bacteriophages (phages) are viruses that naturally infect prokaryotic cells. Only virulent (lytic) phages are used as therapeutic agents due to the need to avoid possible horizontal transmission of antibiotic resistance determinants and genes encoding bacterial toxins [8]. Phage preparations have some advantages over antibiotics. Thus, virulent bacteriophages are capable of lysing bacteria regardless of their sensitivity to antibiotics. This makes phages a powerful tool for combating resistant strains. Another advantage is no side effects on the patient's body. This enables safe use of virulent bacteriophage preparations even in complex clinical cases [9].

Currently, the use of bacteriophages is one of the promising approaches to treatment of staphylococcal infections caused by MDR strains [10]. Successful implementation of these approaches has been confirmed by clinical experiments, both animal [11] and human [12]. We should also mention the effectiveness of bacteriophage preparations against biofilms formed by *Staphylococcus aureus* [10].

The combined use of bacteriophages and antibiotics is considered the most promising strategy for treatment of disorders caused by drug resistant strains [13, 14]. A number of papers about various pathogens report that the combined use of median lethal doses of antibiotics and bacteriophages is more effective compared to separate use [13, 15]. Beneficial effects of such combination were first reported in 2007 [13]. Studies have now shown that the combined use of bacteriophage and antibiotic may also result in neutral and adverse effects [16, 17].

The increased efficiency associated with the combination use of antibacterial agents (mutually enhancing actions) can be explained by one of the following effects: additive or synergistic. More active suppression of bacterial growth associated with additive effects is achieved through summing up antibacterial effects exerted by the agents. Synergism happens when the efficiency of the combination is significantly higher compared to the separate use of individual components or their sum. Neutral effects happen when there are no significant differences between the combination use of drugs and the use of at least one antimicrobial agent. Antagonism happens when the effects of one agent suppress the effects of another one. It should be noted that only isolated cases of antagonistic interactions between bacteriophages and antibiotics have been reported [17].

To date, the described effects were observed when using the combinations of bacteriophages and some antibiotics (vancomycin, daptomycin, oxacillin) against *S. aureus* [12, 17]. However, taking into consideration the genetic and phenotypic heterogeneity of the pathogen, even the laboratory strains, it is important to test suitability of the phage-antibiotic pairs using the larger set of bacterial isolates to reveal the patterns underlying the emergence of this or that resulting effect.

The study was aimed to assess the combined effects of the lytic bacteriophage of *Herelleviridae* family and antibiotics of various classes on the multidrug-resistant (MDR) clinical strains of *Staphylococcus aureus*.

METHODS

Bacterial strains

The study used *S. aureus* strains (SA64, SA413, SA1050, and SA515/1) obtained from the collection of the Laboratory of Molecular Genetics of Microorganisms, Federal Research and Clinical Center of Physical-Chemical Medicine of FMBA of Russia. Bacteria were grown in the LB (lysogeny broth) culture medium (Oxoid; UK) for 18–24 hrs at 37 °C. Typing of the strains was performed by multilocus sequence typing (MLST) using the standard scheme [14]. Minimum inhibitory concentrations (MICs) of antibiotics were defined by the CLSI serial dilution method [18]. MICs of six antibiotics (oxacillin, vancomycin, gentamicin, tetracycline, levofloxacin, linezolid (Sigma-Aldrich; USA)) were defined.

Bacteriophage

Bacteriophage vB_SauM-515A1 (*Herelleviridae* family) was earlier isolated from the commercial complex phage preparation "Staphylococcal bacteriophage" P332 (Microgen; Russia) on the SA515 S. aureus host strains. The detailed bacteriophage characteristics were reported earlier [19, 20].

