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Antibiotic Resistance of Staphylococcal Strains Isolated from Patients with Purulent-Septic Infections and Creation of an Anti-Staphylococcal Phage Preparation

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Keywords
Antibiotic resistance, Infectious diseases

Introduction

One of the main causes of purulent-septic diseases in humans is a staphylococcal infection [1-4]. Staphylococci cause infectious diseases such as bacteremia, endocarditis, pneumonia, arthritis, etc. St.aureus causes co-infections and superinfections with different microbial pathogens. *S. aureus* also releases toxins in the process of vital activity, which have super antigenic activity. They can cause diarrhea and vomiting if toxins enter the gastrointestinal tract and food poisoning if food is contaminated with staphylococci.

Super antigens non-specifically stimulate cells without any normal antigenic identification. Cytokines are released in large quantities and cause a toxic shock syndrome. Exfoliative toxin causes a severe destruction of the granular layer of the epidermis.

St. epidermitis causes infectious complications after surgery on the background of endocarditis, peritonitis, urinary tract infection, otitis media and wound infection. St. saphrophyticus is associated with urethritis, cystitis, pyelonephritis. It occurs during clinical infections such as valve endocarditis, septicemia, peritonitis, urinary tract infection. The expectation of staphylococcal diseases increases in the hospital, where are concentrated many patients and staff.

The resistance of staphylococci to antibiotics complicates the prevention and treatment of purulent-septic diseases, since staphylococci, like other microorganisms, are capable of a rapidly developing resistance to new therapeutic drugs [5-9].

All of the above explains the relevance of studying the mechanisms and characteristics of resistance of staphylococci to new generation antibiotics, identifying the most effective therapeutic drugs and, at the same time, searching for alternative means of the treating staphylococcal infections.

In this context, therapeutic bacteriophage drugs, which are an effective means of antimicrobial therapy, are of great importance, which makes it relevant to create and study new anti-staphylococcal bacteriophages [10].

The aim of the work

The study of antibiotic resistance of staphylococci that cause purulent-septic diseases of humans, the creation and study of anti-staphylococcal bacteriophage drugs.

Research tasks

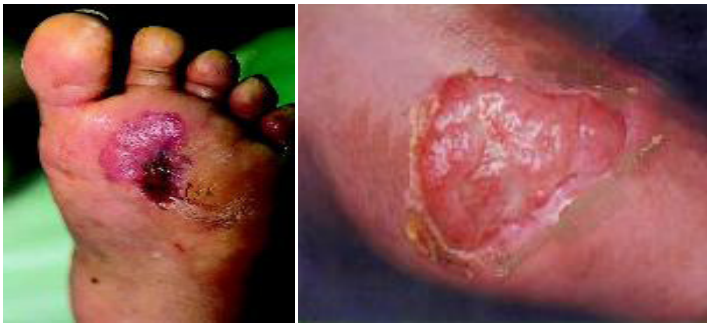
1. Isolation of clinical strains of staphylococci from patients with purulent-septic diseases.
2. Study of the sensitivity of these strains to methicillin, amoxicillin, cephalosporin, teicoplanin, ciprofloxacin, metronidazole, azithromycin
3. Study of R-plasmids that cause antibiotic resistance of the studied strains
4. Creation of bacteriophage preparations against clinical strains of staphylococci

Research Material and Method

S. aureus strains were isolated from material taken from patients with purulent-septic diseases treated in hospitals N N 1, 2, 3 and 4. In particular, 300 strains were isolated, including 80 from trauma patients, 71 from surgical patients, 96 from patients with purulent-

septic diseases and 56 strains from patients with sepsis.

For the cultivation and study of bacterial cultures, meat-peptone broth (1.5%, 0.7% and 2%), agar, L broth, yeast extract 5, bactotrifton 10, table salt, Giss medium, agar of yolk salts pH 7.0-7.2 were used.



Picture 1, 2 Purulent wound

Identification of pathogen

All microbial growths in bloodstream cultures were reported .Positive cultures were Gram stained and subcultured to the sheep blood agar, chocolate agar, Mac Conkey agar and Columbia colistin – nalidixic agar (Clinical Microbiology Procedures Handbook, Henry D. Isenberg). Isolates of bacteria were identified by conventional biochemical, serological methods. Confirmation of species identification was performed by API technique (API-System, bioMerieux, La Balmes-les Grottes, France).

Isolates of *Bacillus* spp., *Corynebacterium* spp., and coagulase-negative staphylococci recovered from a single culture device were considered as contaminants.

