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NOVEL APPROACH FOR ISOLATION OF LYTIC BACTERIOPHAGES AGAINST STAPHYLOCOCCUS AUREUS

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Strictly lytic or virulent bacteriophages against *Staphylococcus aureus* belong to order *Caudovirales* and comprise predominantly two genera: *Kayvirus* and *Rosenblumvirus*. Representatives of both taxonomic groups are well known as safe and viable antibacterial agents. Phages of the genus *Kayvirus* show usually a broad host range activity against clinical isolates of *S. aureus*. Nevertheless continuous isolation of new phages against these bacteria is required in order to provide sufficient activity for therapy of biofilms or to prevent phage inactivation by neutralizing antibodies. In the present study, we describe an approach for isolation of lytic bacteriophages of the genus *Kayvirus* by mitomycin C induction of clinical isolates of *S. aureus*. Our findings suggest the clinical isolates of *S. aureus* a reach source for lytic bacteriophages. However taking into account the inducibility of the *Kayvirus* representatives it have to be evaluated a presence of further unknown life cycles for these bacteriophages such as pseudolysogeny.

Key words: *bacteriophage isolation, mitomycin C, Staphylococcus aureus, Kayvirus, lytic bacteriophages, pseudolysogenic bacteriophages.*

Introduction. Increasing antibiotic resistance is a major global issue, especially in medicine. The emergence and spread of antibiotic resistance leads to the appearance of multi-resistant and even pan-resistant bacteria. In February 2017, the World Health Organization (WHO) published a list of 12 bacteria for which new antibacterial agents are urgently needed. Methicillin- and vancomycin-resistant *Staphylococcus aureus* are in fifth place on this list. The frequency of detection of staphylococci with multiple antibiotic resistance currently reaches 40-57 % [22], especially in nosocomial infections.

The efficacy of antibiotic therapy for nosocomial infections caused by *S. aureus* can be reduced or completely eliminated by the bacterial biofilm. Bacteria in biofilms are incorporated into their own exopolysaccharides (EPS), which protect them from the penetration of antibiotics and significantly increase the mechanical stability of the biofilm. Such tolerance to antibiotics can be observed in the absence of their genetically determined antibiotic resistance. Therefore, bacteria in biofilms can often be tolerant to antibiotics, despite the antibiotic sensitivity of their planktonic forms [14, 21].

The use of virulent bacteriophages is an alternative to antibiotic therapy in the presence of a healthcare associated infection (HAI) and an infection caused by bacteria resistant to conventional antimicrobial drugs [3]. Bacteriophages (phages) are viruses that infect only bacteria with host specificity and growth inhibition activity for replication and reproduction. Virulent phage undergoes only a lytic cycle for host lysis and thus has the potential to develop as a new biocontrol agent against a specific host pathogen [4]. In addition, the phage takes advantage of the infection and lysis of antibiotic-resistant strains without affecting other bacteria or human cells.

However, in the case of infections associated with biofilms, phage therapy can last more than 8 weeks [2]. In addition, long-term or repeated courses of phage therapy may be ineffective due to the appearance in patients of neutralizing antibodies against the used bacteriophage strains [6].

In addition, the population of bacterial cells in the biofilm contains a small population of dormant cells - persistent bacteria, whose metabolism is significantly reduced. Therefore, they are more resistant to antimicrobial agents, including antibiotics, preventing the complete destruction of the biofilm even after prolonged high-dose antibiotic therapy. In these cases, the use of passive phage therapy is especially promising, which consists in achieving a sufficiently high number of bacteriophages adsorbed on bacterial cells, causing their so-called lysis from without due to penetration and subsequent depolarization of the persister cell membrane [1]. This dictates the need for a sufficiently active destruction of the biofilm matrix, which is necessary to increase the concentration of phages in the environment surrounding the bacteria.

Therefore, isolation of bacteriophages that most actively destroy mature biofilms of *S. aureus* is promising for increasing the efficiency and shortening the time of phage-mediated eradication of *S. aureus* biofilms. At the same time, the selection of virulent bacteriophages that are lytically active even against lysogenic isolates of this type of staphylococcus, which are more resistant to phage therapy, is relevant [7].

More than 90 % of clinical isolates of *S. aureus* carry one or more temperate phages in the form of prophages in their genome [15].

Lytic phages kill their bacterial host cell through lysis, while mild bacteriophages (or lysogenic phages) either integrate into the bacterial genome (forming a so-called prophage). In the case of environmental stress, functional prophages can be excised and enter the lytic cycle [16]. Another form of phage-bacteria coexistence is known as a pseudolysogeny when for example phage DNA exist as a plasmid in the bacterial cytoplasm. The phages of the genus *Rosenblumvirus* are known to coexist in equilibrium with their host staphylococci [9].

