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Fersis-Phage against Purulent-Inflammatory (*Staphylococcal*, *Streptococcal*) Pathologies

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Abstract

The problem of prevention and treatment of infectious diseases in infectious and non-infectious clinics is one of the priorities in practical public health. The application of phage preparations, as antimicrobial medioprophyllactic remedies against *staphylo*- and *streptococcal* infections seems to be an alternative to antibiotics and sulfonamide preparations. Pronounced tendency of circulating pathogenic bacterial strains towards reducing sensitivity to antibiotics, also caused by irrational use of the latter, further confirmed our decision to create a new phage preparation. Based on the conducted studies the new variant of Fersis, an active, multivalent phage preparation, consisting phages against *staphylococci* (*S. aureus*, *S. epidermidis*) and *streptococci* (*S. pyogenes*, *S. viridians*, *S. sanguis*, *S. salivarius*, *S. agalacticae*) causing oral cavity infectious diseases, has been prepared.

also group diseases. Pyogenic *streptococcus* is the causative agent of pyogenic invasions of the mucous membranes, skin and deep tissues. A typical expression of invasion is phlegmon. In the last decade of the twentieth century, there was a return of invasive *streptococcal* infection, characteristic of the 20s of the century. The reason for the prevalence of pathogenic *streptococci* is immunodeficiency state among people and ecological problems [2-4]. However, it should be noted that along with mono infection, complex mixed infections are very common.

Antibiotic resistance in human pathogenic bacteria is a threat to public health that has grown immensely in the last years. It is therefore all the more urgent to secure alternative treatment strategies. Phage therapy is one of the alternatives to antibiotics that for a long time has been underexplored in medicine [5,6].

Phage therapy, the use of phages to cure bacterial infections, has received much attention in recent years due to the emergence and rapid spread of antibiotics resistance. In fact, resistance genes towards last resort treatments for multidrug-resistant bacteria are reported to be circulating all around the world. This highlights an urgent need to coordinate a global effort in the search for antibiotics adjuvants or alternative improved treatments [7].

The successful use of phage preparations as antimicrobial medio prophylactic remedies, with *staphylococcosis* and *streptococcal* oral cavity infections, seems to be alternative to antibiotics and sulphanilamide preparations. Scientific research has been published on phage therapy, and review articles are of particular interest [8-11].

In the study of bacterial flora of the oral cavity, along with mono infection, mixed *staphylo*- and *streptococcal* infections often occur in parallel. The association of these two microbes caused complex forms of diseases and antibiotic resistance was expressed most clearly.

The aim of the presented work was production of new, the most optimal phage preparation Fersis (*staphylococcal* and *streptococcal*)-highly active, multivalent, purified diphage with

Keywords: *Staphylococcus*; *Streptococcus*; Bacteriophage; Phage preparation Fersis

Introduction

In clinical medicine, special attention is paid to purulent infections, which retain a leading role in human dental infectious pathology. The infection is a causative not only various inflammatory diseases, but also numerous complications such as periodontitis, gingivitis, stomatitis etc.

The most common etiological agents that cause purulent-inflammatory diseases are *staphylo*- and *streptococci*.

The spectrum of staphylococcal diseases is very wide, since in any part of the body where staphylococcus invades, a pathological process can form. There are many species of *staphylococcus* known [1]; however, two species-*S. aureus* and *S. epidermidis*-predominate in humans.

Streptococcus, namely *S. pyogenes* for a number of characters are closer to pathogenic bacteria, causing not only sporadic, but

wide range of action for local and oral administration, during infections such as periodontitis, gingivitis, stomatitis etc.

Materials and Methods

Strains of *staphylococci* and *streptococci*, which cause pathological process in the human body, were isolated from patients with periodontitis, gingivitis and stomatitis diseases. The strains occurred in association (simultaneously) and in case of *staphylococci* belonged mainly to the following species: *S. aureus*, *S. epidermidis* and *S. saprophyticus* and in case of *streptococci*: *S. pyogenes*, *S. sanguis*, *S. salivarius*, and *S. agalacticae*.

To isolate and identify the causative agent of the disease, the most common bacterial identification methods- microbiological, biochemical, serological and API system were used (Figures 1-3) [12].