All isolates were saved on agar slants and sent for susceptibility testing methods.

Susceptibility testing

Antimicrobial agents were obtained from the respective manufacturersıo. The sensitivity of experimental bacterial strains to the following antibiotics was studied: methicillin, amoxicillin, cephalosporin, teicoplanin, ciprofloxacin, metronidazole, azithromycin.

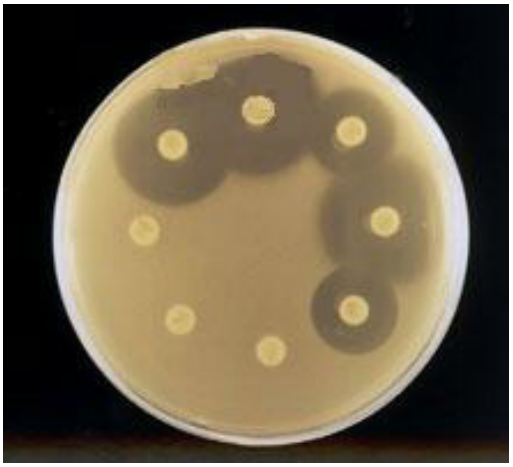
The antibiotic susceptibility for each pathogen was determined in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) guidelines.

Antimicrobial susceptibility testing of isolated pathogens to clinically used antimicrobials was performed by using Kirby Bauer disk diffusion method and ATB susceptibility systems. Antimicrobial agents were obtained from the respective manufacturers (bioMerieux).

Disk diffusion method

For the evaluation of the antimicrobial susceptibility the bacterial suspension previously compared to the 0.5 McFarland standard (McFarland turbidity standard, bioMerieux, Marcy l'Etoile, France) was applied to the Mueller-Hinton agar Petri dishes. Disks were individually placed with sterile forceps and then gently press down onto the agar. Diffusion of the drug in the disk prevented the growth of the bacteria around the disk and the zone of growth inhibition developed.

After that the disks were placed on the plate, the plate was inverted and incubated at 37°C for 18 - 24 hours. After incubation, measure of the diameter of the zones of complete inhibition (including the diameter of the disk) in millimeters was recorded.



Picture 3: Antimicrobial susceptibility of *S. aureus* performed by disk difusión method.

ATB system method

Antimicrobial susceptibility pattern of isolated bacteria also was done using ATB system (bioMerieux) that consists of strips of 15 microdilution wells, including 1 growth control well (without drug) and 14 sample test wells with the various critical concentrations of antibiotics calibrated. Incubation of the strip was done at 37°C for 18 - 24 hours after the inoculation of the strip with suspension with turbidity equivalent to 0.5 McFarland standards. Results were read by using specific threshold values that were empirically defined by them any facture based upon National Committee for Clinical Laboratory Standards.

Phage susceptibility

Phage isolation

Phage was isolated from the waste water, concentrated broth and 24 hour culture of respective microorganism was added to filtered waste water, test tube was placed in incubator for 24 hours, and Millipore filter. Filtrate was poured in broth, where *Klebsiella, Emterobacter, Syaphylococcus* cultures were added respectively. Eventually, broth with filtrate was transparent, but tube with only culture in it was seen to have growth in it. Next step includes inoculation of 100 ml tubes in order to produce more quantity of phage and examination of bacteriophage titer by Apelman and Gracias method. Our aim was to evaluate negative colony shape and size. According to Apelman method to determine the titer,

bacteriophage was diluted from 10^1 to 10^{10} degrees and 0.2 ml of 24 hours culture was added in each tube. Tubes were placed in incubator for 24 hours. The phage titer was 10^7 degree. In order to determine bacteriophage titer by Gracias method, bacteriophage was diluted from 10^1 to 10^{11} degrees.0.7% semisolid agar with quantity of 4 ml and 0.2 ml culture was added to 1 ml of diluted phage , mixture was shacked and applied to Petri dishes, when cooled dishes were placed in incubator.

Results of our investigations

Study of antibiotic resistance of Staphylococcus strains

Figure 1 shows that 87% of the strains isolated from hospital No. 1 were resistant to one antibiotic. 78% were resistant against two antibiotics, 60% to three, and 52, 41, 20, and 15% to four, five, six, and seven antibiotics, respectively.

The maximum number of strains (91%) isolated from hospital No. 2 (Diagram No. 2) showed resistance to one antibiotic. A small number (9%) turned out to be resistant to seven antibiotics. 75, 53, 48, 30, and 14% of the strains were resistant to two, three, four, five, and six antibiotics, respectively.