The presence of prophages in the bacterial genome acts as an additional gene pool of horizontally transferred genes, which makes bacteria more adaptable, for example, due to the presence of virulence genes, antimicrobial resistance genes, or survival factors [5, 12, 19].

Prophages without defects are able to be induced when exposed to factors damaging the host's DNA, when the so-called SOS response occurs, which confirms the lysogenicity of the host microorganism culture. One of the most widely used inducers of moderate bacteriophages is mitomycin C.

A potentially effective approach to the search for virulent (strictly lytic) *S. aureus* bacteriophages is their isolation from mature biofilms of *S. aureus* strains formed under in vitro conditions, isolated from the most typical biotopes of the human body for this pathogen and causing a chronic recurrent inflammatory process at the sites of their colonization. For example the most common infectious disease caused by *S. aureus* is recurrent tonsillitis. Its etiological significance is associated with its resistance to antimicrobial drugs and the ability to form biofilm in the tissues of the tonsils [8].

The aim of the study was to isolate strains of virulent staphylococcal bacteriophages from the microbiota of the tonsils of patients with chronic tonsillitis, possessing lysing activity against circulating antibiotic-resistant *S. aureus* isolates forming mature biofilms.

Materials and methods. The studies were carried out on 106 surgically removed tonsils in patients with chronic adenoiditis or chronic tonsillitis (recurrent tonsillitis) at the Astrakhan branch of the Scientific and Clinical Center of Otorhinolaryngology of the Federal Medico-Biological Agency of the Russian Federation.

Sectional material was delivered to the laboratory within two hours after the planned tonsillectomy. The tonsil tissues were homogenized by grinding in a sterile porcelain mortar with the addition of sterile sand and saline. The homogenized product of each sample was spitted in two parts. The first part was plated on yolk-salt agar according to the Drygalski method with a sterile spatula to obtain isolated colonies. The inoculations were incubated at 37°C for 20-24 hours. After 20-24 hours, suspicious colonies with a golden yellow color and surrounded by a halo of lecithinase activity were subcultured onto nutrient agar slant in order to isolate a pure culture. Crops were incubated for 20-24 hours at 36-37°C. The isolates were identified using standard gram-microscopy, cultural methods and specific PCR. Isolates showing coccal morphology, gram-positive staining, beta-hemolysis and producing catalase, coagulase and DNase were tested for biofilm formation.

The biofilm biomass was measured using a standard technique for staining with crystal violet according to the method described by Stepanović S. et al. [20].

LB broth was used as a nutrient medium. For the formation of biofilms, overnight broth cultures were used, brought with fresh LB broth to optical density (OD) ≈ 0.2. The measurements were carried out in 96-well flat-bottomed plates in a volume of 200 µl at a wavelength of 595 nm on an iMark plate photometer (Bio-Rad, USA). Cultures standardized by OD were inoculated in a volume of 200 µl into the studied microtiter plates, followed by incubations at 36-37°C for 20-24 hours, and titrated to determine the amount of CFU of introduced planktonic broth culture.

To check for the presence of inducible prophages, fresh daily cultures from solid media were transferred in a loop into 5 ml of LB broth and incubated for 2-2.5 hours at +36-37°C to reach the log phase. A solution of mitomycin C was prepared by adding sterile 10 ml of water for injection to a vial with powder for every 10 mg of the drug. The solution was sterilely poured into cryovials (100 µl) and stored at –20°C (after thawing, it was stored at +4 C for up to 1 month). The preincubated suspension was added in an amount of 1-10 µg of the prepared solution of mitomycin C to achieve a final concentration of 1 µg/ml and incubated for 2-2.5 hours at + 37°C with periodic shaking. The cultures were inactivated with chloroform in a volume of 0.5-0.6 ml and the supernatant was obtained by centrifugation at 4000-5000 rpm for 20-30 minutes. The supernatant was collected in sterile tubes with vented stoppers and incubated at room temperature for 1-2 hours. Supernatants in undiluted form and in dilutions 10^{-2}, 10^{-8} were tested for the presence of phages by the Gratia double agar layer plaque assay on the culture of *S. aureus* 2072 strain, free from inducible bacteriophages [13].

The second part of the homogenized tissue was suspended in 5 ml of saline, incubated for 2-2.5 hours at room temperature followed by the chloroform inactivation and centrifugation at 4000-5000 rpm for 20-30 minutes. The resulting supernatant was tested for a phage presence by the plaque assay on the culture of *S. aureus* 2072 strain.