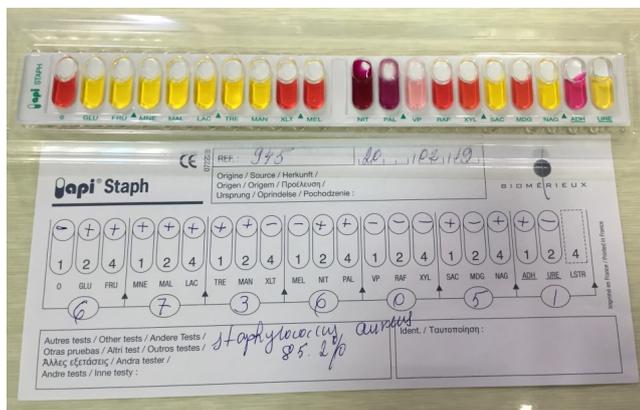


Figure 1. Using API system for diagnostics of *Staphylococcus aureus* strains.

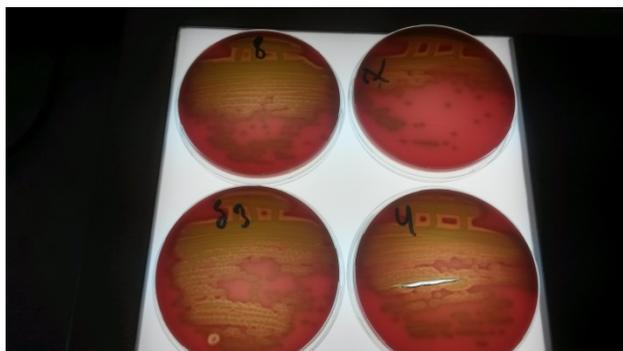


Figure 2. Hemolytic activity of *Staphylococcus aureus* strains isolated from oral cavity.

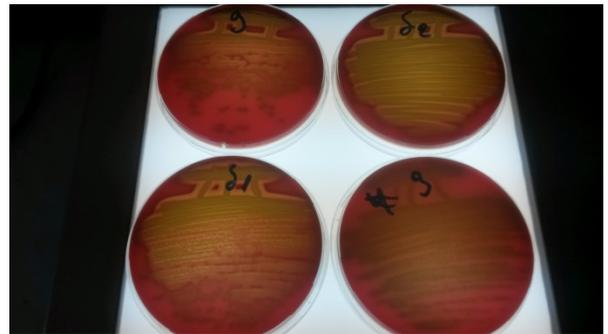


Figure 3. Hemolytic activity of *Streptococcus pyogenes* strains isolated from oral cavity.

Determination of phage sensitivity of freshly isolated clinical and standard microorganisms was carried out using a technique modified.

The antibiotic sensitivity of *Staphylococcus* and *Streptococcus* strains isolated during oral infectious pathologies have been studied for the following antibiotics: Ampicillin, Gentamicin, Kanamycin, Penicillin, Streptomycin and Tetracycline.

Bacteriophage isolation

Races of bacteriophages were isolated from pus, wastewater and other sources and were passaged systematically on the production strains of microorganisms. In addition, works were conducted to select race of phages on phage-resistant and weakly lysed strains. "Mother bacteriophage" was prepared from selected races. Upon controlling of "mother bacteriophage" according to Appelman's method, lysis occurred and activity of all selected production strains corresponded to a titer of no less than 10^7 .

Phage should cause specific lysis of the corresponding strain. Specific activity of the preparation according to the Appelman's method in case of *staphylococci* should be 10^6 and in case of *streptococci* -10^5 .

The bacteriophages were isolated from waste water by filtration. Isolation of bacteriophages was carried out as follows: 100 ml of concentrated broth was added to 90 ml of wastewater. The required culture was added to the mixture and incubated at 37°C for 24 hours. After 24 h incubation in a thermostat, the mixture was filtered through filters with a pore diameter of $0.45\ \mu\text{m}$ (Millipore, United States), and the filtrates were spot-tested for the presence of phages by application of the filtrate (0.1 mL) on the lawn of the test strain on a solid nutrient medium (Figure 4).



Figure 4. Wastewater filtration.

The result was considered positive if there was a lysis zone on the lawn in 18-24 h of cultivation at 37°C [12,13]. The highly specific bacteriophage strains were screened and selected by the plate method according to Gracia [14].

Cultivation, concentration and purification of bacteriophages on synthetic and semisynthetic liquid media were carried out according to the regulations for the production of liquid bacteriophage. The culture was grown to a titer of 5×10^8 cells/ml, adding a phage with a multiplicity of infection of 0.2; aeration was continued for 8-10 h (aeration rate 1-2 l/min).