In the material taken from hospitals No. 3 and 4, the antibiotic resistance of staphylococcus strains decreased as the number of antibiotics increased.

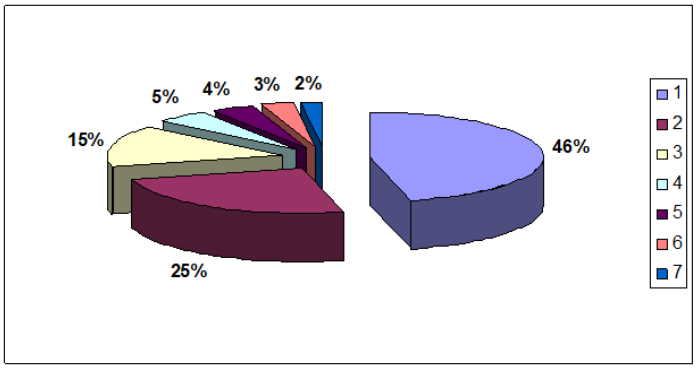


Diagram No 1: Percentage of antibiotic resistance of Staphylococcus strains isolated from hospital No. 1.

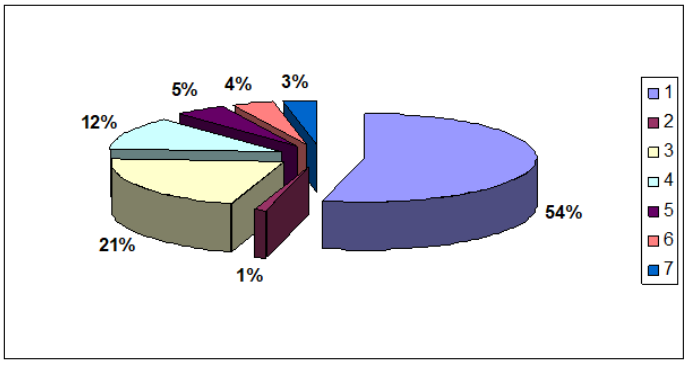


Diagram No 2: Percentage of antibiotic resistance of Staphylococcus strains isolated from hospital No. 2.

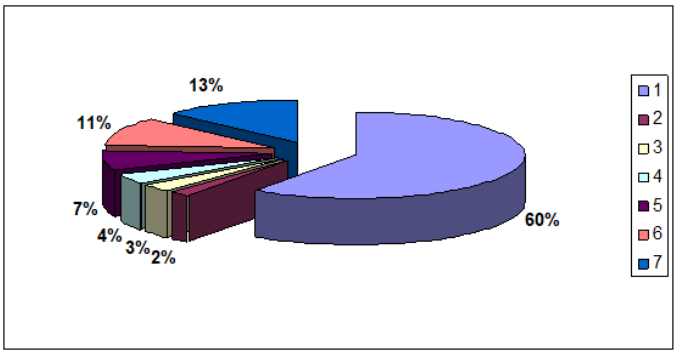


Diagram No 3: Percentage of antibiotic resistance of Staphylococcus strains isolated from hospital No. 3.

In particular, in the material from the third hospital, 96% were resistant to one antibiotic; in the second hospital this indicator was equal to 74%. In both cases, 6% showed resistance to seven antibiotics. For six antibiotics, 8 and 7% were resistant, respectively, for five – 25 and 12%, for four – 46 and 32%, for three – 48 and 35%, and for two antibiotics - 48% and 62%.

In particular, in the material from the third hospital, 96% were resistant to one antibiotic; in the second hospital this indicator was equal to 74%. In both cases, 6% showed resistance to seven antibiotics. For six antibiotics, 8 and 7% were resistant, respectively, for five – 25 and 12%, for four – 46 and 32%, for three – 48 and 35%, and for two antibiotics - 48% and 62%.

The combination of cephalosporin with amoxicillin (2 in the diagram) and ciprofloxacin with amoxicillin (3 in the diagram) produced a stronger effect.

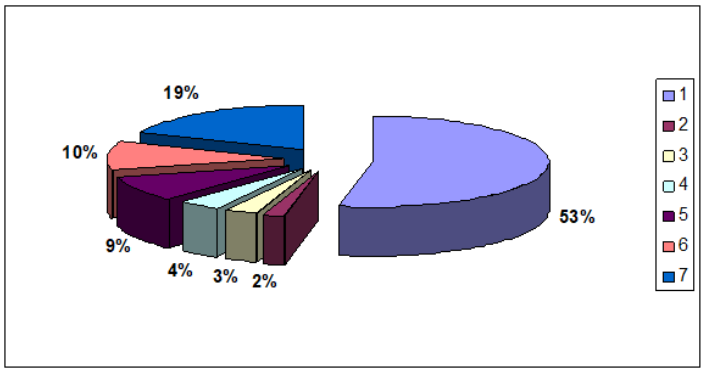


Diagram No 4: Percentage of antibiotic resistance of Staphylococcus strains isolated from hospital No. 4.