Determining the studied bacteriophage titer on the tested strains

The titer was determined by the previously reported method of Grazia [21]. For that aliquots (5 μL) of the bacteriophage preparation ten-fold sequential dilutions (stock 2 × 10^9 plaque-forming units (PFU)/mL) were applied onto the surface of plates with semi-solid LB agar (0.6% agar) containing 0.1 mL of the tested strain overnight culture (10^6 colony-forming units

Table 1. Characteristics of the *Staphylococcus aureus* strains

Strain	ST	EOP	Susceptibility to antibiotics, μg/mL					
			Oxacillin	Vancomycin	Gentamicin	Tetracycline	Levofloxacin	Linezolid
SA64	1	267%	< 0.125 (S)	8 (I)	128 (R)	64 (R)	8 (R)	4 (I)
SA413	8	283%	< 0.125 (S)	0.5 (S)	128 (R)	32 (R)	4 (R)	8 (R)
SA1050	121	72%	< 0.125 (S)	8 (I)	< 0.125 (S)	64 (R)	< 0.125 (S)	4 (I)
SA515/1	8	100%	4 (R)	8 (I)	128 (R)	32 (R)	< 0.125 (S)	4 (I)

Note: R — resistant strains, I — strains showing intermediate resistance, S — susceptible strains.