The lysate was left overnight, after which chloroform (0.4%) was added and centrifuged at 5000 g for 30 min at +4°C; concentration and purification of liquid lysates were carried out by two methods, either by PEG-6000 or by ion-exchange chromatography through a DEAE-cellulose column, followed by twofold differential centrifugation [15]. A total of 523 patients with periodontitis, gingivitis, stomatitis were examined.

Results and Discussion

A study of species composition of microorganisms during different human diseases isolated from oral cavity has shown the prevalence of *staphylococci* and *streptococci* over other pathogens (**Table 1**). Among the 745 strains isolated, 304 belonged to *staphylococci*, 297 to *streptococci* and only 144 strains to other pathogens. The percentage ratio made up 40.8%, 39.9% and 19.3%, respectively.

As for the intraspecific differentiation of the isolated staphylococcal strains and the studied cases, *staphylococci* were mainly represented by *S. aureus* and *S. epidermidis* and *streptococci* by *S. pyogenes*, *S. sanguis*, *S. viridians*, *S. salivarius*, *S. agalacticae*.

The isolation frequency of individual types of *staphylococci* and *streptococci* was distributed as follows: *S. aureus* was most frequently diagnosed from all the organs studied-24.1%, then *S. epidermidis*-21.8%.

Streptococci, isolated from the oral cavity covered all the species we met: along with *S. pyogenes* and *S. viridians*, there were *S. sanguis*, *S. salivarius*, *S. agalacticae*. These species also occur in the case of mucous throat pathology. Thus, in the cases studied by us, different types of *staphylococci* and *streptococci* were found most frequently.

Along with mono-infections caused by *staphylococci* and *streptococci*, associated infections, such as intraspecific associations of *staphylococci*, e.g. *S. aureus* and *S. epidermidis* and *streptococci*, e.g. *S. pyogenes*, *S. sanguis*, *S. viridians*, *S. salivarius*, *S. agalacticae*, are common. In addition, an association of different genera of *staphylococci* and *streptococci* with specific variations is frequently observed.

Table 1. The basic composition of microorganisms isolated from various human organs with certain diseases.

Microorganisms	Absolute number and percentage of isolated microorganism strains													
	Strains in all		Mucus				Discharge				Vaginal		Oral cavity	
			Nasal		Throat		Ear		Eye					
	abs	%	abs	%	abs	%	abs	%	abs	%	abs	%	abs	%
<i>Staphylococci</i>	304	40.8	38	13	58	19	51	17	53	17	48	16	56	18
<i>Streptococci</i>	297	39.9	51	17	69	23	44	15	2	0.7	13	4	118	40.3
Other microorganisms	144	19.3	11	7.6	25	17.7	26	18	25	17.7	38	26	19	13
A total of	745	100	100	13	152	20	121	16	80	11	99	13	193	27

The study of susceptibility of microorganism strains isolated by us during different diseases to the traditionally used antibiotics and to a number of new ones of different groups has shown that presently circulating strains are characterized mainly by high level of resistance, as well as multidrug resistance to individual antibiotics (**Figure 5**).

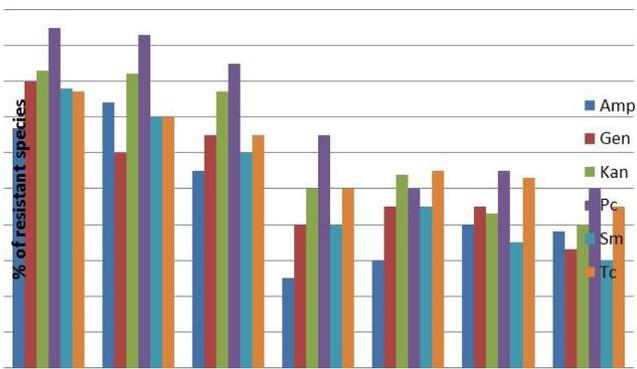


Figure 5. Sensitivity of bacterial strains to antibiotics isolated from oral cavity.

Amp-ampicillin, Gen-gentamicin, Kan-kanamicin, Pc-penicillin, Sm-streptomycin, Tc-tetracyclin.