The combination of the three antibiotics had the strongest effect on the elimination of antibiotic resistance of bacteria. In particular, the combination of ciprofloxacin + amoxicillin + cephalosporin had an effect on 63% of the strains (4 in the diagram). The most powerful effect was achieved by a combination of methicillin + amoxicillin + cephalosporin (5 on the chart), methicillin +amoxicillin + metronidazole (6) and amoxicillin + metronidazole + azithromycin (7).

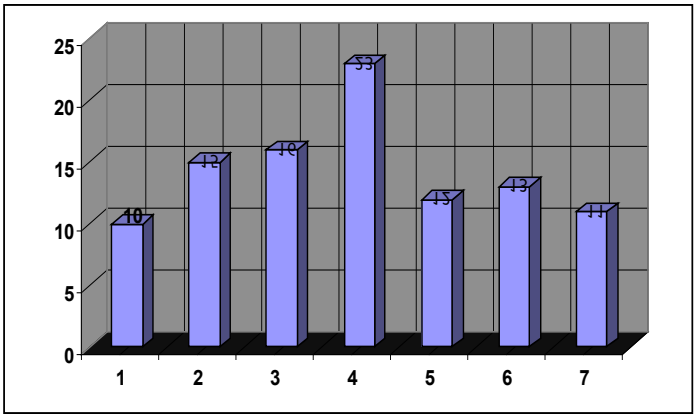


Diagram No 5: Elimination of resistance of staphylococcus strains isolated from hospital No. 1.

Both combinations of antibiotics were used on Staphylococcus strains, selected in hospital No. 2, in this case, significant low indicators of antibiotic resistance elimination were combinations of cephalosporin + amoxicillin (diagram 1), ciproxacillin + amoxicillin (diagram 3), ciproxacillin + amoxicillin + cephalosporin (4 on chart), methicillin + amoxicillin + cephalosporin (5 on chart). The maximum elimination effect was achieved by the elimination of methicillin +amoxicillin + metronidazole (6) and amoxicillin + metronidazole + azithromycin (7).

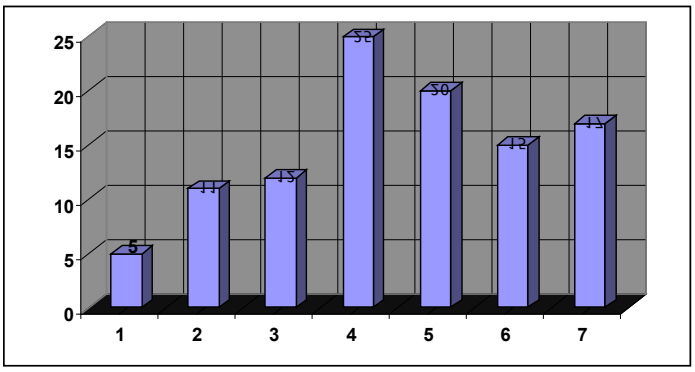


Diagram No 6: Elimination of resistance of staphylococcus strains isolated from hospital No. 2.

The following diagram shows the results of the study of the material taken from hospital No. 3. The low rate of elimination of antibioticresistance had a combination cephalosporin + amoxicillin (1 in the diagram), zyprexaonline + amoxicillin (3 in the diagram), zyprexaonline + amoxicillin + cephalosporin (4 in the diagram), a methicillin + amoxicillin + cephalosporin (5 in the diagram). The maximum elimination effect was achieved by the elimination of methicillin +amoxicillin + metronidazole (6) and amoxicillin + metronidazole + azithromycin (7).

