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Isolation and characterization of virulent phages of *Staphylococcus epidermidis*.

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Abstract
Staphylococcus epidermidis is a coagulase-negative species of staphylococci (CoNS) commonly found on human skin and mucous membranes as part of the normal flora. Nowadays, *Staphylococcus epidermidis* is considered as an important opportunistic pathogen, and the most common source of infections is medical equipment's. Since *Staphylococcus epidermidis* is rapidly becoming resistant to antibiotics, our aim was to isolate active bacteriophages against them. As a result of the studies, four active virulent bacteriophages against Staphylococcus epidermidis were isolated by screening of *Staphylococcus aureus* phages: vB_S.e.22, vB_S.e.23, vB_S.e.39 and vB_S.e.40. The morphology of all four virions was studied, and it was determined that these phages belong to the Myoviridae family according to the Ackermann classification. These phages are characterized with lithic activity, range of action and stable lysis.

Keywords: Bacteriophages, infections, *S. epidermidis*

1. Introduction

T Coagulase-negative staphylococci, and in particular *Staphylococcus epidermidis*, are now becoming more common causes of infections in humans[1]. *Staphylococcus epidermidis* is a coagulase-negative species of staphylococci (CoNS) commonly found on the skin and mucous membranes of humans and other animals. Clinically, CoNS are becoming an important group of pathogenic staphylococci [2]. As a group, coagulase-negative staphylococci (CoNS) are among the most frequently isolated bacteria in the clinical microbiology laboratory and are of increasing importance, especially as causes of nosocomial infections [3,4]. CoNS is the most common cause of nosocomial infections, with a frequency about the same as that of its more virulent relative *Staphylococcus aureus*. Some strains of epidermal staphylococcus, being safe on the skin, cause infectious diseases that are deadly for humans after surgical interventions [5]. In the past, coagulase-negative staphylococci were generally considered contaminants of little clinical significance. However, over the past four decades, these organisms have become an important agent of human disease. *Staphylococcus epidermidis* is currently regarded as an important opportunistic pathogen, the most common source of infections on permanent medical devices. This is due to the fact that *S. epidermidis* is a constant and ubiquitous colonizer of human skin and, as a result, is characterized by a high probability of contamination of the device during insertion [6].

The most common victims of *S. epidermidis* infection are premature newborns, those with leukemia or other malignancies, intravenous drug abusers, and patients with permanent polymer bodies such as prosthetic devices or intravenous catheters. *S. epidermidis* - in children it can be activated already in the first days of life, in older children it can manifest itself against the background of a series of diseases that impair immunity [7].

Treatment of staphylococcal infections at the present stage is an acute problem related to the genetic mechanisms determining the multiple resistance of antibiotics to these microbes [8,9].

As shown by the literature of recent years, Staphylococcal strains are rapidly acquiring resistance to antibiotics. Studies conducted by us in 2014-2015 have shown that *S.epidermidis* strains are characterized by multiple antibiotic resistance [10].

In order to stop the spread of staphylococci in humans, mainly antibiotic-resistant forms, to prevent and treat infected people, it is possible to fight microorganisms not only by physicochemical factors, but also by harmless means such as bacteriophages. The aim of our study was to isolate active virulent bacteriophages against coagulase negative *S. epidermidis* and to study them. To achieve this task, it is important to select phages with high lysine activity and a wide range of action.

Materials and method

Bacterial strains

In these studies were used 34 strains of *S. epidermidis*, 31 strains of *S. aureus*, 19 strains *of S. haemoliticus*, 11 – of *S. saprofiticus*, 5 – of *S. hominis* and one by *one S. warneri, S. xylosis, S. cohnii, S. capitis, S. capnoe* strains from G.Eliava institute collection. Cultivation of strains was performed on Brain heart infusion (BHI) medium and selective Mannitol salt agar.

Bacteriopheges

To determine activity against *S. epidermidis* strain by spot test were used 4 freshly isolated phages– vB_S.e.22, vB_S.e.23, vB_S.e.39, vB_S.e.40 of *S. aureus* from Department of Phage and Strain collection of G.Eliava institute.

For isolation of new active phages from environmental source 25 different samples were collected in from rivers in Georgia (rivers Mtkvari and Digmula).