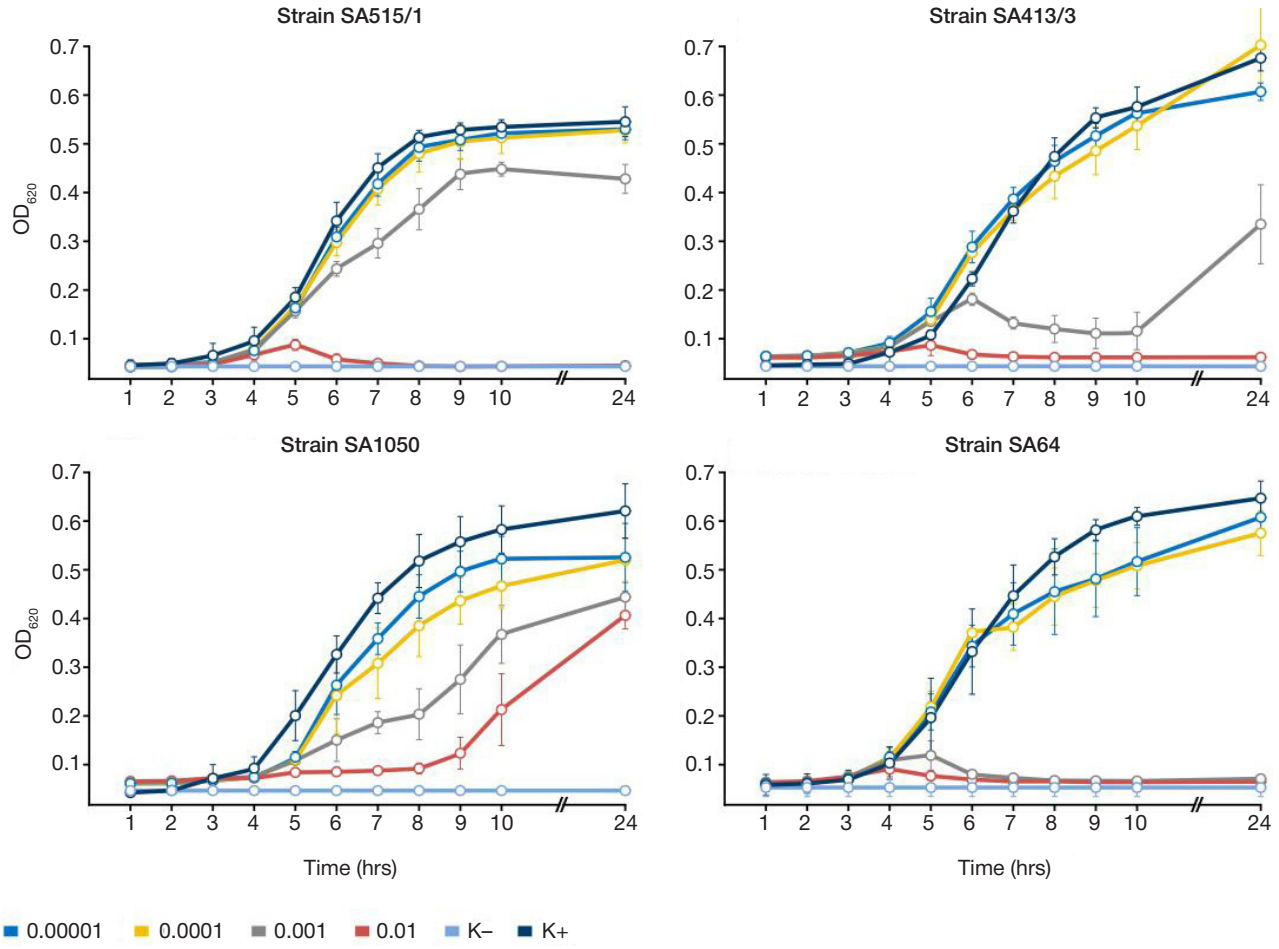

Fig. 1. Growth curves of the *S. aureus* infected with bacteriophage vB_SauM-515A1 with various MOI values

(CFU/mL) and incubated at 37 °C for 24 hrs. The concentrations of phage particles for the tested strains were measured in PFU/mL. The effectiveness of the tested strain lysis by bacteriophage was assessed based on efficiency of plating (EOP) [19]. EOP is defined as a relationship of the phage titer on the tested strain to the phage titer on the host strain (SA515/1), expressed as a percentage. Plating efficiency was tested three times.

Studying the combined effects of antibiotics and bacteriophage

The combined effects of antibiotics and bacteriophages were assessed as previously described [17]. Experiments were carried out in the 96-well flat bottom plates (Thermo Scientific; USA) in 200 µL in the LB medium. Bacterial cells were inoculated during the exponential growth phase (OD_{620} = 0.2; 5 × 10^8 CFU/mL) to the final concentration of 10^4 cells per well. Bacteria were infected separately with the phage at four multiplicity of infection (MOI) values (0.01; 0.001; 0.0001; 0.00001), then exposed to different antibiotics and a combination of two antibacterial agents in various concentrations. Antibiotic concentrations of 1/8 MIC, 1/4 MIC, 1/2 MIC were used. Inoculated culture medium with no added antibacterial agent was used as a positive control, while pure growth media was used as a negative control. The dynamics of the phage and antibiotic effects on bacteria were defined by continuous measurement of optical density (OD) at 620 nm for 10 hrs and after 24 hrs of incubation at 37 °C using the Multiscan Ascent Microplate Reader (Thermo Electron Corporation; Finland). Growth curves for the S. aureus strains infected with bacteriophage at various MOI values were plotted based on the OD values. In certain cases, mutually enhancing activities were confirmed by comparison of the finite OD values in the final point (24 hrs) as previously reported [15].

Statistical analysis

Statistical analysis was performed in the Graph Pad Prism software package, v. 8.0.1 (GraphPad Software Inc.; USA) based on the *t*-test. The analysis involved comparison of OD values obtained after 24 hrs of incubation for samples exposed to only one antimicrobial agent (antibiotic/bacteriophage) with similar values of the samples simultaneously exposed to both agents.

RESULTS

Bacterial strains were characterized based on the sequence types (ST) and tested for susceptibility to bacteriophage and antibiotics (Table 1). MLST showed that *S. aureus* strains fell into sequence types ST1, ST8, and ST121. All samples were MDR, there were strains resistant to oxacillin (SA515/1), gentamicin (SA64, SA413, SA515/1), levofloxacin (SA64, SA413), and linezolid (SA413) among them. All the studied bacteria showed resistance to tetracycline. Intermediate resistance to vancomycin and linezolid of three strains (SA64, SA1050, SA515/1) was revealed. Bacteriophage vB_SauM-515A1 lysed all the studied bacteria. The highest efficiency of lysis exceeding the value obtained for the host strain (SA515/1) more than 2.5 times was shown for strains SA64 (267%) and SA413 (283%). Bacteriophage lysed strain SA1050 less actively (72%).