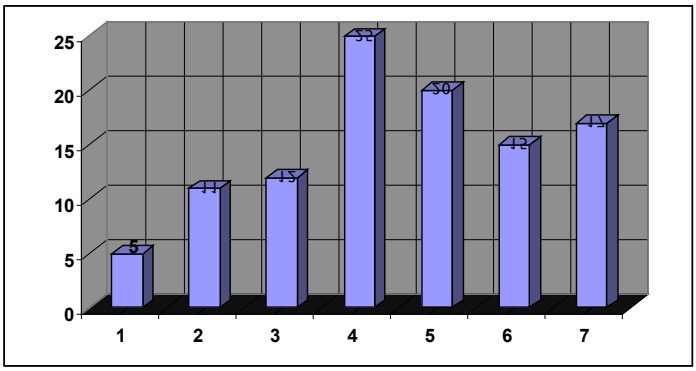


Diagram No 7: Elimination of resistance of Staphylococcus strains isolated from hospital No. 3.

The elimination of antibiotic resistance of staphylococcus strains isolated in hospital No. 4 differed in nature from the previous elimination variant only in that the strongest effect was provided by the combination of methicillin + amoxicillin + cephalosporin.

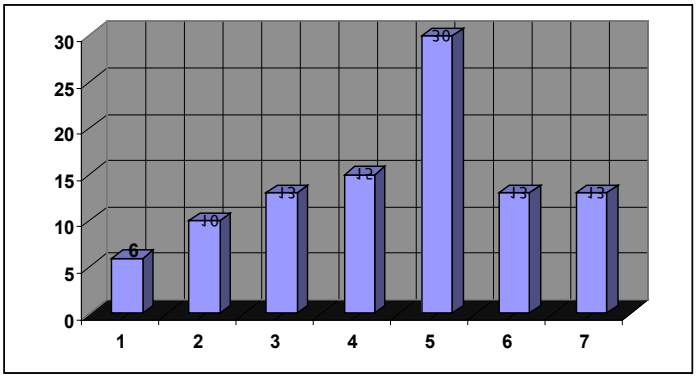


Diagram No 8: Phage sensitivity of staphylococcal strains from traumatological (A) and surgical (B) patients (phage M1 clone).

Thus, the elimination of the strains of staphylococci isolated by us, occurred most effectively under the influence of a combination of three antibiotics, in comparison with the effects of two drugs or one antibiotic. Combinations of antibiotics that can eliminate the antibiotic resistance of staphylococci include methicillin + amoxicillin + metronidazole, amoxicillin + metronidazole + azithromycin.

The Minimum suppressive dose of antibiotics

Table 1 show that the minimum suppressive dose did not exceed µg/ml. In the smallest doses, amoxicillin and methicillin showed an overwhelming effect. In relatively large doses, azithromycin showed an overwhelming effect.

As mentioned above, the study of an antibiotic resistance revealed a pronounced resistance to one antibiotic. In this regard, there

Table 1: The minimum inhibitory doses of antibiotics to strains of Staphylococcus.

Antibiotic	Minimum suppressive doses of antibiotics (µg / ml) for staphylococcal isolates (1-14)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Amoxicillin	0.1	0.1	0.3	0.1	0.2	0.1	0.1	0.1	0.06	0.06	0.1	0.1	0.1	0.1
Methicillin	0.2	0.1	0.08	0.1	0.1	0.1	0.1	0.1	0.06	0.1	1	0.1	0.1	0.1
Azithromycin	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

is a significant difference between antibacterial drugs. Data analysis showed that the majority of staphylococcal strains were resistant to ciprofloxacin (Figure 9) 62% and cephalosporin 57%. Relatively fewer strains showed resistance to teicoplanin (51%) and metronidazole (52%).

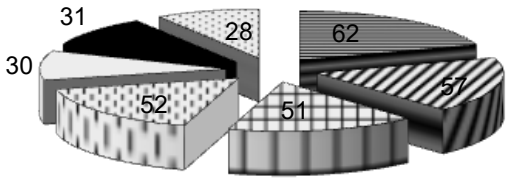


Diagram No 9: Phage sensitivity of staphylococcal strains from traumatological (A) and surgical (B) patients (phage M1 clone).

Statistically significantly less was the number of those strains that showed resistance to amoxicillin (30%), methicillin (31%) and azithromycin (28%).

The data obtained by us confirm the literature data on the strong antistaphylococcal effect of methicillin [11,12]. At the same time, the data obtained indicate the presence of strains resistant to this antibiotic, which also confirms the information available in the literature regarding the methicillin resistance of some strains of staphylococci [8,13,14].

It is necessary to emphasize the strong effect of azithromycin on staphylococci, since the information available in the literature mainly concerns the anti-streptococcal effect of this drug.

Based on the data obtained, we conclude that today, for the purpose of anti-staphylococcal therapy, it is advisable to use amoxicillin, methicillin and azithromycin, although some strains of staphylococci already have resistance to these drugs.