Electron microscopy

The morphology of the phage particles was using an electron microscope JEM x100 (JEOL). Parlodion plates were overlaid with 1010 PFU/mL phage suspensions with uranyl acetate as a contrast agent.

Phage biology study

Biological properties, mainly phage-host interaction parameters including adsorption, stability, latent period, lysis time and average burst size, were calculated by standard methodology and frequency of formation of phage resistant mutants were done by standard methodology [12].

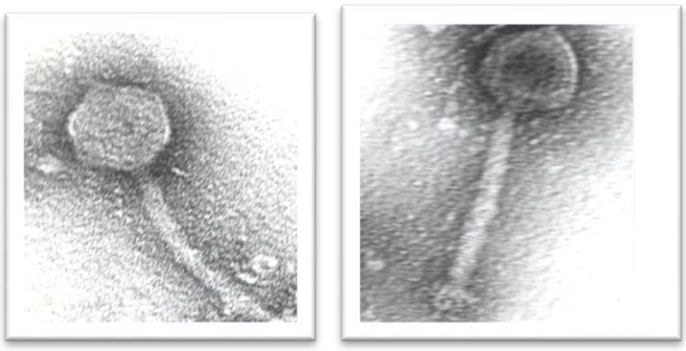
Results and Discussion

In order to isolate bacteriophages specific to *S. epidermidis* microorganisms, samples were taken from the Mtkvari River and Digmula, at different period of the year as well as from the wastewater of Tbilisi clinics. Despite many attempts bacteriophage could not be isolated. Therefore, in laboratory the phage susceptibility of epidermal staphylococcal strains were tested on 42 phages of S. aureus. Study of phage susceptibility shoued that 5% of *S. epidermidis* strains were sensitive to phages and 95% of them were resistant. As a result, of screening *S. aureus* phages 4 active bacteriophages, were selected. *S. epidermidis* 202 was taken as a host cell for these 4 phages. To determine whether the *S. epidermidis* 202 strain contained phage, we have used Fisk method. As a result, it was determined that bacterial strain *S. epidermidis* 202 did not contain phage. The virion morphology of 4 clones: vB_S.e.22, vB_S.e.23, vB_S.e.39, vB_S.e.40 was studied by electron microscopy.

These phages are characterized with an icosahedral head and a long tail. The vB_S.e.22 phage head is 65x65 nm and the tail 217x17 nm, the vB_S.e.23 phage head is 72x90 nm in diameter and 22x18 nm in length. Head of vB_S.e.39 - 81x95 nm, tail - 204 x 22nm; vB_S.e.40 phage head size 91 x 91 nm, tail size 81 x 22.7 nm. according to the Ackermann classification [11], the above-mention phages belong to the Myoviridae family.

1. 2.

3. 4.



Pic.1: Virion morphology of *S. epidermidis* phages - 1) vB_S.e.22; 2) vB_S.e.23;3) vB_S.e.39; 4) vB_S.e.40

We propagated epidermal phages, prepared phage concentrates to study their biological properties. Phage stability were tested by the Appelman method [12], which is different for all 4 phages and was ranging from 10^{-3} -10^{-8}.

The activity range of given phages: vB_S.e.22, vB_S.e.23, vB_S.e.39, vB_S.e.40 was studied to the 105 staphylococcal strains (*S. aureus*, *S. epidermidis*, *S. warneri*, *S. xylosis*, *S. cohnii*, *S. capitis*, S. *saprofiticus*, *S. hominis*, *S. haemoliticus*, *S. capnoe*) existing in the collection of G. Eliava Institute.

According to the Table 1 the activity range of the given phages to *S. epidermidis* strains was as follows: vB_S.e.22 – 23.5%, vB_S.e.23- 32.3%, vB_S.e.39 – 26.4% and vB_S.e.40- 11.7%. The activity range of the same phages to *S. aureus* strains was as follows: vB_S.e.22 – 64.5%, vB_S.e.23- 70.9%, vB_S.e.39 – 67.7% and vB_S.e.40- 61.2%. The lysis spectrum of these phage clones is 26.6–38%.

Table 1. Study of the lysis spectrum of *S.epidermidis* bacteriophages.