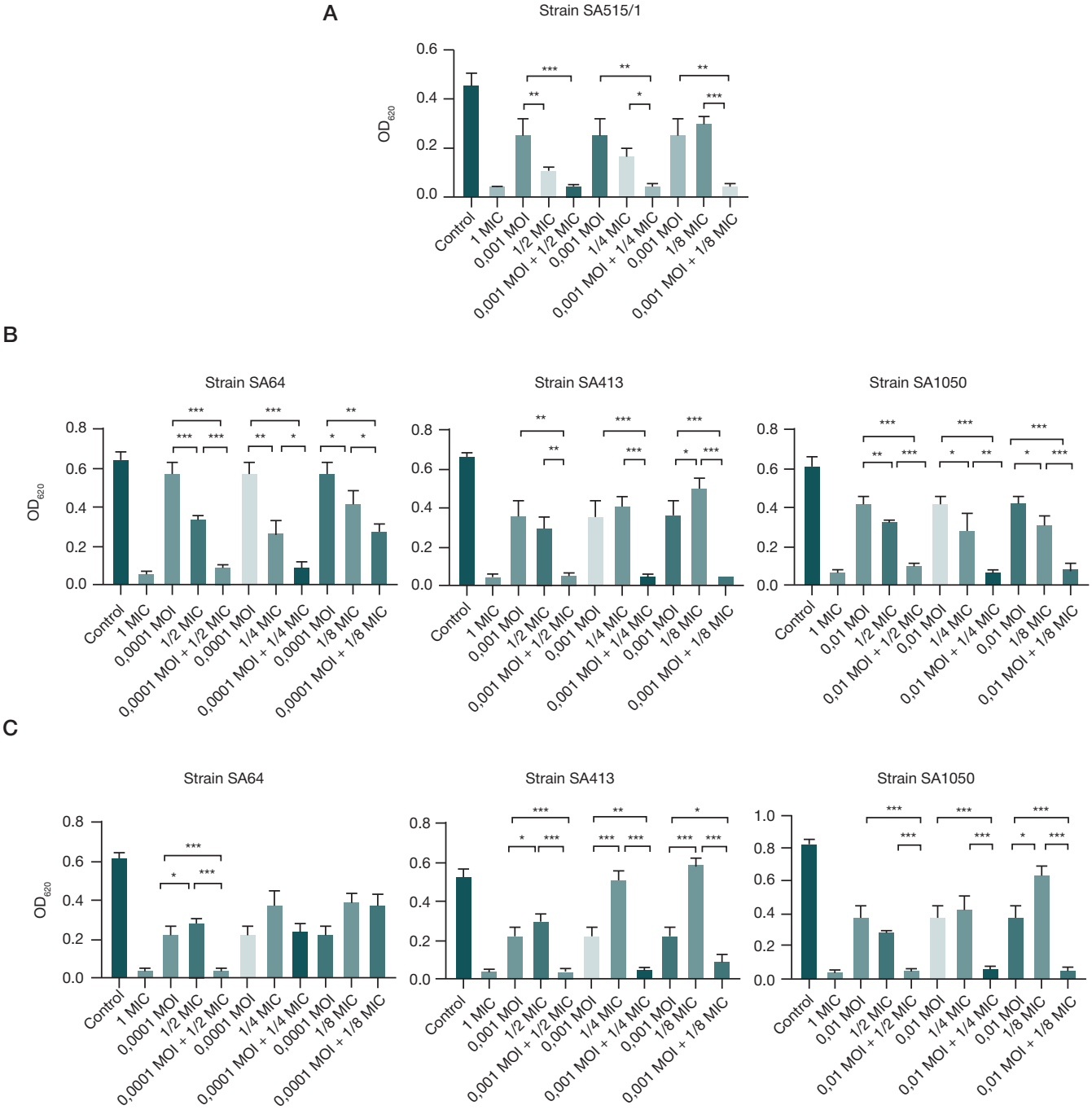

Fig. 2. Combined effects of the lytic bacteriophage vB_SauM-515A1 and antibiotics (oxacillin (**A**), tetracycline (**B**), linezolid (**C**)) on the *S. aureus* strains at optimum MOI values. Statistical significance: * — $p \leq 0.05$; ** — $p \leq 0.01$; *** — $p \leq 0.001$