The data obtained by us also indicate the effectiveness of the combined use of antibiotics. In particular, the most effective in this regard are combinations where methicillin, amoxicillin and azithromycin appear. Consequently, for the elimination of antibiotic resistance, not only the number of antibiotics is important, but also the composition of the used combination of antistaphylococcal drugs.

We have obtained a Fersis phage, which is effective in the fight against staphylococcal infection.

Table 2: The electron-microscopic characteristics of staphylophage.

N	Phage Clone	Morphological group	Head size		Size of the tail	
			Length	Width	Length	Width
1	*S. aureus M1*	*myoviridae*	600Aº	600Aº	150Aº	1500Aº
2	*S. aureus D1*	*myoviridae*	600Aº	600Aº	150Aº	1500Aº
3	*S. aureus 140*	*myoviridae*	750Aº	750Aº	180Aº	2000Aº

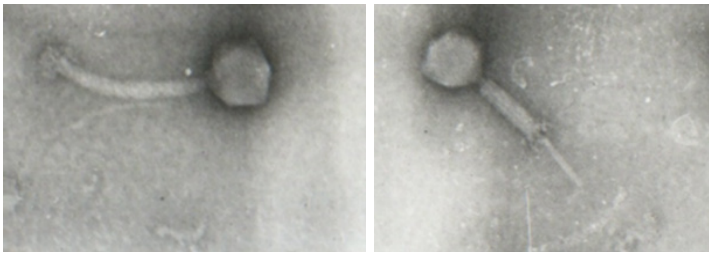
The study of phage clones under an electron microscope showed that they belong to *S. aureus* M1, *S. aureus* D1 and *S. aureus* 140. The size of the phage head varied between 600Aº-750Aº, and the size of the tail showed greater variability depending on the clone. In particular, in *S. aureus* M1 and *S. aureus* D1, the tail width was 150Aº, and in *S. aureus* 140-1800Aº. At the same time, in this clone, the tail length was the smallest-200Aº.

Table 3: Indicators of the biological activity of staphylophages.

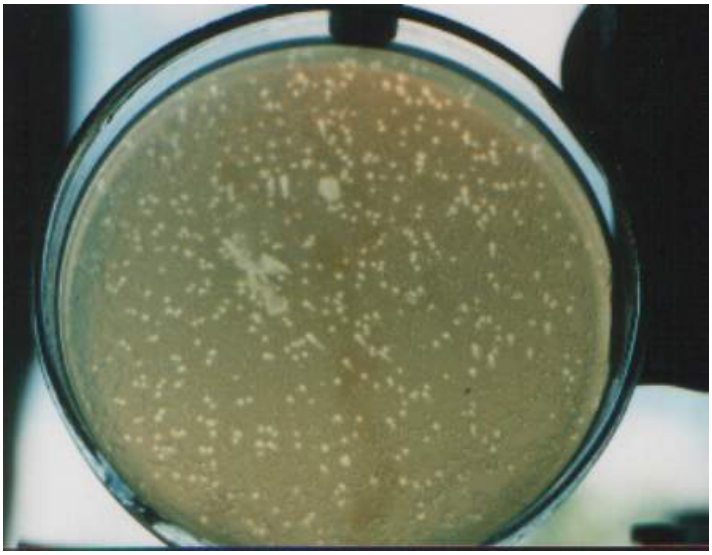
Phage Clone	The period of adsorption min.	The latency period min.	Yield
S. aureus M1	10-12	20-22	100-130
S. aureus D1	10-12	20-22	100-120
S. aureus 140	12-14	22-24	110-130

It should be noted that *S. aureus* 140 was distinguished by the shortest adsorption period and the longest latent period.

Below is an electromicroscopic image of the phage in 200,000-fold magnification.



Picture 4,5: Phage *S. aureus myoviridae.* X 200000.



Picture 6: *S. aureus* 140 negative colonies on agar.

The titer of the phage (according to the method of GRATSIA) It was equal in *S. aureus* M1-9 x 10^9, in *S. aureus* D1 -9 x 10^9, in *S. aureus* 140-5 x 10^9, the size of the negative phage colony on the lawn is 1 mm, 1 mm and 1.5 mm, respectively.

Most of the staphylococcal strains (57%) isolated from trauma patients underwent complete lysis under the influence of the M1

phage, in 28% there was partial lysis and in 15% the phage had no effect on staphylococcal strains (Diagram No. 15).

The strains isolated from surgical patients underwent the complete lysis in 70% of cases, partial lysis in 13%, and remained intact in 11% of cases.