Isolated *S. aureus* strains showed a certain resistance to Penicillin (95.0%), while they were sensitive towards Ampicillin (67%). *S. epidermidis* strains showed high resistance to Penicillin (93%) and Kanamicin (82.0%). Among the *Streptococcal* isolates, there was shown intermediate resistance with most of the antibiotics used in test.

The pronounced tendency of circulating strains of pathogenic bacteria with reduced sensitivity to antibiotics, caused by irrational use of the latter, further confirmed the correctness of our decision to create a new alternative phage preparation.

Proceeding from the obtained results, the presented work was aimed at the creation of a new phage preparation containing phages against the species of microorganisms isolated by us, namely, *S. aureus*, *S. epidermidis* and *S. pyogenes*, *S. sanguis*, *S. viridians*, *S. salivarius*, *S. agalacticae*, which cause periodontitis, gingivitis, stomatitis etc. (Figures 6-9).



Figure 6. New phage isolated against *Staphylococcus aureus* strain.

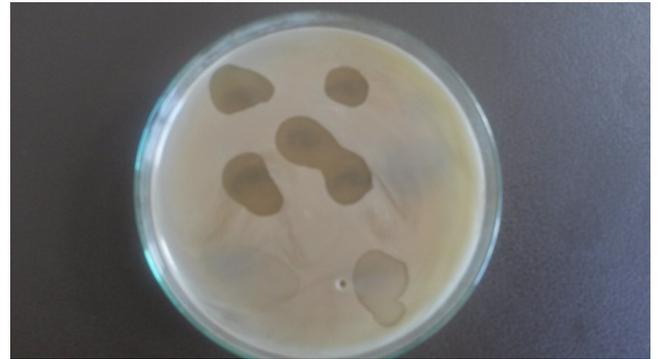


Figure 7. New phage isolated against *Streptococcus pyogenes* strain.

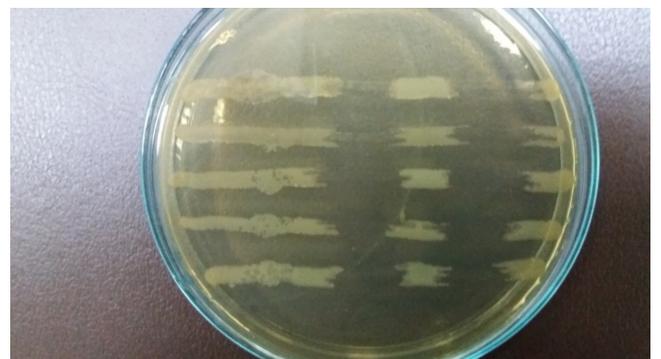


Figure 8. Sensitivity of new phage against *Staphylococcus aureus* strains isolated during oral infectious pathologies.

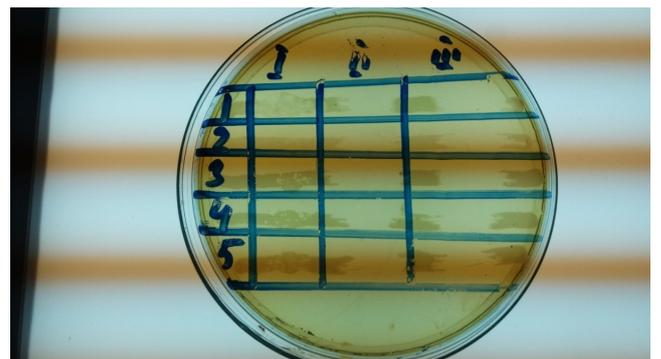


Figure 9. Sensitivity of new phage against *Streptococcal pyogenes* strains isolated during oral infectious pathologies.

The titers of phage preparations separately corresponded for *staphylococci* to 10^6 and for *streptococci* - 10^5 ;

In order to prepare the Fersis-diphage, thus obtained purified phagolysates with the appropriate activity were mixed and then tested for the activity both on typical strains of *staphylo-* and *streptococci* and on freshly isolated strains of the same bacteria.

Table 2. Specific activity of bacteriophage.

Phages	Average titers by Appelman *	
	<i>Staphylococcal</i> strains **	<i>Streptococcal</i> strains **
1. Fersis	10 ⁵ -10 ⁶	10 ⁴ -10 ⁵
2. <i>Staphylococcal</i> component	10 ⁶	–
3. <i>Streptococcal</i> component	–	10 ⁵

* -titers are given in the inverse values;

** -the number of strains studied -50 (for each).

The results of the investigation of the specific activity of the phage components, used to control the Fersis-phage and the

product, are presented in **Table 2**. As can be seen, in the Fersis-phage, the titres of both the *staphylo*- and *streptococcal* components were by a factor of ten higher than in individual cases.