Optimum MOI values were defined using the growth curves of bacterial cultures infected with bacteriophage in order to assess the combined effects of antimicrobial agents (Fig. 1). Reduced OD compared to non-infected control with MOI values of 0.01 и 0.001 was reported for the host strain SA515/1, moreover, bacteriophage-cell ratio that corresponded to MOI = 0.01, completely suppressed growth by hour 24. Thus, experiments involving the use of MOI = 0.001 were the most interesting in terms of assessing mutually enhancing activities of bacteriophage and antibiotic against the SA515/1 strain cell culture. The efficiency of the SA1050 strain lysis by bacteriophage vB_SauM-515A1 was lower than that reported for the host strain, therefore, only partial suppression of cell growth was achieved with MOI = 0.01 and MOI = 0.001: in contrast to the non-infected control, OD dropped from 0.6 to 0.44 and 0.4, respectively, by hour 24. More effective lysis was reported for strains SA413 and SA64 than for the host strain, the most optimal vB_SauM-515A1–SA413 cell ratio was MOI = 0.001, and the most optimal ratios for strain SA64 were 0.0001 and 0.00001.

The efficiency of the combination use of antibiotic (oxacillin, vancomycin, tetracycline, gentamicin, levofloxacin and linezolid) and bacteriophage vB_SauM-515A1 was assessed for strains resistant to the selected antibiotic or showing intermediate resistance. The mutually enhancing activities of oxacillin and bacteriophage vB_SauM-515A1 were considered using the only oxacillin-resistant strain SA515/1 as an example. Bacteriophage enhanced the effects of antibiotic with the MOI value of 0.001 that was optimal for this strain and the concentrations of antibiotic of 1/4 and 1/8 MIC (Fig. 2, Table 2).

Table 2. Resulting effects of the combination use of various vB_SauM-515A1 bacteriophage and antibiotic concentrations on the *S. aureus* clinical strains

Strain	MOI	Oxacillin, MIC share			Vancomycin, MIC share			Gentamicin, MIC share			Tetracycline, MIC share			Levofloxacin, MIC share			Linezolid, MIC share		
		1/8	1/4	1/2	1/8	1/4	1/2	1/8	1/4	1/2	1/8	1/4	1/2	1/8	1/4	1/2	1/8	1/4	1/2
SA64	0.00001	S																	
	0.0001										+	+	+						+
	0.001				L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
	0.01				L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
SA413	0.00001	S			S														
	0.0001											+	+						+
	0.001										+	+	+				+	+	+
	0.01							L	L	L	L	L	L	L	L	L	L	L	L
SA1050	0.00001	S						S						S					
	0.0001												+						
	0.001											+	+					+	+
	0.01										+	+	+				+	+	+
SA515/1	0.00001													S					
	0.0001																		
	0.001	+	+																
	0.01	L	L	L	L	L	L	L	L	L	L	L	L				L	L	L

Note: + — mutually enhancing activities; empty cell — lack of mutually enhancing activities; L — culture completely lysed by bacteriophage; S — antibiotic-susceptible strain.

Similar effects were observed for oxacillin concentration of 1/2 MIC, however, this finding was non-significant.

The majority of mutually enhancing activities against other strains were reported for the combination use of bacteriophage and tetracycline or linezolid (Fig. 2, Table 2). When combined with bacteriophage, these antibiotics more effectively lysed strains SA64, SA413 и SA1050 that any of antimicrobial agents taken separately: this was true for various combinations of concentrations (Table 2). It should be noted that mutually enhancing activities were most often observed with optimum MOI values for each strain and antibiotic concentration of 1/2 MIC.

When using bacteriophage in combination with vancomycin, gentamicin and levofloxacin, no mutually enhancing activities against the *S. aureus* strains were observed. Furthermore, no antagonism was revealed in any of the strains when using antibiotic (oxacillin, vancomycin, tetracycline, gentamicin, linezolid, and levofloxacin) in combination with bacteriophage vB_SauM-515A1 (Table 2).