The phage sensitivity (clone M1) of staphylococcal strains isolated from patients with purulent infections was similar to that of strains isolated from surgical patients. In particular, 66% of the strains underwent complete lysis, 13% underwent partial lysis, and the phage had no effect on 21% of the strains.

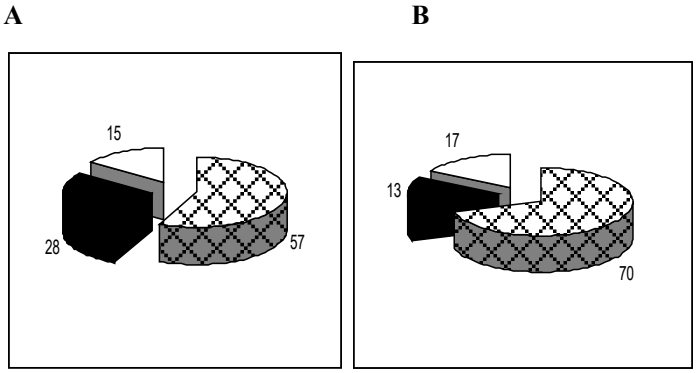


Diagram No 10: Phage sensitivity of staphylococcal strains from traumatological (A) and surgical (B) patients (phage M1 clone).

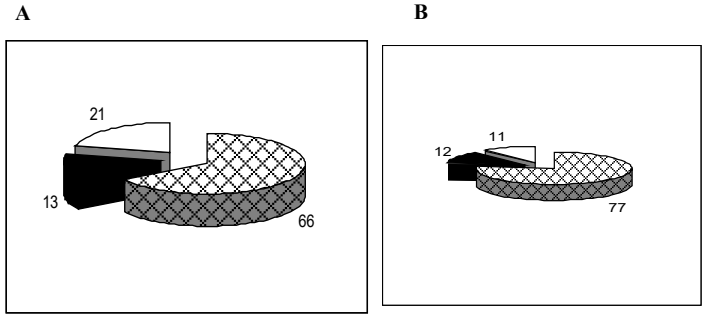


Diagram No 11: Phage sensitivity of staphylococcus strains from patients with purulent infections (A) and sepsis (B) (phage M1 clone).

Staphylococcal strains isolated from sepsis patients underwent complete lysis in 77% of cases, partial lysis was observed in 12%, and phage remained inactive in 11% of cases.

The study of the sensitivity of staphylococcal strains to the D1 phage clone gave the following result.

In strains of staphylococci isolated from traumatological patients, 57%, - underwent complete lysis, 24- partial% and 19% were found to be resistant to phage.

In the strains of staphylococci isolated from surgical patients, 70% underwent complete lysis, 20% underwent partial lysis, and 10% were resistant to phage.

Among the strains of staphylococci isolated from patients with purulent infections, 69% underwent complete lysis, 20% underwent partial lysis, and 11% were resistant to phage.

Of the strains of staphylococci isolated from patients with sepsis, any complete lysis was recorded in 71% of cases, partial lysis in 18%, and 11% were resistant to phage.

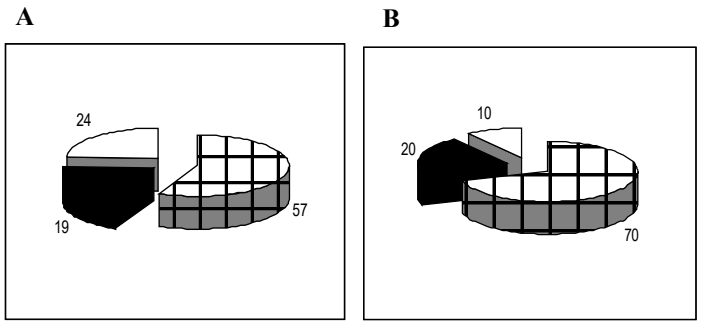


Diagram No 12: Phage sensitivity of staphylococcal strains from traumatological (A) and surgical (B) patients (phage D1 clone).

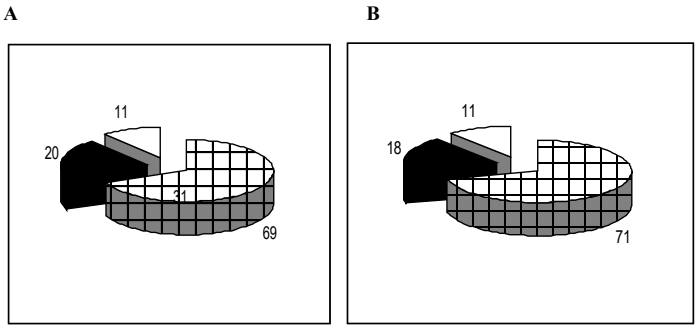


Diagram No 13: Phage sensitivity of staphylococcus strains from patients with purulent infections (A) and sepsis (B) (phage D1 clone).