Staphylococcus strains	Number of strains	vB_S.e.22		vB_S.e.23		vB_S.e.39		vB_S.e.40	
		Quantity	%	Quantity	%	Quantity	%	Quantity	%
S.aureus	31	20	64.5	22	70.9	21	67.7	19	61.2
S.epidermidis	34	8	23.5	11	32.3	9	26.4	4	11.7
S.haemoliticus	19	4	21	5	26.3	3	15.7	4	21
S.hominis	5	-	-	-	-	-	-	-	-
S.saprofiticus	11	1	9	2	18.1	1	9	1	9
S.warneri	1	-	-	-	-	-	-	-	-
S.sylosis	1	-	-	-	-	-	-	-	-
S.cohnii	1	-	-	-	-	-	-	-	-
S.capitis	1	-	-	-	-	-	-	-	-
S.capnoe	1	-	-	-	-	-	-	-	-
All	105	33	31.4	40	38	34	32.3	28	26.6

To determine the general pattern of intracellular reproduction of phages, we studied the individual phases of interaction of 4 bacteriophages (vB_S.e.22, vB_S.e.23, vB_S.e.39, vB_S.e.40) with the host cell: adsorption, latent period and burst size (Table 2).

Table 2. Parameters of one step growth cycle *S.epidermidis* phages

Phage	Strain	Adsorbtion Time in min	Adsorntion %	Latent period in min	Burst size	Lysis time in hr
vB_S.e.22	*S. epidermidis* 202	15	83.1	180	55.3	4
vB_S.e.23		15	55.6	180	59.7	4
vB_S.e.39		10	60	150	157.6	3
vB_S.e.40		10	59.3	150	242.2	3

The percentage of adsorption rate varies between 55.6 and 83.1. The latent period revealed the minimum period of time (150 - 180 min) that lasts from the adsorption of the virus to the moment of lysis. The latent period for phages vB_S.e.22 and vB_S.e.23 is 180 minutes and the lysis time is 4 hours. The latent period for vB_S.e.39 and vB_S.e.40 phages is 150 minutes and the lysis time is 3 hours. The burst size of *S. epidermidis* 4 phages per infected cell is different. Studies have shown that the average burst size of phage particles from an infected host cell ranges from 55.3 to 242. Intracellular multiplication of phages can be divided into 2 parts: 1) 0 to 60 minutes, when the number of bacteriophage particles is stable and their slow growth is observed. 2) A sharp increase in the number of particles within 60 minutes to 3 hours.

During investigation period was determined formation of phage resistant mutant of *S. epidrmidis 202* strain. It was found that frequency of formation of mutants against *S. epidermidis* phages is low $63{,}7x10^{-8}$ – $97{,}5x10^{-8}$.

Table 3. Frequency of phage-resistant mutants.

Phage	Host strain	N	r	a
vB_S.e.22	*S.epidermidis* 202	$2x10^8$	605	$90x10^{-8}$
vB_S.e.23		$2x10^8$	650	$97{,}5x10^{-8}$
vB_S.e.39		$2x10^8$	525	$78{,}7x10^{-8}$
vB_S.e.40		$2x10^8$	425	$63{,}7x10^{-8}$

N - the amount of bacteria in the control
r - Average number of phage-resistant mutants
a - Frequency of mutation

Conclusion

Based on screening of staphylococcal strains, 4 active virulent bacteriophages - vB_S.e.22, vB_S.e.23, vB_S.e.39, vB_S.e.40 were isolated against epidermal staphylococci. Clones of these phages were obtained. 105 different species of staphylococcal strains were fested for lysis spectrum and range activity. Complete resistance was detected against - *S. hominis*, *S. warneri*, *S. xylosis*, *S. capitis*, *S. cohnii* and *S. capnoe* strains. The lysis spectrum of phage clones against other strains - *S. aureus*, *S. epidermidis*, *S. haemoliticus*, *S. saprofiticus*, is 26–38%. Morphologically these phages belong to the Myoviridae family. Intracellular reproduction time of – vB_S.e.22, vB_S.e.23, vB_S.e.39, vB_S.e.40 phages is 3 -4 hours. Burst size per infected cell is ranging from 55.3 to 242.2 units. With the host cell including adsorption it should be noted that low-burst size phages

(vB_S.e.22 and vB_S.e.23) have a longer latent period and lysis time (180min and 4h) than phages vB_S.e.39 and vB_S.e.40 (150min and 3h). However, no correlation among intracellular reproduction of these phages, lysis spectrum and low frequency mutation was observed.

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