For the plaque assay in both types of samples the LB medium with 1.5% agar-agar was poured into Petri dishes with a diameter of 90-100 mm in a volume of 20-30 ml (first layer). After solidification of the medium, the dishes were incubated in a thermostat at +36-37°C for 1.5-24 h to remove excess moisture. In a test tube with 2.5 ml of LB medium melted in a microwave oven and cooled to +45-49°C with an agar-agar content of 0.7% were added 1 ml of the test phage-containing sample and 0.2 ml of an overnight broth culture of *S. aureus* 2072 strain. The contents of the tubes were quickly mixed so as not to solidify the agar, and poured onto the surface of the first agar layer evenly in a second layer. After the agar had solidified, the inoculation was incubated in a thermostat at the temperature optimal for the host strain. The result was taken into account after 18-24 hours. The presence of a bacteriophage was determined by the presence of transparent spots (plaques), clearly visible against the matte background of bacterial growth. Isolated plaques were selected, suspended in sterile saline, then chloroform was added to the suspension, taken in a volume of 0.5-0.6 ml. The resulting suspension was incubated at room temperature for 20-30 minutes, then the incubated suspension was centrifuged at 4000-5000 rpm for 20-30 minutes and the supernatant was taken into a sterile tube.

Confirmation of the taxonomy of isolated phages was performed by genus specific PCRs. Following primers were designed for it (Table 1). Moreover we tested a presence of known integrase genes as described before [11]. The phage samples for the PCRs were consistently filtered via the 0.22 µm PES syringe filter, treated with the DNase I and the resulting phage DNA was isolated using the spin-column K-Sorb kit (Syntol, Russia). The PCR was performed using standard recombinant Taq-polymerase chemistry (Thermo Fisher Scientific, USA) on the CFX96 Real-time amplifier (Bio-Rad, USA).

Table 1

Oligonucleotide primers for phage identification

Primer name	**Oligonucleotide sequence, 5′-3′**	**Size of the amplicon, bp**	**Annealing temperature, °C**	**MgCl concentration, mM**	**Target genus**
KV_caps_fw	gta cgc tga cca att cca ag	276	59	1.8	*Kayvirus*
KV_caps_rv	tca gat act ggt gct act cc				
RV_caps_fw	gat aat cac cgt aag cac gt	580	57	1.8	*Rosenblumvirus*
RV_caps_rv	cgt aat tat cca cgt atg gc				

Results. In the course of the work, 110 staphylococcal isolates with lecithinase activity were isolated and characterized. Of these, 106 isolates were identified by PCR as *S. aureus* and 9 isolates exhibited lysogenic properties. All isolates showed resistance to at least one antibiotic.

A conventional plaque assay showed no bacteriophages within the cultures. However, after mitomycin C induction 7 cultures were phage-positive. The results are shown in Table 2.

Table 2

Results of the efficacy of isolation of phages from cultures of *Staphylococcus aureus*

Name of the biofilm producing *S. aureus* isolate	**OD_{595} of the biofilm, M±SD**	**Result after the conventional phage isolation**	**Result after phage isolation with mitomycin C**	**Phage name**	**Plaque morphology**	**Plaques figure**
3A27	0.053±0.007	Not isolated	Isolated	Ast1	∅1-1.5 mm with Halo	
9зев14	0.803±0.091	Not isolated	Isolated	Ast2	∅1-1.5 mm with Halo	

Name of the biofilm producing *S. aureus* isolate	**OD_{595} of the biofilm, M±SD**	**Result after the conventional phage isolation**	**Result after phage isolation with mitomycin C**	**Phage name**	**Plaque morphology**	**Plaques figure**
7зев14	0.408±0.053	Not isolated	Isolated	Ast3	∅0.5-1 mm with Halo	
4зев14	0.976±0.101	Not isolated	Isolated	Ast4	∅1-2 mm without Halo	
2аден	0.047±0.006	Not isolated	Isolated	Ast5	∅1-1.5 mm without Halo	
125	0.105±0.011	Not isolated	Isolated	Ast6	∅1-1.5 mm with Halo	

Name of the biofilm producing *S. aureus* isolate	OD_{595} of the biofilm, M±SD	Result after the conventional phage isolation	Result after phage isolation with mitomycin C	Phage name	Plaque morphology	Plaques figure
147	0.053±0.008	Not isolated	Isolated	Ast7	∅1-1.5 mm without Halo	

Results from the Table 3 demonstrate different morphology of plaques of bacteriophage isolates isolated as a result of the addition of mitomycin C. However results of the PCR tests showed that all isolated bacteriophages belong to the *Kayvirus* genus. Moreover, no phage genome associated integrases typical for *S. aureus* were detected.