The study of the sensitivity of staphylococcal strains to the phage clone 140 gave the following result.

In the strains of staphylococci isolated from trauma patients, 76% underwent complete lysis, 10% underwent partial lysis, and 14% were resistant to phage.

In the strains of staphylococci isolated from surgical patients, 59% underwent complete lysis, 25% – partial lysis, and 16% were resistant to phage.

Among the strains of staphylococci isolated from patients with purulent infections, 60% underwent a complete lysis, 20% underwent a partial lysis, and 20% were resistant to phage.

In the strains of staphylococci isolated from patients with sepsis, complete lysis was recorded in 62% of cases, partial lysis in 20%, and 18% were resistant to phage.

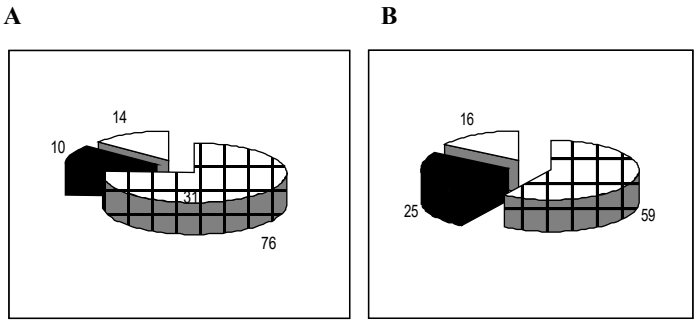


Diagram No 14: Phage sensitivity of staphylococcal strains from traumatological (A) and surgical (B) patients (phage clone 140).

Our study showed that the obtained phages actively affected the strains of staphylococci, causing lysis of the latter.

By comparing the sensitivity of staphylococcal strains to individual phage clones, it is possible to make sure that there is no significant difference in the number of strains that have undergone complete or partial lysis under the influence of the phage. In our opinion, great importance should be attached to the fact that the number of staphylococcal strains that showed resistance to phage clones in any case did not exceed the average of 13, which indicates a high anti-staphylococcal activity of the phage drug obtained by us.

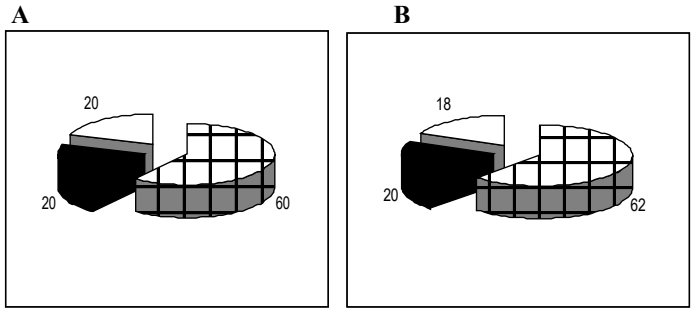


Diagram No 15: Phage sensitivity of staphylococcus strains from patients with purulent infections (A) and sepsis (B) (phage clone 140).

Thus, we can say that the phage preparation obtained by us exhibits high antistaphylococcal activity and can be used in the fight against purulent-septic infections.

Based on the data we received and their analysis, we considered that it is possible to draw the following conclusion.

Conclusion

Strains of staphylococci isolated in purulent-septic diseases have a pronounced antibiotic resistance. The most strongly developed resistance to a single antibiotic. The number of resistant strains decreases in parallel with the increase in the number of antibiotics.

The majority of strains of staphylococci are resistant to zyprexaonline, cephalosporine and metronidazole. The number of strains resistant to methicillin, amoxacillin and azithromycin does not exceed 30%.

For the treatment of purulent-septic diseases of staphylococcal genesis, it is advisable to use the antibiotics methicillin, amoxicillin and azithromycin.

Antistaphylococcal bacteriophage Fersis consists of clones of *S. aureus* M1, *S. aureus* D1, *S. aureus* 140, which are characterized by titers (according to Grazia) 9 x 10^9, 9 x 10^9, 5 x 10^9.

The bacteriophage Fersis causes active lysis of *S. aureus* strains isolated from patients with purulent-septic infections. It is advisable to use phage for the treatment of purulent-septic diseases.

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