DISCUSSION

High prevalence of infections caused by MDR *S. aureus* strains is a major challenge faced by modern health care. The combination use of antibiotics and bacteriophages is a solution. We used an earlier characterized member of the family *Herelleviridae*, the lytic bacteriophage vB_SauM-515A1, to study the combined effects of two agents on the MDR *S. aureus* strains [20, 22]. Staphylophages of the family *Herelleviridae* are one of the most effective for therapy [19]. These obligate virulent phages show a broad spectrum of lytic activity [19]. The latter is in line with our findings: bacteriophage vB_SauM-515A1 effectively suppressed growth of all the studied *S. aureus* strains belonging to highly prevalent clinically significant sequence types (Table 1) [23, 24].

Medications used for treatment of various infectious diseases caused by staphylococci (oxacillin, vancomycin, gentamicin, tetracycline, levofloxacin, linezolid) were selected to assess the combined effects of the lytic bacteriophage and antibiotics [25, 26]. The above-mentioned antibiotics belong to different classes, each of them is characterized by specific mechanism underlying the effect on bacterial cells. It is important to note that the study involved both bacteriostatic (tetracycline, gentamicin, linezolid) and bactericidal (oxacillin, vancomycin, levofloxacin) medications. The studied strains were generally resistant to these antibiotics.

The study revealed cases of mutually enhancing activities shown by medications (oxacillin, tetracycline, linezolid) and bacteriophage vB_SauM-515A1, which is consistent with the reports by other authors. Thus, it was shown that the use of oxacillin and linezolid in combination with the lytic bacteriophage Sb-1 more effectively inhibited growth of the *S. aureus* strains in the majority of cases [17, 25]. In its turn, the combination of tetracycline in certain concentration and bacteriophage of *Herelleviridae* family ensured more effective growth suppression in the *S. aureus* biofilm-forming strains than the phage [27].

The results of the recent study conducted by colleagues were opposite [28]. The authors showed that simultaneous use of antibiotic and lytic bacteriophage never significantly increased the efficiency of bacterial growth inhibition, regardless of the antibiotic type. Such discrepancies may be explained by the outcome dependance on the target bacterial strain [9]. Thus, in our study, strain SA515/1 exposure to the combination of tetracycline, linezolid and the phage never resulted in growth suppression, while the same combination showed mutually enhancing activities against other strains.

Upon detection of beneficial effects associated with the combination use of antibiotics and bacteriophages, it is important to select optimal median lethal doses of both agents. When used in appropriate concentrations, their antibacterial effects are probably summed up, as observed during the study (Fig. 2, Table 2). The effectiveness of combining bacteriophages and antibiotics may be also due to bypassing the mechanisms underlying antibiotic resistance during interaction between cells and virus particles. It has previously been shown that the lytic phage of the resistant *Pseudomonas aeruginosa* strain uses the

membrane protein, porine, essential for efflux of antibiotics, as a receptor. To acquire resistance to phage, the bacterium gets rid of the efflux system and becomes antibiotic-susceptible again [29]. Therefore, the effects of bacteriophage on the cell may provide clone selection, thus increasing the bacterial culture susceptibility to antibiotics.

It is important to note, that no antagonism, i.e., reduced efficiency of some antibacterial agent (antibiotic/bacteriophage) in presence of another one, was observed in any of the studied combinations. Low rate of such negative cases has been also reported in other papers [17, 27].

For now, it remains unclear, what is the basis of mutual activities of phages and antibiotics against bacterial cells. Higher efficacy may be explained by both simple summation of effects exerted by individual antibacterial agents and more complex mutual effects resulting in more active suppression of cell growth. In-depth study of the causes of emerging mutually enhancing activities is essential for further practical use of phages as therapeutic agents in combination with antibiotics.

CONCLUSIONS

The findings show that the combination use of the lytic bacteriophage vB_SauM-515A1 and antibiotics of various classes can be more effective than the separate use of antibacterial agents. Thus, the studied phage can be considered as a promising therapeutic agent for the *S. aureus* MDR strains. The data obtained may be used for further study of effects resulting from the combination use of two antibacterial agents of different types.

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