Table 3

Taxonomy identification of isolated bacteriophages

Phage name	Genera of lytic *S. aureus* phages		*S. aureus* integrase genes [20]						
	Kayvirus	*Rosenblumvirus*	Sa1int	Sa2int	Sa3int	Sa4int	Sa5int	Sa6int	Sa7int
Ast1	+	-	-	-	-	-	-	-	-
Ast2	+	-	-	-	-	-	-	-	-
Ast3	+	-	-	-	-	-	-	-	-
Ast4	+	-	-	-	-	-	-	-	-
Ast5	+	-	-	-	-	-	-	-	-
Ast6	+	-	-	-	-	-	-	-	-
Ast7	+	-	-	-	-	-	-	-	-

Conclusions. Results of the present work demonstrate the chronically *S. aureus* colonized tonsils as a new source for isolation of bacteriophages against these staphylococci. In particularly the cultures of the *S. aureus* themselves can be considered as a source of lytic phages. This novel finding correlate good with recent results of works of Głowacka-Rutkowska A. et al. [9, 10].

The fact of mitomycin C induction of the *Kayvirus* bacteriophages, that were previously known only as lytic, bring new direction of both development of new phage isolation methods and optimization of phage therapy. According to the NCBI database currently at least 56 representatives of this genus have annotated genome (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=1857843). Since the first publication of the type species phage K genome (S. O'Flaherty et al., 2004) a number of studies of the *Kayvirus* phages demonstrate strongly the absence of integrases or other factors able to provide the temperate life cycle [17, 18]. However, the coexistence of these bacteriophages and clinical isolates indicates a possibility of the pseudolysogenic life cycle for the representative of the genus *Kayvirus* and matches to the recent hypothesis [10]. Further, we are planning to present complete genome sequences of the isolated Kayviruses.

In general the pseudolysogeny is not a contraindication for therapeutic application of bacteriophages but under this condition efficacy of phage therapy can be decreased. Thus further evaluation of the factors provoke the pseudolysogeny by isolated phages as well as evaluation of known *Kayvirus* phages and *S. aureus* coexistence is required in order to develop clinical recommendations for optimal phage therapy.

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АНАЛИЗ УРОВНЯ ВИТАМИНА D У ПАЦИЕНТОВ С ХРОНИЧЕСКОЙ РЕСПИРАТОРНОЙ ПАТОЛОГИЕЙ И У ЗДОРОВЫХ ДЕТЕЙ В АСТРАХАНСКОЙ ОБЛАСТИ

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Изучен статус витамина D у когорты пациентов с хроническими заболеваниями легких, конкретизированы показатели сывороточного кальцидиола у группы контроля, состоящей из условно здоровых детей Астраханской области. Выявлена закономерность значений сывороточного кальцидиола, обусловленная этиологией возникновения хронических заболеваний легких, определены показатели уровня витамина D в сыворотке крови у детей контрольной группы. Проанализирована ось значений уровня витамина D в зависимости от возрастного аспекта и гендерного фактора у пациентов с хроническими заболеваниями легких и в группе контроля.

Ключевые слова*: хронические заболевания легких, хронический бронхит, муковисцидоз, иммунодефициты, витамин D, дети.*

ANALYSIS OF VITAMIN D LEVELS IN PATIENTS WITH CHRONIC RESPIRATORY PATHOLOGY AND HEALTHY CHILDREN IN ASTRAKHAN REGION

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Vitamin D status was studied in a cohort of patients with chronic lung diseases, serum calcidiol indicators were specified in a control group consisting of conditionally healthy children of the Astrakhan region. The regularity of serum calcidiol values is revealed due to the aetiology of chronic lung diseases, the serum vitamin D levels in the children of the control group are determined. The axis of vitamin D levels according to age aspect and gender factor in patients with chronic lung diseases and the control group was analyzed.

Key words: *chronic lung diseases, chronic bronchitis, cystic fibrosis, immunodeficiencies, vitamin D, children.*

Введение. Хронические заболевания легких (ХЗЛ), представленные большим разнообразием нозологических форм, являются наиболее сложным разделом детской пульмонологии. Хроническое поражение органов дыхания ассоциировано с постоянным интоксикационным синдромом, гипоксией, изменениями в системе иммунитета, что ведет к ранней инвалидизации больных, необходимости