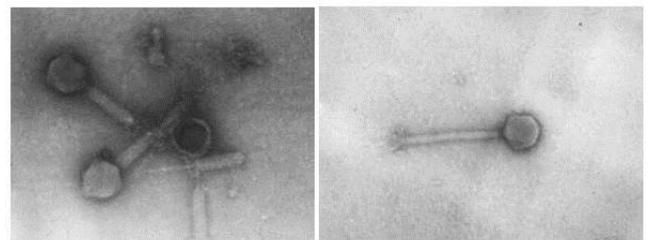
The next stage of the study was to determine the phage sensitivity of freshly isolated strains of *staphylococci* and *streptococci*. The results are shown in **Table 3**. A high degree of sensitivity of *staphylococcal* strains (*S. aureus*, *S. epidermidis*) to both the Fersis-phage and the *staphylococcal* component was found -75-77% and 72-74%, respectively. There was also observed pronounced sensitivity of *S. pyogenes*, *S. viridians*, *S. sanguis*-69-70% and 65-62%, respectively, while *S. salivarius*, *S. agalacticae* had less sensitivity-50-60% and 50%, respectively.

Table 3. Phage sensitivity of *staphylococcal* and *streptococcal* bacterial strains isolated during certain human diseases.

Bacteria	abs	Sensitivity to bacteriophages					
		<i>Staphylococcal</i> component		<i>Streptococcal</i> component		Fersis combined	
		abs	%	abs	%	abs	%
<i>Staphylococcus</i>	240						
<i>S. aureus</i>	115	86	75	-	-	89	77
<i>S. epidermidis</i>	103	74	72	-	-	70	74
<i>Streptococcus</i>	210						
<i>S. pyogenes</i>	101	-	-	70	69	71	70
<i>S. viridians</i>	80	-	-	52	65	50	62
<i>S. sanguis</i>	15	-	-	11	70	10	70
<i>S. salivarius</i>	10	-	-	5	50	4	60
<i>S. agalacticae</i>	4	-	-	2	50	2	50

Morphological characteristics of phages

Electron microscopy study of phages involving in Fersis phage preparation allowed classifying them (**Figures 10 and 11**). *Staphylococcus aureus* phage 15 had a tubular tail with helical symmetry and the diameter of 15 nm. The tail length of the phage was 12.5 nm, and the head diameter of 65 nm. This phage was referred to Myoviridae family. Phages *Staphylococcus aureus* phage 23, *Streptococcus pyogenes* phage 1 and *Streptococcus pyogenes* phage 2 had relatively thin, long, non-contractile, and flexible tail with an isometric head. The tail length of these phages varied between 20-34 nm, a head diameter was in a range of 60-95 nm. They were referred to Siphoviridae family.

**Figure 10.** *Staphylococcus aureus* phages 15 and 23 (Electronic microscopy image).

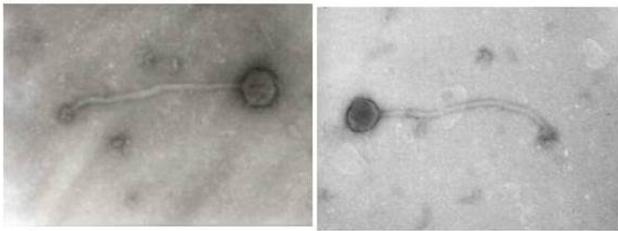


Figure 11. *Streptococcus pyogenes* phage 1 and 2 phages (Electronic microscopy image).

Thus, based on the studies conducted, *staphylococcal* and *streptococcal* infections prevailed in a number of human diseases. These microbes were represented by the species: *S. aureus*, *S. epidermidis*, *S. pyogenes*, *S. sanguis*, *S. viridians*, *S. salivarius*, *S. agalacticae*.

The presence of these bacteria in a number of diseases such as periodontitis, gingivitis, stomatitis etc., both separately and in combination, and a high resistance of pathogens to antibiotics, served as the basis for creating of Fersis, a new version of active, polyvalent phage preparation consisting of *staphylo*- and *strepto* phages, successfully applicable for treatments and prevention infectious diseases caused by *Staphylococcus* and *Streptococcus* genera.

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